Mortality, Malaria, and Mosquitoes: How CRISPR-Cas9 Gene Drive Technologies Can Reduce rates of IUGR in Africa

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Intrauterine Growth Restriction (IUGR) and low birth weight caused by Malaria can lead to neonatal death and future health complications and thus certain measures need to be taken regarding the spread malaria in order to reduce the prevalence of IUGR in Africa. There are many strategies to reduce the spread of malaria in developing countries such as some of the methods employed in Africa, however none seem to be having much of an effect. CRISPR-Cas9 gene drive technology aims to eradicate the female *Anopholes* population by introducing infertile female *Anophoeles* into the population in an effort to limit the female *Anopholes* population. Hence, using the CRISPR-Cas9 gene drive technology as a method to reduce the female *Anopholes* mosquito population in Africa, should provide a superior approach to reducing rates of IUGR relative to intermittent preventive treatment and insecticide treated nets.

Biological Mechanisms of Malaria and IUGR

Malaria is often a fatal disease caused by Plasmodium parasites that are transmitted by female *Anopheles* mosquitos (WHO, 2016). Once a human is infected with the parasites, the body responds with a fever, headaches, vomiting, muscle and joint pains, or the chills within 7-28 days (WHO, 2016). Some more dangerous symptoms include anaemia, respiratory illness, and in adults it is common for many organs to be affected and hypoglycemia or cerebral malaria to result , which are very dangerous as they block blood flow to the brain (WHO, 2016). The Plasmodium parasite is a single-celled organism and is injected into the body by the female *Anopheles* mosquito and finds itself to the liver where it can multiply and an alarming rate at approximately 10 000 times (WHO, 2016). The parasite then enters the blood streams thus infecting red blood cells. It should be noted that there are multiple types of malaria all caused by different types of Plasmodium parasites: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, and Plasmodium malariae*, but all transmitted through the Anopheles mosquito (WHO, 2016). However, *Plasmodium falciparum* is the most dangerous as it results in the most number of deaths (WHO, 2016). This is a very serious problem as it is very easy to get bitten by the anopheles mosquito in countries in Sub-Saharan Africa, and also where they have limited medical treatment.

Typically, malaria can only be spread by being bitten by a female *Anopholes* mosquito that contains the parasite. There is an exception to this rule, congenital malaria (Fried, 1998). This occurs when a pregnant mother with malaria gives it to her unborn baby (Fried, 1998). This is extremely dangerous for both the mother and child as being pregnant increases the severity of malaria. Due to the increased number of steroids in a pregnant woman's body they are more prone to be bitten by the *Anopheles* mosquito as about 6 million women are at risk of being infected with malaria during pregnancy (Okpere et al, 2010).

The primary consequences of pregnant women developing malaria is maternal anaemia and lower birth weight of the baby (Menendez, 2000). This low birth weight is brought on by intrauterine growth restriction (IUGR) and is when a fetus's growth is stunted due to s poor maternal nutrition, or low oxygen to the fetus (Sankaran, 2009). Fetuses are classified as having IUGR when their weight is in the bottom 10^{th} percentile for their gestational age (Sankaran, 2009). It is very serious as about 60% of all newborn deaths in the world are due to low birth weight and it leads to premature births, stillbirths, and long-term health problems (Sankaran, 2009). There are a couple of possible reasons for this, Malaria-induced anemia and placental infection. Placental infection occurs when the Plasmodium parasites interfere with blood circulation in the placenta resulting in reduced efficiency of transporting nutrients to the fetus (Fried, 1998). A study found that acute and chronic placental infection was significantly associated with IUGR related low birth weight ($p<0.05$) (Fried, 1998). In this same study about 20% of children born in a highly malaria-endemic area of Tanzania had developed IUGR leading to low birth weight, and about 20% of these individuals were born premature (Fried, 1998). It was found that that a malaria-infected mother had more than 3 times the risk of giving birth prematurely. (Fried, 1998) Their findings indicated the development of a disease called massive chronic intervillositis where there is a very large reduction in birth weight. This disease can result in placental lesions damage the placental villi thus reducing nutrient transfer to the fetus and can be identified by the type of cytokines that they release (Fried, 1998). Cytokines are molecules secreted by cells, typically immune cells, to have a desired effect on other cells. Some cytokines can be beneficial for pregnancies while others are very dangerous and can stunt the growth of a developing fetus (Fried, 1998). Cytokines such as IFN-y and IL-2 were absent in healthy placentas but were prevalent in

placentas in mothers with malaria. TNF- alpha and TGF- beta were prevalent in both but much more in malaria-infected mothers, and cytokine IL-10 is much more prevalent in healthy mothers (Fried, 1998).

