Refractory gene drive risk assessment: Scoping and hazard analysis for UCMI

Problem formulation and preliminary hazard list

Background

University California Malaria Initiative (UCMI) have completed several pathways to harm workshops (in conjunction with Transmission Zero) to identify hazards associated with a hypothetical field release of a genetic, malaria-vector, population modification strategy. UCMI subsequently completed two problem formulation workshops, facilitated by CSIRO, to identify hazards specific to their anticipated products and staged-release strategy. The problem formulation workshops placed the outcomes of the pathways to harm workshops within the broader context of the hazards (and other related issues) associated with genetic vector control strategies that have been postulated and discussed in the scientific literature.

This document

This document identifies a preliminary hazard list (PHL) for UCMI's potential gene-drive investigational mosquito. The PHL contains the hazards deemed by the UCMI team to be pertinent to an initial, island-based, field-release strategy. The document also lists the potential hazards that were deemed to be non-applicable in the specific context of this strategy. The hazards within the PHL are presented as pathways to harm in the same manner that the United States Environment Protection Agency (USEPA) uses to portray Adverse Outcome Pathways (Ankley *et. al.,* 2010). This approach maintains a graphical structure similar to that adopted in the original pathways to harm workshops, but *inter alia* categorises the evidence base for the steps between the events on the pathways and identifies events that are potentially the best points for laboratory and/or field evaluation.

What's next?

Following the receipt of comments on, and finalisation of, this document, CSIRO and FNIH may explore with UCMI the potential application of additional hazard analysis tools in a more detailed, second-stage analysis. This analysis could identify risk assessment techniques for each of the hazards identified in the PHL and may include the construction of fault trees and/or signed digraphs, for example, to provide additional support for a subsequent risk assessment. UCMI will also review this document following outcomes from their on-going community engagement activities.

PREFACE

The University of California Malaria Initiative is committed to a Relationship-Based Model with respect to the assessment of risks as they relate to regulatory and community engagement (Kormos *et. al*., 2021). The salient feature of this model is that it places stakeholders and community members at the center of decision-making processes, rather than as recipients of predetermined strategies, methods, and definitions. Although the UCMI seeks input for its own internal evaluation of risks, it will not impose this assessment nor endorse the imposition of any other external assessment upon communities and stakeholders at its field sites.

INTRODUCTION

Hazard identification and pathways to harm

Forty years ago, risk assessment was characterised as a four-step process that began with hazard identification (NRC, 1983). At that time hazards were defined as "*a situation that in particular circumstances could lead to harm*" (Royal Society, 1983). Hence a key objective of hazard identification is to describe these circumstances. To do so the analyst must describe the exposure pathway (Wolt *et. al*., 2010), or "pathway to harm" (Devos *et. al.*, 2019) - that is the causal chain of events that link exposure to a substance or activity, such as the use of an insecticide or the planting of a genetically-modified crop, to a harmful outcome.

The United States Environmental Protection Agency (USEPA) advocates a similar approach under the label of "Adverse Outcomes Pathways" (Ankley *et. al.,* 2010), as does Raybould (2006), who calls for risk assessments to be based on a clear description of how a proposed activity may cause harm, during an initial Problem Formulation step, originally described by Norton *et. al.* (1992), but now widely adopted, that precedes risk calculations. So, whilst the terminology has changed over time, the underlying notion that risk calculations be based on, and preceded by, a clear enunciation of the causal linkages (pathways) between a substance or activity and the things we care about, remains a central tenet of the risk assessment method.

Here we document the results of a hazard identification for a hypothetical field release of a novel malaria vector control strategy. The analysis identifies a set of assessment endpoints – potentially harmful changes to human health, animal health and environmental components, process or services that may influence decision makers – as unambiguously as possible; and describes a set of plausible pathways to harm. Each pathway is portrayed using the graphical representation of adverse outcomes as proposed by Ankley *et. al.,* (2010) wherein the pathways are "anchored" at

each end by an initiating event (typically at a molecular level in this case) and a harmful environmental outcome (at a population, community, or ecosystem level).

In the approach advocated by Raybould (2010), each link between the steps in a pathway should be associated with a risk hypothesis of no harm – that is a refutable conjecture that one step cannot lead to the other, or the frequency of the step is too low, or the magnitude of the resulting effect too small, to be harmful. In Ankley *et. al.* (2010), the links between the steps are characterised on a weight-of-evidence basis. In their schema, steps that are mechanistically or empirically known (with well-established supporting data) are distinguished from plausible steps that have limited data to support them, and from hypothetical steps that are biologically possible but may never have been observed. Exposure-response links that can be quantified by modelling are also identified, together with biomarkers that are indicative of exposure.

Here we attempt to apply the approach developed by Ankley *et. al.* (2010) to emphasise the types of evidence that each pathway is based on. However, we substitute for the biomarkers what we believe are the most practical, cost-effective, or safest point in the pathway to make risk predictions that can be tested in the field or the laboratory. These events are often interim steps in the pathway, and hence serve as measurement endpoints – that is proxies for the assessment endpoints that allow risk decisions to be made before environmental or human health values may actually be harmed.

Final biosafety decisions will depend on the regulatory bodies with jurisdiction over potential field sites, but the outcomes of models, tests and experiments conducted at these steps should help inform go/no-go decisions for the proposed intervention. Desired outcomes at these steps might also form part of the Target Product Profile definition and therefore be associated with minimally acceptable efficacy or safety criteria (Carballar-Lejarazú and James, 2017; James *et. al*., 2018). Work on transgenic strains that fail to meet these criteria would likely be terminated or the strains modified to meet the criteria followed by additional testing or modelling for confirmation.

