Exploring the use of attractive sugar bait stations to deliver antiplasmodial compounds to *Anopheles* mosquitoes

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Abstract

Between 2000 and 2015, Sub-Saharan Africa made historic process against the scourge of malaria. Since this time there has been a plateau in the number of malaria cases and mortalities. The decreasing efficacy of the current methods used against this parasitic disease, such as IRS and bednets, is associated with a multitude of interspersed factors; such factors may be overcome with the incorporation of novel strategies. Furthermore, the re-emerging interest in attractive toxic sugar bait stations has been negatively impacted by the suggestion that the toxic compounds used could cause issues for non-target organisms. In this study the aim was to identify a series of methods through which the viability of anti-plasmodial compounds can be investigated for their application into a mosquito bait station. To do so, the potencies of two known sexual-stage active compounds were evaluated, alongside two control compounds, before and after their prolonged exposure to the environmental conditions of an attractive bait station in sub-Saharan Africa. The conditions used in this assessment included pH, temperature, time and light. The inhibition (%) of the compounds against the asexual and sexual stages of the *P. falciparum* (Dd2 strain) lifecycle were determined using luciferase assays and mosquito feeding assays respectively. Promisingly, the compounds maintained their stability within the relevant temperature cycles, however, they were unable to provide the necessary pan-activity against the oocyst and sporozoite stages of the plasmodia lifecycle. MMV011895 displayed some activity against the oocyst development, but not against sporozoite formation whereas MMV666060 displayed some activity against the sporozoite formation but not against oocyst development. Although a compound with the required characteristics has yet to be found, the *in vitro* methods used do provide a way in which the stability of candidate compounds can be screened without the use of more expensive, timely and labour-intensive methods. With the identification of a suitable compound, the exposure of infected *Anopheles* to an anti-plasmodial using bait stations could be a climacteric strategy in the bid for malaria elimination.

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1 Introduction

1.1 The Malaria Burden

Malaria is a serious and occasionally fatal disease caused by the *Plasmodium* parasite that is transmitted by the *Anopheles* mosquito. In 2017, almost half of the global population were deemed to be at risk of malaria, with 87 countries having ongoing malaria transmission (WHO, 2018). Some populations are at woefully higher risk, particularly those in low- and middle-income countries (LMIC). Over 90% of global malaria cases in 2019 were concentrated to the WHO African region; with over half of malaria mortalities occurring in just six countries: Nigeria (23%), Democratic Republic of the Congo (11%), United Republic of Tanzania (5%), Burkina Faso (4%), Mozambique (4%) and Niger (4%) (WHO, 2018) (Figure 1). The geographical distribution of malaria is largely determined by climate and ecology; however, its impact is most significant on the poorest communities (Hay et al, 2004) who are the least able to afford preventative measures and medical treatment. To be successful, therefore, national malaria control programmes must be suitable for and accessible by such groups (WHO, 2018).