Essentially in pregnant women malaria is much more dangerous and this is because the plasmodium parasite is capable of attaching to the trophoblastic villous epithelium, an important part of nutrient delivery in the placenta (Sanaia, 2013). Its attachment is facilitated by chondroitin sulfate A (CSA) (Sanaia, 2013). This is particularly concerning for first-time mothers who do not have any immunological experience with parasites that bind to CSA as studies have found that mothers become more and more susceptible to these CSA-binding parasites with each successive pregnancy (Sanaia, 2013). Placental concentration of IFN-y and TNF-alpha were significantly higher in first-time pregnant mothers giving birth to low birth weight babies compared to normal weight babies ($p=0.0075$) (Fried, 1998). Additionally, researchers found that there were significantly higher concentrations of IFN-y and TNF-alpha in lower weight placentas meaning that low placental weight also contributes to low birth weight (Fried, 1998). Additionally maternal anemia brought on by malaria was significantly associated with low birth weight when TNF-alpha was detected in the placenta ($p<0.05$) (Fried, 1998). TNF-alpha was more prevalent in anemic mothers suggesting that concentration of TNF-alpha may be associated with maternal anemia (Fried, 1998). Therefore, the identification of cytokines provides evidence of the effect that malaria has on the occurrence of IUGR and low birth weight in pregnant mothers.

IUGR can lead to preterm births that result in stillbirths or babies being born with low birth weights. About 14% of births in sub-Saharan Africa result in low-birth weight babies (AHO, 2014). A study found that in developing countries babies born with low birth weights have a much higher risk of mortality compared to babies born at healthy weights (Sanaia, 2013). They found that 26% of neonatal mortality can be attributed to IUGR induced low birth weight (Sanaia, 2013). Now even if a baby survives into childhood it is not free from the long term risks associated with being born with a low birth weight caused by IUGR. This can lead to multiple long-term problems such as heart disease, diabetes, and high blood pressure. If the conditions inside the womb or the nutrients being delivered to the fetus, the fetus will adapt so it can optimize its chances to survive in that environment (Godfrey, 2002). With these placental lesions preventing efficient nutrient

transfer it is an enormous strain on the fetus and thus it must adapt and this can result in epigenetic changes that will permanently affect the fetus throughout the rest of its life (Godfrey, 2002). These changes can be passed down to subsequent generations (Godfrey, 2002). Some of these changes can include reduced insulin secretion, increased gluconeogenesis, reduced insulin sensitivity, and increased plaque formation (Martin-Gronert, 2007). These together can lead to a low birth rate ultimately leading to impaired glucose tolerance and Type 2 diabetes in adulthood (Martin-Gronert, 2007). A study found that 26% of men born with low birth weight had glucose intolerance and 17% had diabetes, while none of the men born at a healthy weight had diabetes and only 13% had glucose intolerance (Barker, 2005). Thus this suggests that these epigenetic changes that occur during fetal development can lead to long-term health complications.

Therefore there has been evidence that has shown a clear association with Malaria and IUGR along with the risks of developing IUGR. The World Health Organization (WHO) has put forward guidelines to prevent malaria-associated pregnancies which have been implemented into policies in majority of sub-Saharan countries. Yet, these strategies to battle the spread of Malaria in pregnant women exhibit varying success.

Existing Interventions Recommended by the World Health Organization

The safest interventions based on efficacy that is currently recommended by WHO to control for malaria-associated pregnancy in sub-Saharan Africa are intermittent preventive treatment in pregnancy (IPTp), insecticide-treated nets (ITN), combined with malaria case management (World Health Organization [WHO], 2015).

IPTp includes the administration of sulfadoxine-pyrimethamine (SP-IPTp), an antimalarial drug used to treat and prevent parasitic infection by *Plasmodium falciparum,* to all primigravidae mothers regardless if they are infected or not. Dosages are routinely given at antenatal care visits and is administered in the second and third trimester given that SP is dispensed one month apart (Hill et al. 2015). It has been standardized that two doses effectively reduce the incidence of low birth weight. However, all pregnant women, should *ideally* be given at least three doses during pregnancy (WHO, 2015). Based on a meta-analysis conducted on seven trials in sub-Saharan African countries such as Malawi, Mali, Kenya, Zambia, Burkino Faso, three doses compared to two doses demonstrated a 56-gram increase in newborns (Kayentao et al., 2013). By adhering to

three dose regimen, newborn with lower birthweight decreased by 20%, placental malaria infection declined by 50% and hemoglobin levels in mothers increased. Reasons why sulfadoxine-pyrimethamine is administered in the second and third trimester is that fetal growth is critical during the final 6 -10 weeks of pregnancy (Kayentao et al., 2013). Folic acid supplementation is also administered as part of antenatal care to treat anemia associated with malaria. A low dose (0.4 mg) is recommended in combination with SP due to its effects on the efficacy of antimalarial drugs (WHO, 2015).

Insecticide treated nets are also provided by antenatal care (ANC) clinics, child care hospitals, voucher systems as well as sold at pharmacies and stores in areas of moderate to high transmission of malaria (Hill et al., 2015). In African countries such as Kenya and Mali that has implemented WHO recommendations as a policy, ITNs are mostly provided to women for free. Furthermore, net protection while sleeping correlates with a significant decrease in placental malaria infections, fewer stillbirths and a decreased number of newborn with low birthweight (Singh, Brown and Robertson, 2013).