Construct and proposed field-release scenario

The UCMI construct was not specifically defined during the hazard analysis workshops. For the purposes of this document, we assume that the construct comprises an effector gene coupled to, or embedded within, a CRISPR/Cas9 drive component. The effector gene is assumed to comprise an endogenous, cis-acting, DNA control region and an effector region. The control region determines when, where and how much of the anti-malarial product is produced by the effector region. Carballar-Lejarazú and James (2017) identify many possible combinations of various anopheline

mosquito *cis*-acting DNA elements that can be used to express different anti-malarial molecules. The initial field-release scenario envisages a small-scale release on an island where there is minimal human traffic and transport of goods that may harbor mosquitoes to an adjacent mainland area [Lanzaro *et al*. 2021). Two island nations identified during the workshop were the Democratic Republic of São Tomé and Príncipe and the Union of the Comoros. The target organisms are defined as the two mosquito species, *Anopheles gambiae sensu strictu* and *Anopheles coluzzii*.

METHODS

Workshops

UCMI completed a series of pathways to harm workshops facilitated by the Foundation for the National Institutes of Health (FNIH). At this stage, participants included members of the UCMI team, Transmission Zero team and FNIH. CSIRO personnel acted as guest observers during these workshops. The UCMI group discussed the importance of the concerns and perceptions of local people during these initial workshops. They acknowledged that the issues and concerns of local people and stakeholders will affect the way that they perceive the project, interact with the research team, and determine their behaviour and feelings about the project overall. UCMI emphasized that they are committed to thoughtful engagement to ensure that concerns of stakeholders are seriously considered and addressed (Kormos et al. 2021). These concerns are legitimate social risks regardless of whether they are supported by scientific or technical analysis.

The workshops mapped out several pathways by which the release of gene-drive modified mosquitoes designed to modify wild type populations to be refractory to malaria parasites could be harmful to human and animal health and a variety of environment values (such as biodiversity, water quality, etc.).

After these workshops UCMI personnel attended two further workshops facilitated by CSIRO. The first summarised the outcomes of the pathways to harm workshops and reviewed their outcomes in light of the hypothetical hazards associated with gene drive modified mosquitoes identified in the literature. The second workshop compiled the feedback from the first and presented the first draft of a preliminary hazard list for a hypothetical release scenario. The outcomes of the feedback received during this second workshop are presented here.

Literature review

Hypothetical hazards associated with genetically modified mosquitoes (GMMs) are discussed in a growing body of literature (designated hereafter as 'the literature) that can be broadly classified into three types:

- the biosafety regulations of relevant individual authorities.
- documents, produced by respected international or national organisations such as the World Health Organisation (WHO-TDR, 2014), the Secretariat to the United Convention on Biological Diversity (UNEP/CBD, 2016), the United States National Institutes of Health (NIH, 2003), the National Academy of Sciences Engineering and Medicine (NASEM, 2016), the European Food Safety Authority (EFSA, 2013; 2020) and the Australian Academy of Sciences (AAS, 2017).
- the views of individual scientists, or groups of scientists, sometimes published as the proceedings of workshops or in so called "self-governance" documents, such as Benedict *et. al.*, (2008), David *et. al.*, (2013), Hayes *et. al.,* (2018), James *et. al.*, (2018; 2020), Roberts *et. al.* (2017), Rode *et. al.,* (2019), Romeiss *et. al*., (2019) and Teem *et. al*., (2019).

This analysis reviewed all the documents listed in the last two groups and categorised each of the hazards and issues identified within them into one of the seven "risk areas" described by EFSA (2013). Another risk area labelled "Evolutionary and stability considerations" was added to accommodate issues raised in the literature that were deemed sufficiently different to warrant this additional category. This in effect created a hazard checklist to compare against the outcomes of the pathways to harm workshops.

After reviewing the structure of the hazard checklist, and prompted by feedback from the first CSIRO workshop, it was clear that there was considerable overlap among the hazards identified in some of the original EFSA risk areas. Some of the areas were therefore combined resulting in the following five categories:

- Pathogens, infections and diseases, and the impacts of GMMs on human and animal health
- Persistence and invasiveness of GM insects, and interactions of GMMs with target organisms
- Interactions of GMMs with non-target organisms including horizontal gene transfer
- Impacts of techniques used for the management of GMMs
- Evolutionary and stability considerations

The results of this analysis are presented according to this classification of risk areas.

RESULTS

Pathogens, Infections and Diseases & Human and Animal Health

Pathogens, infections, and diseases adversely impact human and animal health, so it seems sensible to combine these two risk areas into a single category. Under this combined category the literature identifies seven hazards:

1. An increase in the vectorial capacity or vector competence for the target pathogen or other nontarget pathogens.
2. The emergence of target pathogens with increased virulence, possibly through the development of resistance to modified physiological mechanisms in the target vector resulting in a population of pathogens that may be transmitted more easily.
3. An increase in the abundance of disease transmitting insects through pathways such as niche replacement or competitive release of another disease vector.
4. The introduction of new pathogens into the receiving environment, including into areas where a non-GMM comparator is not present.
5. Physiological or behavioural differences in the GMMs that effect nuisance impacts, such as increased human biting rate.
6. Transmission of toxic or allergenic substances (related to the components of an engineered gene drive) either directly by biting or indirectly by exposure from such substances released into the environment (e.g. incidental exposure through inhalation or ingestion).
7. Resurgence of disease following loss of immunity in human populations after a prolonged period of low incidence, and hence reliance on continued long-term positive effects of vector suppression or modification strategies.

In addition to these specific hazards, the literature encourages proponents to consider hazards that may derive from possible malfunctioning of the GMM technology, for example through gene silencing, undetected drive elements due to loss of a dominant marker gene or recombination events that disrupt the transgene structure and lead to loss of function. It also poses a somewhat under-specified question: could the GMM mosquitoes release metabolites that alter the pathogen population?

Increase in vector competence or vectorial capacity

According to the underlying principles of the Ross-MacDonald model (Rainer, *et. al.,* 2013), the incidence of human infection can increase through: a) an increase in the vectorial capacity or vector competence of genetically modified mosquitoes as compared to their wild-type counterparts; or b) an increase in the post-release abundance of the vector that changes, perhaps temporarily, the vector-to-host ratio within a defined region.