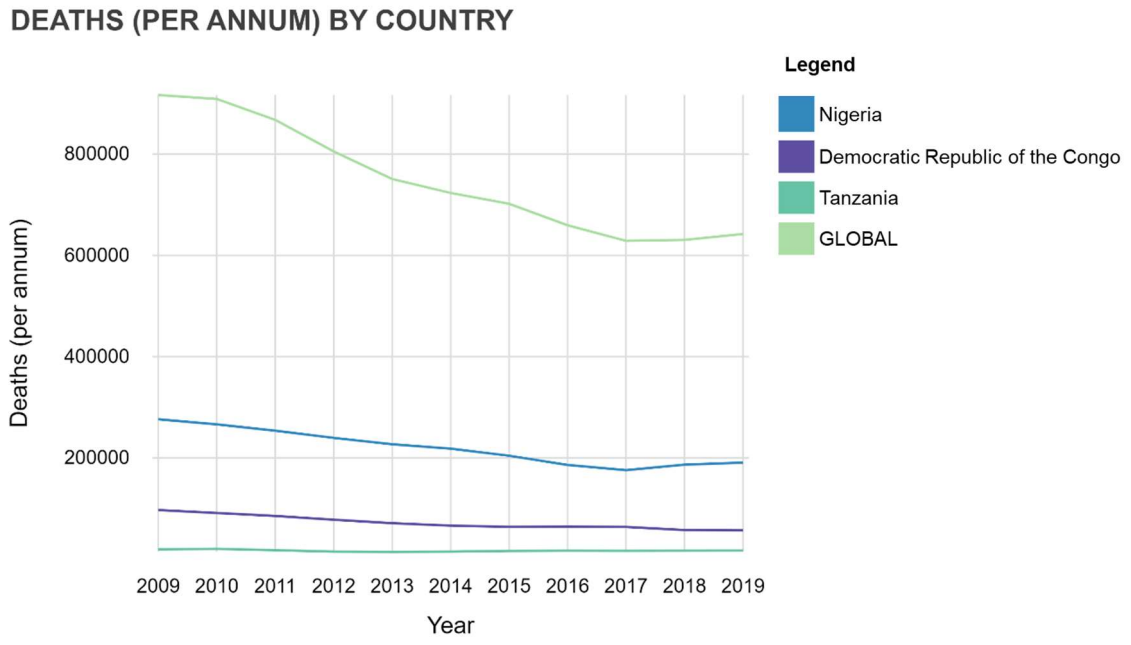

Figure 1. Annual number of deaths caused by Malaria in Nigeria, Democratic Republic of the Congo, Tanzania and globally (MAP, 2020).

In 2015, there were approximately 211 million cases of malaria globally. This incidence value was a result of gradual decline and is about 10% lower than that 2000. In this same period of time, mortality of the disease is reported to have fallen by 29% (WHO, 2018). Despite efforts, the falling numbers of global incidence did plateau; there was increase of 2 million cases more reported in 2017 than in 2016. The plateau in the number of malaria incidence rate continued in 2018 (WHO,

2018) and clearly highlights the ongoing need for more targeted and effective strategies to be developed. The plateau in decline of malaria cases has been associated with several key and interlinked factors which makes it difficult to separate the singular effect of each of them. With this, however, it is generally accepted that human population dynamics, multidrug-resistant *Plasmodium* species, insecticide resistant mosquito species and climate change are some of the major drivers of malaria propagation (Artzy-Randrup et al, 2010).

There have been several highly successful malaria prevention programmes, with many focussing on the development of vector control strategies and others on malaria treatment and prophylaxis targeted at high-risk groups such as pregnant women and young children. Since 2000, eight countries have achieved and sustained malaria elimination, and others have significantly reduced the transmission levels (WHO, 2015). Invigorated by these past successes, elimination is considered to be an attainable goal. The aspiration to eradicate malaria is once again resolutely on the global health agenda, instantiated by the introduction of bold global targets for malaria eradication by 2040 and national targets for malaria elimination: elimination from the four southern African countries (Botswana, Namibia, South Africa and Swaziland) by 2020 (WHO, 2015), a 90% reduction in global malaria incidence, and elimination in 35 countries by 2030 (Gates & Chambers, 2015).

To achieve these goals, WHO launched the 'high burden to high impact' campaign to jump-start malaria eradication in countries of which malaria has exacted a heavy mortality toll. This aggressive strategy quick-started in 2018 focuses on developing coordinated national databases in order to help identify the best intervention strategies, and translating political commitment into tangible actions (WHO, 2018). Both new interventions and new methods of implementing old interventions are continually being developed with the shared target of global malaria incidence decline. Complete malaria elimination is an achievable target which requires goal-orientated, evidence-based and context-specific operational strategies and actions at the local, state and national levels (Gates & Chambers, 2015).

1.2 Pathogenesis

Malaria is a life-threatening communicable disease caused by single-celled parasites belonging to the *Plasmodium* genus (CDC, 2017). It is estimated that 2.4 billion people, almost half the world's population, are at risk of malaria (Costa et al, 2006). Although fever represents the cardinal symptom, clinical findings in malaria are extremely diverse and symptoms may range in severity from a mild headache to serious complications leading to death (Bartolini & Zammarchi, 2012). The more severe cases are often caused by *Plasmodium falciparum* and *Plasmodium vivax* infection; however, *P. falciparum* is the more virulent (Geleta & Ketema, 2016). Prompt diagnosis and

appropriate treatment are thus crucial to prevent severe illness and mortality (Bartoloni & Zammarchi, 2012).