An important factor in deciding on the correct intervention strategy involves its economic value. The intervention provided by WHO have proven to be economically inexpensive which is why it is commonly used in sub-Saharan Africa. For example, for a thousand pregnant women, SP-IPTp and ITNs cost approximately US $13.17 in the rural community of Mozambique. (Securi et al., 2010). A major reason why this is cheap is because antenatal clinic services/established health care systems, provide these interventions decreasing the cost.

Barriers to IPTp and ITNs in sub-Saharan Africa

However, this intervention does have inadequate coverage in malaria prevalent areas. For example, a study conducted on 27 sub-Saharan African countries from 2009 to 2011 indicate that although the coverage of antenatal care visits was 84.6%, the two dose administration of SP was low at 27% (Hill et al., 2013). To add, despite the fact that 96% of sub-Saharan Africa have standard policy to distribute ITNs, coverage was discovered to be 17% of women who were pregnant (O'Meara, Smith, Ekal, Cole, and Ndege, 2011). Therefore, it is evident that there are barriers to accessing this intervention.

This was studied by Hills et al. (2013) who conducted a meta-analysis on twenty intervention studies in sub-Saharan Africa from 1990 to 2013. It was observed that the amount of visits made to antenatal clinics, awareness/knowledge of the effects of IPTp, socioeconomic status and usage of ITNs are a few of the many determinants that affect access to interventions. Barriers included lack of knowledge of malaria associated pregnancy and SP as some mothers developed the notions that SP was a chemical that would be detrimental to the health of the fetus. Environmental barriers prevent the efficiency of distributing IPTp as well since health care facilities are not physically prevalent in remote communities limiting the access to dosage of IPTp-SP to women in rural areas who commit to farming. Culturally, marriage plays a role as attending antenatal clinics are dependent on the husband's financial support and decision making skills for transportation as seen in cases stemming from Nigeria (Hill et al., 2013). In the health care facilities, low supply of IPTp and cups used for direct observed therapy (DOT) along with reduced clinic hours of availability can lead to lengthy waiting periods and increased patient/healthcare provider ratio thus limiting access to IPTp. Many women complained that the health care clinics charged user fees for SP or was charged for attending clinics for the first time late in their pregnancy. It was reported that many healthcare professionals treated women unprofessionally and were confused with the specific period at which to administer the two dosages of SP (Hill et al., 2013).

Insecticide treated nets, an important tool used for protection from mosquitoes, was not being used even if owned. It was also found that government policy for free dispense of ITNs was ineffective for the target demographic, pregnant women. For example, a study conducted in rural areas of Kenya concluded that pregnant women who are qualified for the free intervention packages at antenatal care clinics are as likely to own ITNs as compared to those without pregnant women residing in the household (O'Meara et al., 2011). Low stock of ITNs in clinics, cost, low financial support from husbands and seasonal changes (risk of mosquito prevalence) are additional factors that contribute to the low spatial coverage of ITNs in sub-Saharan Africa (Hill et al., 2013).

Resistance to Antimalarial Drugs: Increasing Concern for IPTp

The main issue with using antimalarial medication to control for malaria-associated pregnancy is mosquito drug resistance. *P. falciparum* have developed modified dihydrofolate

reductase (sextuple mutations) and dihydropteroate synthetase (quintuple mutations) which are connected to the resistance of sulfadoxine-pyrimethamine (Muanda et al., 2015). A meta-analysis published in 2007 indicate that the IPTp including SP administration in resistance areas such as Malawi, Mozambique, Mali and Kenya is consistently beneficial. More specifically, their analysis on four studies with an SP resistance between the limited ranges of 19%-26% were tested for the effect of two dose regimen of SP in primigravidae women and concluded that despite these mutant mosquitoes, IPTp does reduce the incidence of abnormal fetal consequences despite the varying levels of resistance to antimalarial drugs (ter Kuile, van Eijk and Filler, 2007). On the other hand, a recent meta-analysis found that in Eastern Africa where resistance was 50%, the protective effects of SP was not correlated with a decline in newborns with low birthweight. (Muanda et al., 2015). Even in high grade resistant areas such as western Uganda, IPTp were not connected to decreases in low birthweight, anemia and decrease in placental malaria infection with *P. falciparum* (Braun et al., 2015). ACT (artemisinin-based combination therapies) has been recommended over single drug therapies such as sulfadoxine-pyrimethamine, chloroquine and amodiaquine in areas expressing a severe increase in quintuple/sextuple mutations. However, parasites are already developing a resistance to these artemisinin-based compounds, an alternative drug used in resistant areas (WHO, 2015).

Eliminating the Female Mosquito and Its Effect on IPTp and ITN Coverage

The potential of new parasitic strains that can acquire mutations to alternative medication is unattractive. Moreover, considering the barriers to ITN/ IPTp-SP treatment access, one might question the economic value and long term consequences with continuing a policy/ health programs the facilitates free distribution of treatments. In a macroscopic sense, IUGR associated with *P. falciparum* infection would be resolved if malaria transmission experienced a crash overall. Decreasing the prevalence of female mosquito carriers in sub-Saharan Africa, diminishes the chances of physical contact with pregnant women ultimately decreasing the need to buy and distribute nets. This would also indirectly relieve the individual burden to travel long distances to consequently be charged for antimalarial drugs. Furthermore, women would not be inconvenienced by low stock of SP in health care facilities as overtime, the patients who would need antimalarial drugs would decline. There would be less need to displace community volunteers to deliver

packages in rural communities and countries would observe different allocation of government spending into advertising, educating and training the population on other important diseases such as HIV. In addition, there would be lower cases of resistant parasites in the placenta and this would subsequently reduce the time and money into constantly synthesizing and manufacturing new drugs to counteract new resistant phenotypes.