Here we assume that the hypothetical release scenario envisages a release of male-only mosquitoes at an initial population size that lies somewhere between 1% and 10% of the wild type population, on the islands of São Tomé or Comoros. Since male mosquitoes do not bite this should have no direct appreciable effect on the vector-host ratio. However, this analysis acknowledges that sex-separation procedures are not 100% effective and hence some small (conservatively 5%) of the released mosquitoes may be female. This could in theory lead to some increase in pathogen transmission, but in practise this effect is expected to be negligible because laboratory biosafety procedures are designed to ensure that all released cohorts are free of any human or animal pathogens, and density-dependent larval mortality is expected to cause the population of adult female mosquitoes to re-equilibrate within a month or so.

Unlike changes to the vector-host ratio, changes to vector competence (a component of vectorial capacity governed by intrinsic factors that influence the ability of a vector to transmit a pathogen) and some of the other vectorial capacity parameters in the Ross-MacDonald model, such as the intrinsic incubation period, will be pathogen-specific. An unambiguous definition of the risk endpoint therefore requires that the human and animal pathogen(s) addressed by the assessment are identified.

Across Africa, mosquitoes in the *An. gambiae* complex are known to transmit the four historical protozoan species of human malaria parasites: *Plasmodium falciparum*, *P. ovale*, *P. vivax* and *P. malariae*, along with seven other human or animal pathogens, namely Bwamba virus, lymphatic filariasis, Ngari virus, o'nyong nyong virus, *Rickettsia felis*, Rift Valley fever virus and Tataguine virus (Hayes *et. al*., 2020). Searches of a published materials database (Pubmed, PubMed (nih.gov)) using keywords including the pathogen name and country failed to identify any reports of Bwamba, Ngari, o'nyong nyong or Tataguine viruses in either Sao Tome and Principe or the Comoros islands. Both island groups have lymphatic filariasis (transmitted also by *Culex quinquefasciatus*) and the Comoros has records of Rift Valley Fever (Ruiz *et al*., 1994; Sabatinelli et al., 1994; Fan *et al*., 2013; Roger et al., 2014). Sao Tome and Principe also have records of *Rickettsia felis* being present in animals (Tsai *et al*., 2020). Active and passive surveillance confirms the presence of the arboviruses causing

dengue and chikungunya fever in both island nations, but these are *not* transmitted by anopheline mosquitoes (Sang *et al*., 2008; Dellagi *et al*., 2016; Yen *et al*., 2016).

The workshop participants recognized the four major species of malaria-causing protozoa (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* [the primary focus of UCMI is *P. falciparum*]) as target pathogens, and noted that non-target pathogens would be determined by the occurrence of the pathogens in the release site and areas into which the GMMs are predicted to spread, and any pathogens of concern identified by stakeholders or regulators at the release sites.

In this context the participants also noted that:

- currently there is no evidence to suggest that genetic modification has increased the vector competence of GMMs for target or non-target pathogens and the available experimental data support this conclusion (Pike *et al*., 2018).
- the potential for this issue can be designed out of the GMMs through careful selection of the gene drive target gene.
- changes in the vector competence parameters of the vectorial capacity equation can be tested in the laboratory, but these tests should only be considered if there is an expectation that the expression of the novel genes or insertion sites of the modification impact some intrinsic feature of the competence of the mosquito for non-target pathogens.
- indirect effects on vectorial capacity parameters due to changes in the microbiome of GMMs could occur but it is difficult to see how this would lead to an increase in vectorial capacity compared to wild-type mosquitoes.

Emergence of resistant pathogens

The workshop participants noted that a change in virulence to the host may occur through increased transmission (i.e., by a greater infectivity to the host at the liver stage) or through Increased impact to the host (i.e., causing more severe and damaging illness/disease). They also noted that emergence or selection for resistant target pathogens is to be expected with a single effector mechanism, but this will not necessarily lead to an increased virulence, and hence may not necessarily be harmful (Figure 1a).

The possibility of increased virulence, for example by increasing parasite load in mosquitoes or human hosts or by making parasites more competent at progressing through either insect or human host stages, was recognised as a salient issue. However, since the effector genes in the UCMI product are exogenous, even if resistance to these synthetic gene products does arise it is unlikely that these could result in changes to parasite development in the mosquito. Modelling suggests that

multiple effector genes can prevent, or delay resistance and this strategy is employed by the UCMI team.

Resurgence of disease following loss of immunity

The resurgence of disease following the loss of immunity is a potential hazard for any successful malaria intervention strategy, genetic or otherwise. Modelling suggests that rapidly reducing exposure to the malaria parasite can reduce disease prevalence in highly immune populations, but this initial benefit can be offset by a greater disease burden in later years due to a gradual loss of herd immunity (Ghani *et. al*., 2009). However, there appears to be no evidence of this hazard to date occurring with the conventional applied transmission-blocking and disease-mitigation technologies, Insecticide Treated Nets (ITNs), Indoor Residual Spraying (IRS) and malaria mass-drug treatments (Pryce *et. al.,* 2018; Kigozi, *et. al.,* 2020). Furthermore, Sao Tome & Principe and the Comoros, are both hypo-endemic for malaria (there is no malaria at all in Principe), which makes moot the 'rebound effect' as an issue for concern in this scenario.

Implausible hazards

The following hazards identified in the literature were deemed to be implausible by the workshop participants:

- transmission of toxic or allergenic substances.
- an increase in the abundance of disease-transmitting insects through pathways such as niche replacement or competitive release of another disease vector.
- the release of GMMs with increased biting rates.
- the introduction of new pathogens into the receiving environment, including areas where the non-GMM comparator is not present.