There are four species of *Plasmodium* that are considered true parasites of humans given that they utilise humans exclusively as their natural intermediate hosts: *P. falciparum, Plasmodium malariae, Plasmodium ovale* (two species) and *P. vivax* (CDC, 2017). There are also reports of *Plasmodium knowlesi*, a typically zoophagic parasite being found in humans (Costa et al, 2006). There are a number of conspicuous phenotypic as well as geographic differences among the species, however, they exhibit the same principal lifecycle with only minor variations (Sinden et al, 1978).

The malaria infection is initiated when sporozoites are inoculated with the saliva of a feeding infected female mosquito (Figure 2) (Boyd & Kitchen, 1939). Transported by the circulatory system, sporozoites first invade the liver hepatocytes. Here, the parasite undergoes exoerythrocytic schizogony: differentiating into thousands of uninucleate merozoites (Shortt & Garnham, 1948). The infected hepatocytes eventually rupture and merozoites enter the blood and invade the reticulocytes (Nardin & Nussenzweig, 1993). The parasite matures as a metabolically active trophozoite and multiplies asexually into schizonts (Nardin et al, 1982). During the erythrocytic stage, new merozoites are formed by mitosis and released when the erythrocyte ruptures to establish further reticulocyte invasions (Gerald et al, 2011). Alternatively, some of the merozoites can develop into gametocytes, which are taken up during blood-feeding by an *Anopheles* mosquito (Dantzler et al, 2015).

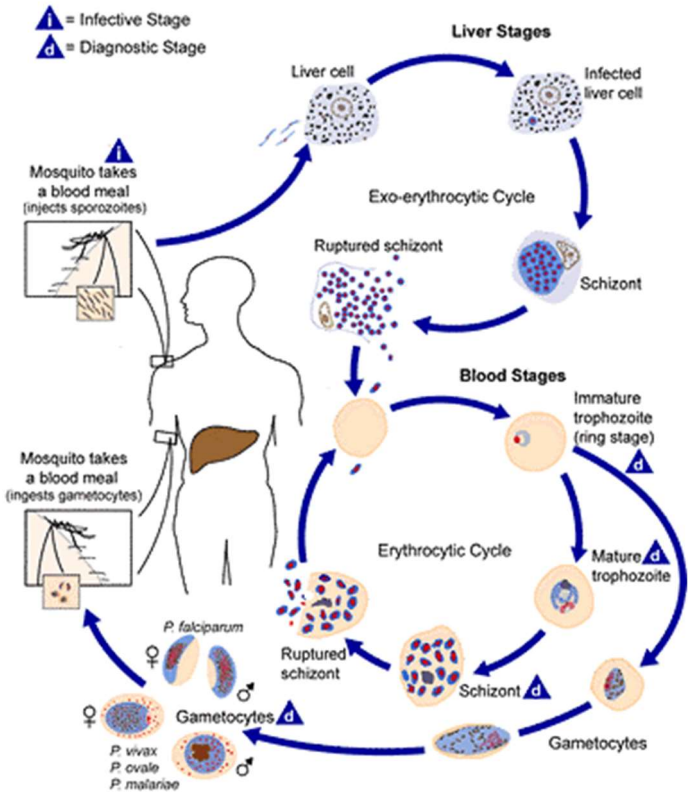

Figure 2. The human stages of the Plasmodium lifecycle following inoculation (adapted from CDC, 2017).

P. falciparum is responsible for the most malaria deaths globally and is the most prevalent and perilous species in sub-Saharan Africa (WHO, 2018). Death from malaria is associated with complications such as cerebral malaria, pulmonary oedema, acute renal failure, severe anaemia and/ or haemorrhage (Burns et al, 2019). The pathogenesis of *P. falciparum* is complex and comprised of parasite-induced erythrocyte alterations and microcirculatory abnormalities (Grau et al, 2003), accompanied by local and systemic immune reactions of the host (Marsh et al, 1995).