Therefore, by eliminating the vector, the female *Anopheles* mosquito, the barriers connected to existing recommendations with antimalarial drugs and ITNs would shrink. CRISPR-Cas9, a prospective technological method, would do just this; eliminate a species in hope of a long term solution with malaria.

What is CRISPR-Cas9 and Gene Drive?

Before providing recommendations on utilizing the CRISPR-Cas9 gene drive mechanism on mosquitoes, the CRISPR-Cas9 system and gene drive must be explained in order to provide background into how this method will reduce the prevalence of malaria in Africa, thus leading to reduced rates of intrauterine growth restriction (IUGR) in Africa.

An Explanation of the CRISPR-Cas9 System

The use of Cas9 RNA-guided endonucleases as a technique in genome editing has come from knowledge of CRISPR (clustered regularly interspaced short palindromic repeats) which is a part of the immune system used in archaea and bacteria against viruses (Hsu, Lander, and Zhang, 2014). The CRISPR-Cas9 system involves Cas9 (an endonuclease), along with a short RNA guide which in terms of the bacterial immune system, is synthesized from stored viral DNA that the bacteria integrates in a long string of DNA called a CRISPR locus (Mali et al., 2013). CRISPR-associated enzymes are responsible for obtaining the spacers from viral protospacer sequences that are then integrated in the CRISPR locus (Hsu et al., 2014). The guide RNA is transcribed by using the CRISPR array (where most of the spacers from the viral DNA are stored) as a template (Hsu et al., 2014). The CRISPR RNAs act as guides for the Cas9 proteins which find complementary DNA sequences associated with active bacterial viruses (Mali et al., 2013). Once Cas9 locates the specific DNA sequence via the RNA guide, base-pairing occurs between the RNA guide and the DNA target that is located, in order to ensure that they complement each other (Hsu et al., 2014). The binding of a protospacer-adjacent motif (PAM) further downstream the target

sequence aids in facilitating a DNA double-stranded break (Hsu et al., 2014). Cas9 then inserts into the break and cleaves the target DNA sequence, leading to natural repair mechanisms to compensate for the removal; usually through homology directed repair (HDR) (Hammond et al., 2016).

In terms of the use of CRISPR-Cas9 in genetic editing/engineering, the system works in similar ways in terms of the function of CRISPR in bacteria. The short guide RNA is naturally complementary with phage DNA but can be introduced/altered into an RNA guide that can cause the retargeting of Cas9 to cut out any desired DNA sequence/gene in different species and organisms (Hammond et al., 2016; Ran et al., 2013). Multiplex targeting of various sequences can now be initiated by inserting a series of short guide RNAs after which Cas9 will knockout the gene associated with a targeted DNA sequence (Hammond et al., 2016). With the CRISPR-Cas9 system, scientists can now also insert desired gene sequences into the genome as well as modify a single nucleotide base in a DNA sequence (Ran et al., 2013). Through HDR, after the targeted DNA sequence is cut using Cas9, the introduction of an exogenous DNA repair template can make changes to the genome (Ran et al., 2013). The delivery of Cas9 and the engineered RNA guides in various species or individual organisms can be made through many viral (adeno-associated virus, lentivirus, etc.) and non-viral methods (Hsu et al., 2014).

How do Gene Drives Work?

Gene drives are natural genetic systems/mechanisms where a particular gene is more likely to be promoted/passed down and spread throughout generations unlike in sexual reproduction (Mendelian genetics) where the chances of passing on a particular gene have a 50% chance (Sinkins and Gould, 2006). Natural gene drives are the result of endonuclease activity such as from homing endonuclease genes (HEGs) or RNA-guided endonucleases (Hammond et al., 2016). For example, HEGs (in single celled organisms) are found in the DNA recognition sequence and can recognize as well as cleave a 15 to 30 base pair DNA sequence (Hammond et al., 2016). When a HEG is associated with a chromosome which has an uninterrupted DNA recognition sequence, there is a double strand break due to the HEG cleaving the chromosome that does not have the gene drive (Hammond et al., 2016). The break is repaired using the homologous chromosome which results in both chromosomes having the gene drive (the offspring becomes homozygous),

increasing the frequency of the gene in the population (Hammond et al., 2016). In summary, endonuclease gene drives cut chromosomes that do not have the gene drive at a particular location, and then the damage is repaired by the homologous gene drive sequence. Any endonuclease such as Cas9 from the CRISPR-Cas9 system can be redesigned as a gene drive system to target or insert specific genes in insect vectors in order to combat vector-borne illnesses.