Whilst the effector mechanism(s) in the hypothetical release scenario were not precisely defined, the workshop participants noted that mode of action does not involve expression of proteins in the salivary glands and does not involve the production of toxins or allergenic substances. Tests of salivary glands protein composition of transgenic mosquitoes were acknowledged to be possible but likely unnecessary because the genetic construct does not involve any salivary gland gene control sequences.

Population modification strategies are deliberately designed to leave the ecosystem structure largely unchanged thereby preventing empty niches and competitive release, whilst also providing on-going protection against re-establishment of unmodified mosquitoes (Carballar-Lejarazú and James, 2017). Nonetheless, genetic modification may incur fitness costs that can be overcome with effective gene

drives (Unckless *et. al.*, 2015), so population modification can still theoretically occur whilst the abundance of the target population is concurrently diminished. However, the UCMI team are aspiring to a target product profile that ensures there will be no significant difference in mosquito abundance or behaviour and will eliminate laboratory strains that display significant fitness or biting behaviour differences.

The introduction of new pathogens into the receiving environment also was deemed implausible because the genetic modification would be introgressed into the wild-type genetic background, the modified mosquitoes released into a location with endemic wild-type comparators, and standard insectary operating procedures (Adelman, *et. al.*, 2017) will be used to ensure that no pathogens will be introduced with the released strains.

Persistence and invasiveness of GMMs & Interactions of GMMs with target organisms

The literature identifies five hazards in these combined risk areas:

8. Unintentional genetic or behavioural changes that might decrease susceptibility to control (or surveillance) measures such as insecticides and attractants.
9. Changes in target organism's population parameters, fitness or behaviour (e.g. altered larval competition or accelerated maturation) that may advantage GMMs as compared to the wild type, causing increased persistence and invasiveness, and possibly leading to the displacement of other insect species.
10. Reduction in the efficacy of the GMM-mediated trait that may result in adverse effects.
11. Changes in interactions with the target organisms arising from an altered genetic diversity of a reared GMM population that may result in adverse effects.
12. Adverse effects arising from not achieving the quality or number of released GMM needed to achieve intended vector or disease outcomes.

In addition to these specific hazards, the literature notes that changes to the habitat or geographic range of the target population, including the potential for long range, trans-boundary dispersal, and the spread of the genetic construct via vertical gene transfer to sexually compatible species in the release area, possibly disrupting their population dynamics, may or may not lead to harmful outcomes.

Several of the workshop participants thought that the use of the terms "persistent" and "invasive" in the risk area title was misleading because the GMM does not persist and invade, rather the

transgene moves into the indigenous wild-type genetic background through mating, and its persistence is beneficial because it provides a sustainable control option that removes the requirement for continual interventions even in the face of re-introduction of wild-type mosquitoes.

Vertical gene transfer

The *Anopheles gambiae* complex consists of nine sibling species (Barron et. al., 2019). Three of these species – *An. gambiae sensu strictu, An. coluzzii* and *An. arabiensis* – are dominant vectors of malaria, whilst the others are either minor vectors or non-vectors due to their localised distribution or animal feeding preferences (White et. al., 2011). Experimental crosses between *An. gambiae sensu strictu* and *An. coluzzii* result in fertile, viable offspring with no obvious fitness costs in laboratory settings. In the field, assortative mating between the two species periodically breaks down resulting in extensive hybridisation (Pombi *et. al.,* 2017). Consequently, hybrids are generally reported to be below 1% but can reach much higher rates episodically and in so-called 'hybrid zones' on the western edge of the species' distribution (Vicente, *et. al.,* 2017). The workshop participants identified *An. gambiae sensu strictu* and *An. coluzzii* as the target species due to the (beneficial) potential for the genetic construct to introgress into either of these two species. In the field sites currently under investigation by UCMI only one or the other of these species is known to occur, *An. gambiae* s.s. in the Comoros (Brunhes 1977) and *An. coluzzii* in Sao Tome and Principe (Loiseau *et al*. 2019).

Although there are reports of introgression between *An. gambiae* (Giles) and *An. arabiensis*, and they mate readily under laboratory conditions, the resulting F1 males are sterile and F1 females are fertile (Slotman *et. al*., 2004, 2005a, 2005b.) Subsequent fertility in backcrosses vary due to incompatible alleles. *An. gambiae* x *An. arabiensis* hybrids in the field are very rare (estimated at less than< 0.1%), probably because of a variety of incomplete prezygotic mating barriers and selection acting against these hybrids (Slotman *et. al*., 2004, 2005a, 2005b; Fontaine, *et. al*., 2015; Pombi *et. al*., 2017). Transfer of the genetic construct to *An. arabiensis* was therefore considered highly unlikely, not necessarily harmful, and not identified as a plausible pathway to harm. Importantly, *An. arabiensis* is not present on either of the island sites being evaluated (Brunhes, 1977; Loiseau *et al*. 2019).

Insecticide resistance

The workshop participants noted that the probability of an enhanced insecticide resistance in GMM as compared to wild-type mosquitoes would be extremely low because any Cas9-induced off-target mutation to a specific codon, such as the voltage gated sodium channel (*Vgsc*) codon, resulting in knock-down resistance (*kdr*) to pyrethroids (Martinez-Torres *et. al,* 1998) would be lower than

spontaneous mutation rates, estimated to be approximately 3 x 10^{-10} per base pair per replication in the vinegar fly, *Drosophila melanogaster* (Drake *et al*., 1998) and there is no reason to believe that this rate would be higher in mosquitoes. Laboratory analyses of transgenic mosquitoes carrying effector genes confirmed this conclusion and showed no changes in insecticide-resistance phenotypes (Pike *et al*., 2018). Nonetheless, the workshop participants noted that laboratory tests for enhanced insecticide resistance may be a procedural requirement imposed by biosafety authorities when permitting the importation or release of GMMs.