During the early stages (rings) of the parasite's erythrocytic cycle, the host's infected cells may remain in circulation (Gerald et al, 2011), while exhibiting only mild modifications of their adhesive and/ or deformability properties (Maier et al, 2009). However, once the parasites enter the later stages of their erythrocytic development (trophozoites, schizonts), parasite-derived ligands are expressed on the surface of infected erythrocytes (e.g. *P. falciparum* Erythrocyte Membrane Protein 1, Subtelomeric Variable Open Reading frame proteins and repetitive interspersed family proteins) (Maier et al, 2009). These ligands cytoadhere to host proteins (receptors/adhesins) on the surface of endothelial cells, placental cells and uninfected erythrocytes. This cytoadherence, also known as sequestration, occurs in the microvascular systems of specific organs, such as the brain, lungs and placenta (Beeson & Brown, 2002). This process, ultimately, hampers blood flow within the vasculature and leads to pathology (Grau et al, 2003).

Anaemia is a common manifestation of *P. falciparum* infection due to obligatory destruction of erythrocytes through a combination of splenic filtration, schizont rupture, macrophage phagocytosis, complement-mediated haemolysis and increased free radical damage (Douglas et al, 2013). In chronic infections, this can coincide with dyserythropoiesis (Mackintosh et al, 2004) caused by the direct inhibitory effects of the parasites and cytokines (Douglas et al, 2013).

The pathology of malaria is caused by the blood stages of the *Plasmodium* lifecycle, this is when clinical symptoms occur, and diagnosis can be made. The antimalarial drugs used in treatment predominantly target this stage of the lifecycle (Burns et al, 2019). The survival and proliferation of the parasite *in vivo* involves a complex series of often irreversible events to interrelate with one another; even small disruptions of such can limit its survival (Castelli et al, 2007). To this end, many compounds display potent selective anti-plasmodial activity, however, do not have the suitable pharmacokinetic and pharmacodynamic properties to be considered candidates for anti-malarial drug development (Cartwright et al, 2010). The aim of this project is to determine whether these compounds could be used to eliminate *Plasmodium* within the mosquito population, therefore circumventing the need for vertebrate efficacy and toxicity studies.

1.3 Treating Malaria

The prompt and effective treatment of all cases of malaria is the mainstay of malaria control (WHO, 2018). The specific medication prescribed and the length of treatment depends on; the infecting *Plasmodium* species, the severity of symptoms and the patient's age, amongst other factors (CDC, 2019a). The most commonly used treatments are shown in Table 1.

Table 1. The recommended antimalarial chemoprophylaxis (adapted from CDC, 2019a).

Plasmodium species	Infecting Region	Recommended Drug	Additional Guidelines
Uncomplicated malaria/ *P. falciparum* or unidentified	Chloroquine resistant or unknown resistance	Atovaquone- proguanil	Not recommended in pregnancy or in infants weighing <5 kg.
		Artemether-lumefantrine	Not recommended in the first trimester of pregnancy or in infants weighting <5kg. May be used in second and third trimesters of pregnancy.
		Quinine sulfate with either Doxycycline, Tetracycline or Clindamycin	Doxycycline and Tetracycline are not recommended in pregnancy or in children <8 years old.
		Mefloquine	
	Chloroquine sensitive	Chloroquine phosphate	If treating *P. ovale* or *P. vivax* primaquine and tafenoquine (after testing for G6PD deficiency) should be given.
		Hydroxychloroquine	
Uncomplicated malaria/ *P. malariae* or *P. knowlesi*	All regions	Chloroquine phosphate	
		Hydroxychloroquine	
Uncomplicated malaria/ *P. ovale* or *P. vivax*	All regions	Chloroquine phosphate with either Primaquine phosphate or Tafenoquine	Not recommended for persons with G6PD deficiency. Primaquine must not be used during pregnancy but may be used in breastfeeding if the infant is found to have normal G6PD levels. Tafenoquine is not suitable for children, pregnant women or breastfeeding mothers.
		Hydroxychloroquine with either Primaquine phosphate or Tafenoquine	
Uncomplicated malaria/ *P. vivax*	Chloroquine-resistant	Quinine sulfate with either Doxycycline, Tetracycline plus either Primaquine phosphate or Tafenoquine	Not recommended for infants weighing <5 kg. Not recommended for persons with G6PD deficiency. Primaquine must not be used during pregnancy but may be used in breastfeeding if the infant is found to have normal G6PD levels. Tafenoquine is not suitable for children, pregnant women or breastfeeding mothers.
		Atovaquone-proguanil with either Primaquine phosphate or Tafenoquine	Not recommended in persons with G6PD deficiency. Primaquine must not be used during pregnancy but may be used in breastfeeding if the infant is found to have normal G6PD levels. Tafenoquine is not suitable for children, pregnant women or breastfeeding mothers.
		Mefloquine with either Primaquine phosphate or Tafenoquine	