Combining CRISPR-Cas9 and Gene Drive

The CRISPR-Cas9 gene drive is precise in gene editing, simple, flexible, and cost effective to use, making it better than previously known gene drive technology (Webber, Raghu, and Edwards, 2015). When the CRISPR-Cas9 system is integrated in a species as part of a gene drive, a mutation or edit that was made by Cas9 and the guide RNA on a genome sequence will make copies of itself (Webber et al., 2015). This will cause heterozygous individuals for the selected mutation/gene to be changed into homozygous individuals for that trait (Webber et al., 2015). The genes for the Cas9 endonuclease and the guide RNA must be included in an organism's genome along with an altered version of the targeted gene in order for the gene drive to take place in subsequent generations (Harvard University, 2015). When mating occurs, the Cas9 protein with the help of the guide RNA will cleave the targeted gene in the other mate's chromosomes, causing the repair mechanism to copy the gene drive to the mate's chromosomes (Harvard University, 2015). This will result in offspring inheriting the altered trait as well as the gene drive (Harvard University, 2015). For some applications of gene drive technology, the focus of the CRISPR-Cas9 gene drive would be to ensure that a deleterious trait is present within an invasive species or a vector species, and to release a certain amount of these species in order for the engineered mutation to spread in the population through the gene drive mechanism (Webber et al., 2015). For the CRISPR-Cas9 gene drive to be applied successfully, the organism that the technology is applied to must have short generation spans (Webber et al., 2015).

How does CRISPR-Cas9 and Gene Drive Relate to Mosquitoes and Malaria?

Since its emergence, CRISPR (clustered Regularly Interspaced Short Palindromic Repeats) Cas9, has become a powerful technology tool for genome editing and is now widely used in basic biomedical research to explore different gene functions. Recently, this technology has been applied to the study and treatment of certain human diseases such as Barth syndrome, Duchenne muscular

dystrophy, hemophilia and cystic fibrosis (Cai, Fischer, Huang, & Xie, 2016). It's ability to modify the genome yields to its powerful utility in correcting disease-causing DNA mutations ranging from a single base pair to large deletions in model systems. The application of this CRISPR system has recently been proposed as a potential strategy for the treatment of malaria, through the genetic modification of the *Plasmodium falciparum* species, which fosters the main vector in malaria transmission.

Malaria

Malaria affects 300-500 million people worldwide and causes 1-2 million deaths annually on average. It's limitations to treatment center largely around drug resistance, limitations in the ability to store and distribute drugs and vaccines, as well as the poverty level of many of the vulnerable populations such as Africa (Hammond., et al, 2015) . In areas where the eradication of malaria has been reported, it has largely been achieved through mediated local control of the mosquito vector population. Notably, malaria caused by blood-borne protozoan parasites of the genus *Plasmodium,* with *Plasmodium falciparum* being the most costly species in terms of its effects on both human life and economic progress, has largely been the main target. Specifically, mosquitoes of the genus *Anopheles* function as vectors for the *Plasmodium* parasite and are crucial for disease transmission. Since the discovery by Ronald Ross (1897) that malaria transmission occurred through the transmission of a vector found in malaria, vector-based malaria control has provided an alternative promising route for disease treatment as the course of malaria infection is highly dependent on the lifecycle of the mosquitos (Lee & Fiddock, 2014).

Controlling Malaria via Vector Control Strategies

Vector control strategies have been designed primarily to reduce the mosquito population, with several others seeking alternate solutions to render the mosquito vector less competent to transmit malaria. Such control methods encompass environment management (involves limiting the mosquito population through draining of neighboring wetlands, removing potential breeding habitats as well as through the use of larvivorous fish), insecticide treatments (utilizes microbials and fungal treatments to reduce insect population) and through molecular entomology approaches (Zamanian & Andersen, 2016) .

While many of these approaches have reduced/been able to control the growing mosquito population and therefore reduce occurrences of malaria – as illustrated in Rome, India, Brazil, Egypt and Zambia – they face many challenges. One of the biological challenges is that at least 150 different mosquitoes have been identified as malaria vectors, making genetic modifications quite difficult. Moreover, the vectors developed are not immune to the development of *Plasmodium* resistance, and thus multiple anti-*Plasmodium* genes would be required to prevent this from occurring. Thus, new emerging technologies are needed to combat the growing concern surrounding malaria (Gantz et al., 2015).

The use of CRISPR-Cas9 as a Mechanism of Treating Malaria

To this degree the use of the new CRISPR-Cas9 system has provided an alternative route to disease treatment and could in turn help reduce rates of IUGR due to the implications malaria may have on the placenta throughout the course of fetus development. Because the CRISPR-Cas9 tool is made of DNA, it is possible to use CRISPR to insert *itself* into the target organism. This creates what is known as a "perpetual-motion machine for gene editing" in that whenever the cells divide, the CRISPR-Cas9 tool emerges spliced into each genome, and whatever genome is selected by the researchers remains. Hence, a genetic sequence can be inserted into a wild type DNA sequence of a mosquito with which it is paired. The "gene drive" proposal comes from the fact that if the genes function as expected, every descendent of the mosquito will possess the genotypic or phenotypic trait conferred by the inserted sequence. Such an idea confers high applicability to mosquitoes as their short generation time allows for rapid genetic changes from generation to generation allowing a particular gene mutation or trait to spread very quickly (Zamanian & Andersen, 2016).