Changes to fitness and population parameters

The term "fitness" is used to describe a variety of life-history parameters associated with viability and vigour (such as life-stage specific mortality rates, adult longevity), fecundity (number of eggs laid, egg hatching rates), and fertility (percent of female laying eggs) and mating competitiveness. The fitness of genetically-modified mosquitoes when compared to a wild-type comparator may be increased by changing one or more of these life-history parameters.

The fitness of GMMs can be lower than that of wild-type comparators due to relatively rapid adaptation to laboratory conditions and the reduced genetic diversity of laboratory populations (Catteruccia *et. al.*, 2003). However, the workshop participants also noted that the microbiome and/or transcriptome of GMMs may be different from wild-type and this can influence fitness (citing differences in mating choice in laboratory studies) and noted that competitive interactions with other species are possible, most likely in the larval aquatic ecosystems. Any changes to the fitness of GMMs were thought likely to be modest.

In direct laboratory comparisons between the *An. gambiae* G3 strain and the gene-drive modified strain AgNosCd-1, Carballar-Lejarazú *et al*., (2020) identified small but statistically-significant increases in fecundity and fertility of outcrossed heterozygotes. The aggregate genetic load of the construct did not affect the gene-drive dynamics in small cage trials but the workshop participants acknowledged that these changes might lead to a small increase in the vectorial capacity of non-target pathogens by increasing the vector to host ratio in isolated island settings (Figure 1b). It was also noted that this pathway could lead to an increase in the vectorial capacity of target pathogens but only in the situation that the effector gene concurrently fails, or resistance emerges (Figure 1a).

Implausible hazards

Hazards 10, 11 and 12 were all deemed implausible in this context. The genetic construct would be introgressed into the wild-type genetic background prior to release and is then expected to spread into the endogenous wild-type genetic background. Introgression of any other genes responsible for laboratory-induced phenotypic behaviour was considered to be unlikely based on the mechanistic

biology of Cas9-based gene drives. Hence the participants were unable to identify any harmful pathways due to the altered genetic diversity of the GMMs.

The participants also were unable to identify any adverse effects that may occur following a reduction in the efficacy of the GMM-mediated trait or any adverse effects arising from not achieving the quality or number of released GMM needed to achieve intended vector or disease outcomes. This hazard in particular seems more directed at Sterile Insect Technology (SIT), as they did not foresee any difficulty in achieving the relatively small number of transgenic mosquitoes necessary for the field trial, and they could not see how failure of the construct in the field trial would result in the GMM being able to transmit malaria better than wild-type mosquitoes.

Interactions of GMMs with non-target organisms, including horizontal gene transfer

The literature identifies horizontal gene transfer as a potential mechanism leading to adverse effects on non-target organisms. This mechanism was similarly invoked in several of the pathways to harm identified by the workshop participants, so these two separate EFSA risks areas are combined here. The literature identifies a further 5 hazards under this combined category:

13. Adverse effects on insectivorous vertebrates due to toxins or allergens associated with the GMM.
14. Change in the abundance or species composition of pollinators and the pollination service they provide, or changes in other ecosystem services such as decomposition of organic matter, nutrient cycling, water regulation and purification (e.g., reduced larval consumption of algae causing levels of algae to increase and their associated toxins produced from algal bloom).
15. Reduction in the abundance (or composition) of species of ecological, economic, cultural and/or social importance through competitive release if the GMM population is reduced, or from trophic consequences of species that rely on mosquitoes for food at specific times of the year.
16. Adverse effects on the reproduction of non-target organisms through sterility or mutation.
17. Potential adverse effects arising from the exchange of genetic information between GMMs and symbionts/parasites associated with them

The literature also highlights the possibility of horizontal gene transfer to micro-organisms, noting that *a priori* this could be expected to be more likely than horizontal transfer to other insects (or

eukaryotes more generally), and in this context poses the following questions: (i) do transgenes contain components that could confer a selective advantage to micro-organisms with which the GMMs will interact; and, (ii) are there any undesirable consequences should the transgene persist in the ecosystem?

Implausible hazards

Hazards 13 to 17 were all deemed to be implausible by the workshop participants. As noted previously the construct is not anticipated to produce toxic or allergenic substances in the mosquito salivary glands (or elsewhere), and the introgression of the construct into wild-type mosquitoes is not anticipated to cause any significant change in the abundance, fitness parameters or behaviour of mosquitoes.

Harmful outcomes due to horizontal gene transfer to prokaryotes or other eukaryotes, including adverse effects on reproduction of non-target organisms, or other harmful effects following the transfer of genetic information to the symbionts and parasites associated with GMMs, were also deemed to be implausible for several reasons:

- the UCMI team recently completed an intensive review of published materials and found evidence for a few documented examples of HGT between prokaryotes and eukaryotes supporting the conclusion that is rare. Over evolutionary time frames, HGT from *Wolbachia* to their hosts has been documented in *Aedes* mosquitoes (Klasson *et. al*., 2009), but the team found only one report of horizontal transfer occurring in the opposite direction, that is transfer of latrotoxin genes from spiders to their bacterial endosymbionts (Bing, *et. al.,* 2020). However, the original citation for this report states that "it appears likely that the spider latrotoxins were acquired via lateral transfer from a bacterial endosymbiont" (Zhang *et. al.,* 2012).
- even if the construct was transferred to prokaryotes it is highly unlikely to be functional because the transgene promoters are exogenous, eukaryotic and highly divergent.
- the team has conducted a search in every eukaryote genome that has been sequenced to-date for DNA sequences that are identical or complementary to the guide-RNA used in their construct and did not find any (Carballar-Lejarazú *et al*., 2020).