Uncomplicated malaria: alternatives for pregnant women	Chloroquine-sensitive	Chloroquine phosphate	
		Hydroxychloroquine	
	Chloroquine-resistant	Artemether-lumefantrine	
		Quinine sulfate with Clindamycin	
		Mefloquine	
Severe Malaria	All regions	Intravenous Artesunate treatment followed by one of the following: Artemether-lumefantrine, Atovaquone-proguanil, Doxycycline or Mefloquine	

Most commonly, the treatments used are the highly efficacious artemisinin-combination therapies (ACTs) of which there are several different types: artemether-lumefantrine (Kokwaro et al, 2007), artesunate-amodiaquine, artesunate-mefloquine (Taylor & White, 2004), artesunate-pyronaridine (Ramharter et al, 2008), artesunate-sulphadoxine-pyrimethamine (Cui & Su, 2009) and artesunate-chlorproguanil-dapsone (White, 2008). ACT describes a combination of two or more drugs that act via different mode of actions (MoAs). It is well-accepted that dosing medications in combination limits the probability of developing resistance to both drugs at the same time (Nosten & White, 2007).

Artemisinin is a sesquiterpene lactone characterised by an endoperoxide trioxane moiety. It is effective against nearly all asexual and sexual *Plasmodium* parasite stages (Skinner et al, 1996). Being a pro-drug, artemisinin must be chemically activated into its bioactive metabolite-dihydroartemisinin, which is eliminated via glucuronidation (Ilett et al, 2002). It is widely believed that the metabolism of artemisinin and its derivatives is primarily mediated by the liver cytochrome p450 (CYP) enzyme CYP2B6 (Svensson & Ashton, 1999). Despite being able to quickly initiate their potent effects on the asexual intraerythrocytic forms of *Plasmodium*, artemisinin derivatives have very short elimination half-lives *in vivo* (Svensson et al, 2003). They therefore should be co-administered with compounds with longer half-lives (Belete, 2020).

The exact mechanism of artemisinin's MoA is an active area of research (Heller & Roepe, 2019). It is hypothesised that a Fe^{2+}-catalysed cleavage of the intramolecular bridge generates cytotoxic free radicals which in turn, alkylate intracellular targets (Meshnick et al, 1993). Heme, generated from the digestion of haemoglobin in the parasitophorous vacuole, is toxic to *Plasmodium* (Francis et al, 1997) and must be detoxified through polymerisation to form hemozoin (malaria pigment).

Artemisinin derivatives react with the free hemin and readily oxidise the erythrocyte membrane thiols (Heller & Roepe, 2019). The high reactivity of artemisinin-derived radicals has been demonstrated *in vitro* (Scott et al, 1989), however, the compound's extremely low toxicity suggests that its derived radicals selectively damage cellular targets (Wu & Liu, 1989). Despite being the fastest acting compound against all erythrocytic stages of *Plasmodium*, artemisinin derivatives have a very short elimination half-life (Heller & Roepe, 2019), which precludes ACT for malaria prophylaxis.

Artemisinin-combination therapies are recommended for the treatment of uncomplicated malaria caused by *P. falciparum* and in cases where the infecting malaria species is not known with certainty (CDC, 2019a). The decision of which ACT to use should be based on the result of therapeutic efficacy studies against the local *P. falciparum* strains. Pregnant women with uncomplicated *P. falciparum* malaria must not be prescribed ACT within their first trimester. Instead, they should be provided with alternative treatments such as quinine and clindamycin (CDC, 2019a). In areas of uncomplicated malaria infection not associated with *P. falciparum*, patients may be treated with either ACT or chloroquine. In areas of known chloroquine resistance, ACT is used (WHO, 2015).