A group at the imperial college of London identified three genes (*AGAP005958*, *AGAP011377* , *AGAP007280*) that confer a recessive female-sterility phenotype (in the *Anopheles gambiae* type that transmits malaria in sub-Saharan Africa) when disturbed and inserted into a CRISPR-Cas9 gene construct, designed to target and edit each gene (Hammond et al., 2015). This gene was designed using CRISPR technology to inactivate genes involved in egg production in female mosquitoes resulting in a primarily male mosquito population. For each targeted locus, a transmission rate of progeny of 91.4% to 99.6% was observed indicating strong gene drive at the molecular level and the ability of such a vector to modify the mosquito genome. This gene drive

has the potential to spread through wildlife populations, eradicating the female mosquito's species *Plasmodium falciparum* and essential wiping out the entire mosquito species. The elimination of the entire mosquito species would serve to eliminate malaria transmission entirely (Hammond et al., 2015).

Despite being in its early development stages, the notion has sparked much interest amongst many scientists and politicians for its potential to serve as a treatment strategy against malaria. Driving the mosquito population into extinction would not only lower economic burdens associated with controlling and treating malaria but will eliminate the burden of malaria transmission entirely. Specifically, in the context of fetal programming, eliminating malaria could help reduce instances of IUGR in many areas such as Sub-Saharan Africa where it continues to be a cause of infant deaths. Hence, while it still harbours disadvantages and challenges that may need to be addressed, it serves as a newly growing innovation for the potential treatment of not only malaria but other parasitic diseases and its implications could go beyond just treating that single disease as in the case of IUGR. The remainder of the article will go on to discuss the advantages and disadvantages of such a tool in eliminating diseases such as malaria and the implications it has on reducing rates of IUGR in comparison to other current treatments.

Proposed Implementation Method of using CRISPR-Cas9 Modified Mosquitoes in Africa

This section will discuss the hows. Specifically, how to implement a strategy to use CRISPR-Cas9 modified mosquitoes to get rid of mosquitoes in the "best" way possible. However, it is very hard to motivate the individual without first addressing the "why". In brief, the "why" for NASA spending billions of dollars to go on daring quests to space is the same "why" here the argument is to use CRISPR-Cas9 as a new solution to the malaria problems. New advancements and understandings has always brought people more tools to control the future. Just as the bicycle is a tool for going fast physically. The computer as a bicycle for our minds. CRISPR-Cas9 is the bicycle for our species.

It is very evident that releasing mosquitoes carrying a gene drive that would turn all offspring into males would over time drive a species to extinction. This would impose negative externalities on some people. Not everyone will benefit from having a mosquito-less world. So, how do society as a whole help those people, who have "lost", out? This raises another more

difficult question. Who is considered the "losers"? Are families who lose more than 50% of their income due to the eradication of mosquitoes considered to be on the losing side? Does the gain to those families from the decreased risk of contracting malaria, somehow, justify the lost of income? In theory, the people who impose the negative externality should pay those who loses. However in reality, transaction costs are too high. To find and locate the winners and the losers would take tremendous work and high costs. It's almost just as hard to ask the winners to pay to the losers. The benefits of a malaria-less world is defused, and the costs concentrated. The most sensible action is not to aim to make a society equal, with the winners paying the losers, as we will not get equality by aiming for equality.

From a game theory perspective, it matters not whether or not all countries consent to releasing CRISPR-Cas9 modified mosquitoes. We are entering an era of warfare, with CRISPR-Cas9, that is much deadlier than nuclear, and much more evasive than cyber. A war of disastrous consequences could be initiated by rouge organizations rather than rogue nations. If one country, or even for that matter one person, chooses to release these modified mosquitoes, then it would be all over for that species of mosquitoes. This reality is sadly just a matter of time. This is why It may not be wise to wait for all countries in the world to sign a consent form to releasing these modified mosquitoes. Countries almost never agree. Instead, given that these mosquitoes would be released sooner or later, wouldn't it be better to release them sooner rather than later in a controlled setting. We as world citizens have a duty to take protective measures to plan, protect and preserve wild mosquitoes in facilities ahead of time.

This paper proposes a strategy that have a selected set of free nations and some in Africa build bomb proof facilities to house wild type mosquitoes. This project would take several years to build. In the meantime, wild type mosquitoes should be kept in non-bomb proof but secure facilities as a backup. At the same time, nations opted-in in this effort would collectively raise some money and give out grants and hire scientists to come up with hypothesis of the impact that CRISPR-Cas9 modified mosquitoes would have on different facets of life on Earth. From these hypothesis, scientists would then be given funds to set up data collection mechanisms using cameras, probes, etc to test their hypothesis. The idea is to collect as much data as possible. A distributed data centre would be set up in opt-in nations. All collected data would be pooled into

those data centres. These data would then be made freely available to scientist all over the world in real time so that more people can do data analysis on them.