It is worth noting at this point that similar reviews of published materials conducted previously by CSIRO have identified other examples of eukaryote to prokaryote gene transfer over evolutionary time scales. For example, Le *et. al*., (2012), cite an apparent example of horizontal gene transfer from *Ae. aegypti* to *Wolbachia* (these authors also cite a plant-to-bacteria example), and Duplouy *et.*

al., (2013) report two possible cases of gene transfer from a variety of possible insect sources to *Wolbachia*, concluding that this, and other cases previously reported, "raises the possibility that *Wolbachia* genomes are able to receive, harbour, transfer, and possibly use protein coding genes of eukaryotic origin". Gabaldón (2020) also provides a recent review of this topic.

Impacts of techniques used for the management of GMMs

The literature does not identify specific hazards in this risk area but poses the following situations, initiating events or questions and asks if these may lead to adverse outcomes**:**

- Resource usage and waste production of GMM production facilities
- What are the implications of a potential reduction in conventional vector control to mosquito population dynamics, humans health and the wider environment, including altered management and control measures of other (secondary) vector or pest species that arise as a consequence of the control of the primary vector or pest species?
- Could changes in land management in the receiving environment (e.g., wetland drainage, irrigation practices), exploitation of environmental resources or use of different control/recovery systems occur as a result of the introduction GMMs?
- Management responses to reduced efficacy of GMMs.
- What are the potential impacts of program activities in the release site related to mosquito surveillance and trapping?

No pathways to harm were identified by the workshop participants after considering these issues. The participants noted that there could be some increase in vector monitoring activities, but parasite monitoring activities would remain largely unchanged, and the anticipated reduction in insecticide use (if the trial was successful) could have positive environmental outcomes.

Evolutionary and stability considerations

Here the literature again poses situations, initiating events or questions and asks if these may lead to adverse outcomes:

- Is the phenotype conferred by the modification, including its marker and other expressed genes, if any, consistently expressed after numerous generations of propagation, whilst under environmental selection?
- Is the modification undergoing rearrangement or other mutation at a measurable rate?

- Synergistic genetic interactions and unexpected phenotypic consequences of multiple "stacked' transgenic modifications

Discussions around this risk area identified a second pathway to harm (Figure 2) wherein the Cas-9 endonuclease reliably makes off-target cuts in every generation. Reciprocal chromosomal translocation might then occur at these break points at rates that are higher than baseline rates associated with spontaneous mutation. This enhanced rate of chromosomal translocations could encourage speciation, and lead to phenotypes with a range of possible adverse characteristics, such as increased vectorial competence or vectorial capacity.

Non-homologous end-joining repair of off-target, CRISPR/Cas-9 double stranded DNA breaks are known to give rise to chromosomal rearrangements such as deletions, inversions and translocations (Cho *et al*., 2014). These types of rearrangements can present strong barriers to gene flow between populations because they reduce recombination in heterokaryotypes, and hence encourage reproductive isolation and speciation (Navarro and Barton, 2003). However, the extent to which this speciation process may lead to phenotypes with undesirable attributes is unknown and hypothetical at this stage. It is also unknown how the scale of such effects would compare to rearrangements events generated by normally acting genetic processes that lead to speciation.

Further discussion around synergistic genetic interactions did not identify a specific pathway to harm but the workshop participants noted that it is theoretically possible for different constructs to interact, for example through template switching due to target sequence homology between the two, and hence in practice different research groups may need to consider ways to design orthogonal drives that are not able to interact.

DISCUSSION

The academic literature and reports from respected international bodies identify 17 hazards together with a number of associated situations and initiating events, that may lead to adverse outcomes on human health and environmental values following the production, release, reliance on and long-term use of genetic control of mosquito vectors. Informed by this information, and the outcomes of a series of hazard identification workshops, this analysis identifies 3 pathways to harm for a hypothetical release of genetic construct designed to make mosquitoes refractory to the *Plasmodium* parasites that cause human malaria. The presentation of these pathways emphasises the weight of evidence that supports each step, distinguishing well-established relationships from hypothetical ones, and attempts to identify the most cost-effective, practical or safest point in the

pathway to gather laboratory or field-based observations to test risk hypothesis of no harm and support subsequent risk assessment calculations.

Three important choices must be made before and during this type of analysis. The first pertains to the problem at hand and the range of solutions that are available for solving it. The second choice pertains to the risk assessment endpoints and the choice of environmental values that are deemed to be important and worth protecting. The third pertains to the choice of risk hypotheses that are deemed important enough to carry through to the risk-calculation stage versus those that are not. It is essential that the stakeholders and communities that stand to lose or benefit from any proposed solution to a given problem are involved in all of these choices (Nelson *et. al.,* 2004; Stirling *et. al,* 2019), and indeed the UCMI model for engagement requires that these communities make these choices (Kormos, et al. 2021).

Our problem of interest is the on-going burden of malaria in sub-Saharan Africa (WHO, 2018), but we focus here on only one possible solution: the use of a genetic control technique that in theory can modify mosquito populations so that they are refractory to the main malaria-causing parasite *Plasmodium falciparum* (Carballar-Lejarazú and James, 2017). This analysis has not canvassed the opinions of scientists or stakeholders on the viability or attractiveness of other solutions, but studies that have addressed this issue in Africa suggest that community members, policy makers and regulators are generally supportive of genetic control techniques whereas scientists tend to be more sceptical (Okorie *et. al.,* 2014, Finda, *et. al.,* 2020).

The analysis reported here was not conducted with, or informed by, any formal stakeholder engagement activities. While the technology described in the release scenarios in this document is at a relatively advanced stage of discovery, and identification and consultation with relevant community groups and stakeholders is underway, the endpoint definitions and distinctions between plausible versus implausible pathways reflect the judgement, beliefs, and values of the research team who participated in the workshops. UCMI will use these as a guide to inform future work, however the final process for risk assessment will be determined by the appropriate authorities and communities at the UCMI field sites.

Nonetheless, African community engagement activities independent of this assessment have taken place (Finda *et. al*., 2021), and there is overlap among the concerns expressed in these community consultations and the issues addressed here, for example around the possibility of an increase in disease transmission. A potentially important point of difference is the possibility of HGT causing adverse impacts to humans. Harmful outcomes due to HGT in this analysis are deemed implausible

whereas the community expressed concerns about the genetic modification transferring to humans through biting.