Atovaquone, is a substitute 2-hydroxynaphthoquinone and is used in both the prophylaxis and treatment of *P. falciparum* malaria. Atovaquone possesses a novel MoA against the parasite via the inhibition of ubiquinol (Kessl et al, 2003). Ubiquinol is an essential component of the mitochondrial electron transport chain; it binds in the centre of the cytochrome *bc1* complex (Fry & Pudney, 1992) and is oxidised to ubiquinone. Atovaquone, therefore, disrupts the mitochondrial electron transport chain, thereby preventing ATP synthesis (Srivastava e al, 1997). It is also believed that by preventing the regeneration of ubiquinone, the drug's MoA includes the inhibition of pyrimidine biosynthesis, halting DNA synthesis and leading to protozoal death (Birth et al, 2014). Atovaquone as a monotherapy, however, is not very effective in curing malaria (30% treatment failure in clinical trials) (Looareesuwan et al, 1996) and preventing recrudescence (Chiodini, et al, 1995). It is, however, effective in killing the active liver stages and, taken in combination with proguanil, is typically utilised as a prophylactic drug (Nixon et al, 2013).

Proguanil, if used alone, is metabolised by the host to a cyclic triazine molecule, cycloguanil. This metabolite implements it's anti-plasmodial action by inhibiting the parasite's dihydrofolate reductase enzyme (Peterson et al, 1991) which is involved in the reproduction of the parasites within infected erythrocytes (Kain, 2003). The rapid detection of resistance to proguanil monotherapy (Blasco et al, 2017) was shown to be mediated by point mutations of dihydrofolate reductase (Kain, 2003). This widespread nature of the resistance-imparting mutations is suspected to be central to the 90% treatment failure of proguanil monotherapy in clinical trials (Looareesuwan et al, 1996). Proguanil, however, displays synergism when used in combination with atovaquone

(Shanks et al, 1999): it acts as a biguanide, enhancing atovaquone's effect on the mitochondrial membrane (Canfield et al, 1995) and effectively lowering *in vivo* concentration of atovaquone required to disrupt the parasite's mitochondrial membrane. Used in combination with proguanil, atovaquone therefore, results in parasite clearance at concentrations that would otherwise be considered suboptimal (Srivastava & Vaidya, 1999). Clinical trials using atovaquone-proguanil combination provided encouraging results, with cure rates approaching 100% (Looareesuwan et al, 1996) and little evidence of the emergence of resistance (Radloff et al, 1996). The slow activity of proguanil in comparison to that of cycloguanil is not associated with dihydrofolate reductase inhibition (Srivastava & Vaidya, 1999). While proguanil's MoA is not completely understood when used alongside atovaquone (Kain, 2003), evidence suggests that it is linked to an ATP synthase function that only becomes essential when the mitochondrial electron transport chain is inhibited (Painter et al, 2007).

Chloroquine, like other quinoline derivatives i.e. mefloquine, quinine and quinidine, acts by inhibiting *Plasmodium* polymerisation activities (Herraiz et al, 2019). It too, is used as a prophylactic as well as a treatment for malaria. At a physiological pH of 7.4, 18% of chloroquine becomes monoprotonated, but due to its amphiphilic properties is still lipid-soluble and able to traverse the biological membrane (Browning, 2014). Due to its diprotic weak base properties, it becomes biprotonated (to CQ2+) in the acidic parasitophorous vacuole (pH 4.7) digestive vacuole (Hempelmann, 2007). Membranes are not permeable to charged species; chloroquine, therefore, accumulates in the compartment and commences its antimalarial functions (Hempelmann, 2007). The compound caps the hemozoin molecules, leading to the formation of a heme-chloroquine complex (Herraiz et al, 2019). This highly toxic conformation disrupts the cell membrane, ultimately leading to cell lysis and parasite cell autodigestion (Veignie & Moreau, 1991). In areas with chloroquine-susceptible infections, individuals with uncomplicated malaria infection caused by *Plasmodium* species other than by *P. falciparum* are treated with either ACT or chloroquine (WHO, 2015).