For example, a common foreseeable consequence of eliminating mosquitoes would be its effect on the food web. Specifically, negative consequences such as decreases in crop yields. The next section will focus more on foreseeable consequences. It has been observed that fish in a nearby pond could affect pollination of the surrounding area (Knight et al, 2005). A part of small fish's diet is mosquito larvae. If mosquitoes were absent, then these fish could substitute to something else such as dragonfly larvae. A big part of dragonflies diet is bees. So, the absence of mosquitoes could lead to a decrease in dragonfly population which could lead to more bees and more pollination. This is one hypothesis. The results could go the opposite way. We are not sure, but with cameras, probes and the proper experimental setup, we can find out.

The benefit of this approach is that it is reversible. If it turns out that mosquitoes do play a huge positive role in our ecosystem, then we can reintroduce mosquitoes again because we have back ups. If it turns out that mosquitoes don't play a significant positive role in our ecosystem, then we still didn't lose a species as we have back ups. The other benefit of this approach is the knowledge that we would gain from such an event . This could be informative in many ways such as the impact of losing a species, how species are interconnected, who is impacted the most from eliminating mosquitoes. Based on the knowledge gained, charitable nonprofits could go and help people who "lost" from the extermination of mosquitoes such as certain farmers near fish ponds. It is estimated by UNICEF that malaria resulted in 12 billion dollars in direct costs in Africa in 2010 and the total cost could be many times more (UNICEF, 2004). UNICEF also claims that malaria cost Africa 1% GDP growth rate per year. This means that Africa's year over year GDP could be 50% more in 40 years if there is simply no malaria. In turn, not only would rates of malaria be reduced but rates of IUGR and still births amongst pregnant mothers would also decrease significantly.

The cost of this implementation strategy is the cost of data centres and bomb proof facilities. These costs are estimated to be 1-2 billion USD. This is an initial fixed cost. The marginal cost per year from operating data centres and bomb proof facilities are significantly less. The hiring and grants to scientists could cost somewhere from 100-500 million USD a year. The

countries who have the most to gain are expected to foot most of the bill. As we can see, the fixed cost plus the marginal costs are significantly less than the present loss of GDP of 12 billion a year. Just from an economic point of view, this is well worth it.

This section has talked briefly about one approach, that we can take, to implement the strategy to releasing CRISPR-Cas9 modified mosquitoes to the wild. The next section will talk about what could go wrong, and what are some foreseeable consequences of using this strategy.

Potential Considerations and Consequences of CRISPR-Cas9 Gene Drive implementation

Gene Drive Resistance

The transmission of the CRISPR-Cas9 gene drive in *Anopheles* mosquitoes is a heterogeneous and dynamic process (Gantz *et al.*, 2015). Mosquitoes transfected with the gene drive could adapt by breaking the drive when incorporated in the chromosome (e.g. via endonucleases) and display resistance (Borel, 2016). The increase of resistance in gene drive systems has been proposed to include inactivation or potential safety mechanisms (Unckless, Clark, & Messer, 2017). Homologous repair (HR) of DNA breaks – that is, the usage of the genetic template for DNA ligation – could reinstate the gene drive (Unckless *et al.*, 2017). However, non-homologous repair (NHR) involves random joining of DNA ends, which could induce resistance over time by preventing re-ligation of the gene drive DNA (Unckless *et al.*, 2017). An example of NHR is when the maternal and paternal alleles after fertilization may cause the homologous template to be too far for DNA repair (Gantz *et al.*, 2015). Researchers have suggested using multiple gene drives targeted near genes necessary for survival to evade NHR (Noble, 2016). According to the National Academies of Sciences, Engineering and Medicine (NASEM), the frequency of both DNA repair mechanisms would depend on the species, developmental stage, and population of *Anopheles*, revealing the complexities of implementing the CRISPR-Cas9 gene drive.

Other options for gene drive resistance include the existence of targeted gene variants that are not identified by the guide RNA and avoid excision (Borel, 2016). These gene variants may still exist in the population and pose as resistant (Borel, 2016). Consequently, female mosquitoes with the variants can continue to persist and infect expecting mothers in malaria-endemic African regions, which could increase IUGR frequencies in the population. Another possibility could be

spread and a sudden stop of gene drive transmission in *Anopheles* to suppress the female population, which could impose environmental and health-based issues (Borel, 2016). However, as a new technology, this CRISPR-Cas9 system still harbours an alternative approach to malaria treatment and overall reduction in rates of IUGR as it would directly impact the disease spreading organism.