A general analysis such as the one here is likely to be adapted by stakeholders to reflect local values and social practices. Thoughtful and intentional engagement with stakeholders, as pointed out above, should occur to ensure that the perceived risks (concerns) of local people are seriously considered and addressed. Perceived risks that may not be likely to eventuate, are legitimate social risks that may affect the way that the stakeholders and community members view the project and will affect their behaviour and decisions. These considerations provide an opportunity to recalibrate and adapt the hazard analysis to new endpoints while documenting the rationale for their inclusion relative to the current characterisation.

All hazard analysis exercises must discern plausible and implausible pathways to keep the overall risk assessment tractable. The judgement of plausibility is often (even if implicitly) based on the perceived probability of an event, whereas probability is not (at least explicitly) considered until the subsequent risk calculation step. These types of exercises may therefore be criticised as qualitative risk assessment masquerading as hazard analysis; such criticism is valid to the extent that hazard analysis is a qualitative process that supports the structured, articulated and defensible development of a quantitative risk assessment. Ultimately, any risk-based decision process requires a judgement to be made at this point based on the best currently-available evidence, in a way that is sensitive to the concerns of the relevant stakeholders.

A related criticism that might then be raised at this juncture is that any subsequent analysis will be incomplete because the list of possible hazards is infinite, whereas the list of hazards carried through to the risk calculation stage must be finite. However, this argument cannot be used to support or prevent any specific proposal because the same criticism can be levelled at a risk assessment performed for any alternative proposal (Kaplan and Garrick, 1981), or indeed to an assessment of the risks associated with “do nothing” or “business as usual” options.

A possible solution proposed by Kaplan and Garrick (1981) is to consider an “other” risk hypothesis that includes all the hazards that are not yet thought of. By monitoring outcomes during the operating experience, the likelihood of any individual outcome within this aggregated “other” group can be calculated, including in those situations where no adverse outcomes are observed, using the methods for the so-called zero-numerator problem, reviewed by Hayes *et. al*., (2015), and an appropriate statistical model for the potentially relevant observations.

A staged-release strategy, with genetic and geographical containment, lends itself to this approach by providing an opportunity for operating experience to grow in a contained and thereby safer

manner. However, it is important to recognise that this approach assumes that adverse outcomes (caused by the release) that were not previously thought of would be detected by post-release monitoring activities if they did in fact occur. This may not be true for a variety of reasons, and even if it were, such a detection would be fortuitous because the outcomes in this "other" group would not have contributed to the objectives of any post-release monitoring design.

In an entirely novel situation, such as the release of an unlimited gene-drive product for which there is no directly relevant operating experience, the problem of a possibly incomplete hazard analysis, and hence by implication risk assessment, can never be eliminated entirely. Society accepts this in order to enjoy the benefits of novel technology. The best we can do is a careful, systematic and rigorous hazard analysis that comprehensively addresses known concerns expressed by scientists, regulators and stakeholders. This is one reason why staged-release strategies that provide relevant field-based observations are so important, and why hazard analysis based on checklists such as those generated here from the literature, should ideally be complemented by other methods that are designed to help proponents imagine how adverse outcomes might occur.

The pathways to harm analysis conducted here should thus be viewed as the first step in an iterative process of desk-top based analysis and modelling, followed by limited field release and observation, together with formal engagement and collaboration with stakeholders, and potentially complemented by additional hazard identification methodologies. The field and laboratory tests identified in this analysis should also be viewed as an ideal minimum set, that does not preclude additional tests and experiments.

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Fig 1a Increase in malaria due to emergence of target pathogens with transmission advantage