Other behavioural and genetic consequences

Additional concerns for implementation of CRISPR-Cas9 gene drive include the potential of the gene to undergo horizontal gene transfer, where transgene is transmitted to other species (Ledford & Callaway, 2015). Undesired non-specific targeting of populations with the gene could result in lethal effects. For example, transgenes in *Anopheles* could be passed on to other species that may not induce disease or that normally benefit the ecosystem (Lunshof, 2015). It would be advantageous if a reversible drive or an immunization strategy be utilized as a form of remediation in the case of these occurrences (Lunshof, 2015). Possible transmission of these genetic drives to embryos of expectant women in Africa (e.g via transgenic female mosquitoes that have not expressed the male-inducing phenotype) could pose serious viability issues or cause developmental defects such as IUGR. Another issue is the possibility that populations could stop breeding with the transgenic species (Borel, 2016). This form of behavioural resistance could arise when the wild type Anopheles female mosquito avoids mating with the transgenic male or preferentially mates at certain time periods when the transgenic males are absent (David, Kaser, Morey, Roth & Andow, 2013). Thus, assessing the stability and potential of the gene drive and the impact on ecosystems and populations are critical practices for proper usage.

With the existence of legitimate concerns and propositions regarding CRISPR-Cas9 gene drive implementation, it must be noted that models are not equivalent to experimentation to identify potential effects (Borel, 2016). These resistance experiments should be highly controlled and relevant in determining the role of utilizing CRISPR-Cas9 gene drive in malaria-endemic areas in Africa by targeting *Anopheles* mosquitoes. Past studies have included fluorescent studies of the gene drive by researchers at Cornell University (Borel, 2016). Preventing the release of transgenic mosquitoes with unknown phenotypes into areas where reproduction and proliferation could occur would be optimal.

Consequences of eradicating malarial mosquito vectors.

The risk of eliminating malaria-transmitting mosquitoes in African regions via the CRISPR-Cas9 gene drive should also be considered in application of the technology. Removing mosquitoes as a food source in the ecosystem can potentially increase the number of predators and disrupt the food web (David *et al.*, 2013). An example of a predator is the East African jumping Spider, *Evarcha culicivora*. The East African jumping spider selectively eats malaria-transmitting mosquitoes to consume blood in an indirect fashion (Nelson & Jackson, 2006). A lack of mosquitoes in the environment could decrease the population and affect the insectivorous role of *Evarcha culicivora* (Nyffeler, 2000). Reducing the population of *Anopheles*, a nectar-consumer, could also reduce the number of pollinators in many African ecosystems and affect crop production (Fang, 2010). Targeting a specific *Plasmodium* species (e.g. *P. falciparum*) in *Anopheles* by the CRISPR-Cas9 gene drive could cause possible competition between local species and increase the prevalence of certain mosquito populations (David *et al.*, 2013). Lastly, induction of the gene drive could result in *Anopheles* becoming a better host to another pathogen that could harm human health, or another insect disease vector to fill the gap by suppression (Pennisi, 2015). Thus, consideration of how species eradication on the ecosystem as whole will provide greater insight in the process of CRISPR-Cas9 gene drive development in Africa.

The Ecosystem, Environment and the Community

Utilising the gene drive in Africa for malaria and IUGR intervention affects communities in addition to the ecosystem and the environment. According to NASEM, most of the individuals affected by malaria tend be in low income countries and/or experience low socioeconomic status. In addition, their healthcare needs tend to be overlooked by wealthier and more developed countries. In Sub-Saharan Africa, costs of current malaria treatments are about $300 million annually (*FAQs: Gene Drives*). As a result, the construction of risk assessments and the development, modulation and usage of gene drives, requires the involvement of combined discourse, whether it be with communities, stakeholders, and/or the government. This public discourse should also include socially excluded individuals that are directly or significantly impacted by malaria. The purpose of this discourse would not be to come to a consensus with regards to the issue of gene drive and malarial interventions in Africa but to establish an informed

awareness and provide reasons as to the decisions made. Stakeholders and governments can collaborate to assist with the financial costs for implementation and perhaps establish local research facilities to foster complementarity versus social coercion. Amidst public skepticism and perceptions, individuals should be informed that that the benefits may outweigh the uncertainties and consequences of the gene drive. Persons in areas that more severely impacted by malaria, may be more focused on implementation. Ultimately, effective utilization and evaluation of the CRISPR-Cas9 gene drive for intervention in African malaria and IUGR incidence rates requires a targeted, collaborative, and holistic effort.

Conclusion

The prevalence of malaria in Africa is one of the highest in the world and has been implicated in congenital developmental abnormalities caused by IUGR. Many of the current interventions are restricted by cost limitations or the buildup of treatment resistance. Thus, the CRISPR-Cas9 gene drive was proposed to be a tool to prevent the spread of malaria and reduce IUGR incidences in endemic African countries, relative to commonly implemented treatments. The CRISPR-Cas9 gene drive is a new and potent genetic technology developed to reduce the spread and pathogenesis of the *Plasmodium* parasite via the *Anopheles* mosquito population. By insertion of a transgene into the wild type female, targeted removal and conversion of female *Anopheles* to male would reduce reproduction rates and result in elimination of the *Anopheles* population. Application of the gene drive requires financial, governmental and scientific cooperation in nations external and internal to Africa along with an risk evaluation of implementation on a genetic, organismal, ecological, environmental and societal level. Overall, the CRISPR-Cas9 gene drive is a technology that offers great promise in the elimination of malaria and decrease IUGR as a result, possibly even beyond the capability of current treatments.

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