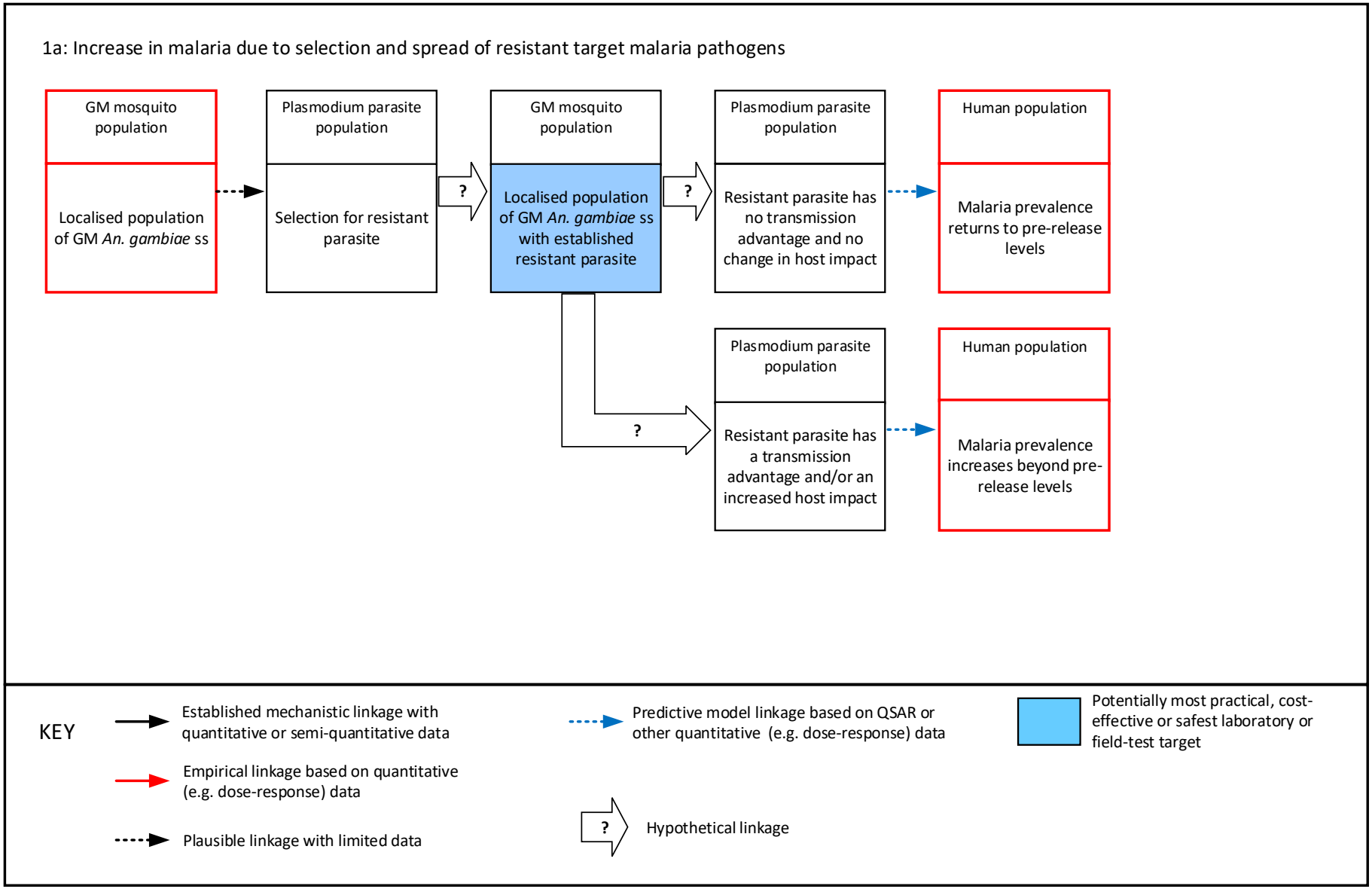

Fig 1b Increase in vectorial capacity of not-target pathogens

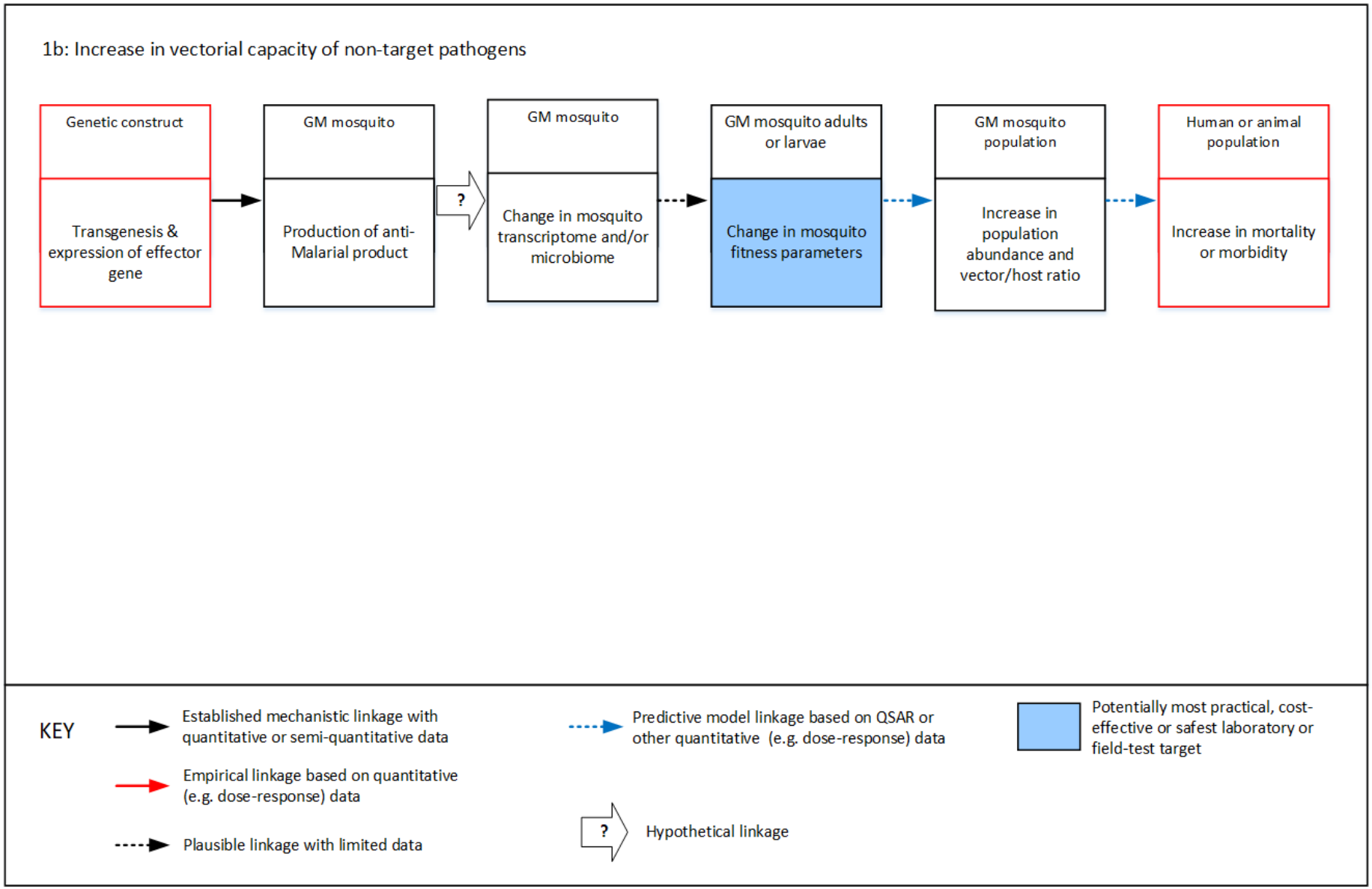

Fig 2 Emergence of new mosquito phenotype through enhanced chromosomal translocation.