In other *Plasmodium* infections erythrocytic schizogony occurs within the host's hepatocytes, but with *P. ovale* and *P. vivax*, only a portion of the sporozoites develop into schizonts. These *Plasmodium* species also form small, non-replicating hypnozoites (Campo et al, 2015). The dormant parasitical stages cause relapses of infection weeks to years after the initial infection and are not eradicated by schizonticidal compounds (Markus, 2010). Primaquine, an 8-aminoquinoline, is currently the only marketed drug for this indication (Campo et al, 2015) and is typically administered concurrently with chloroquine (Beeson & Brown, 2002). The metabolism of primaquine primarily occurs in the liver (Campo et al, 2015) and is presumed to follow two distinct pathways catalysed by CYPs and monoamine oxide enzymes (Ganesan et al, 2009). The metabolites generated is thought to reduce glutathione, which plays a significant role in regulating the intracellular redox

homeostasis (Camp et al, 2015). Individuals who have glucose-6-phosphate dehydrogenase (G6PD) deficiency cannot produce adequate levels of G6PD, which in turn results in low levels of NADPH and glutathione (Ganesan et al, 2009). This means the erythrocytes of this group are more susceptible to oxidative damage to haemoglobin and the cell membrane (Campo et al, 2015). Primaquine treatment may therefore lead to clinically relevant reductions in erythrocytes in individuals who are G6PD deficient and must not be prescribed to these individuals (WHO, 2015).

To effectively treat all those infected, including asymptomatic carriers, a well-established infrastructure is required. Secure surveillance systems, a predictable domestic funding, robust political engagement, and a dependable health care system are necessary to accurately diagnose and treat all those infected (Mendis et al, 2009). Because these factors are not always prevalent, many national programmes focus on more feasible means of malaria control: intermittent preventative treatment (IPT) and integrated vector management (IVM).

1.4 Intermittent Preventative Treatment

Anti-malarial chemoprophylaxis includes artemether, lumefantrine and quinine amongst other compounds (Lalloo et al, 2016). These drugs act on different stages of the *Plasmodium* lifecycle, both on the pre-erythrocytic forms (causal prophylaxis) and the erythrocytic blood forms (suppressive prophylaxis) (Castelli et al, 2007). Whilst they have been proved to be effective for use by travellers to malaria-endemic areas, this medication is largely unaffordable for individuals living in low socioeconomic conditions (Feachem et al, 2010). Most adults living in hyper- or holoendemicity possess partial immunity to malaria, therefore, chemoprophylaxis should not be sustained for prolonged periods as this would interfere with the development of this naturally acquired immunity and put them at risk of catastrophic rebound (Korenromp et al, 2016). Furthermore, giving such treatment to an entire community would facilitate the emergence and spread of drug resistant *Plasmodium* strains (Aponte et al, 2007).

Intermittent preventative treatment (IPT) programmes aim to eliminate malaria infection in high-risk groups i.e., pregnant women, infants or at periods of the year when transmission is at its greatest (WHO, 2004). Supported by systematic reviews of randomised controlled trials, WHO (2004) recommends prophylaxis for those who visit malarial areas and IPT for pregnant women and infants in these areas. In areas of marked seasonal variation of malaria transmission, IPT of children under five has also been operationalised (Rehman et al, 2019) and is hoped to reduce the infectious reservoir on a community-wide scale (Staedke et al, 2018).

Studies have shown community volunteers within existing antenatal and vaccination clinics can competently and cost-effectively assume the role of IPT administration (Greenwood, 2010). IPT with sulphadoxine/pyrimethamine (SP) provides 4-6 weeks protection against sensitive isolates and

therefore expectant mothers must be treated on two or three occasions during the second and third trimesters (Kalanda et al, 2006). A trial in Tanzania, in which three doses of SP were given within the first year of life at the time of routine immunisation, showed highly encouraging results with approximately 50% reduction in clinical attacks of malaria and anaemia (Schellenberg et al, 2001).

Unfortunately, IPT programmes face two major limitations: the development of resistant *Plasmodium* strains and a decreasing burden of malaria which results in a disbalance of the cost benefit ratio (Greenwood, 2010). A Tanzanian study in an area of very high resistance to SP, investigated mefloquine and chlorproguanil as an alternative regimen for IPT. Although highly effective, mefloquine treatment resulted in a high incidence of side effects, especially vomiting (Gosling et al, 2009). There are many alternative compounds that exhibit anti-parasitic properties, however, there is concern over their limited chemical diversity. The current compounds used can be attributed to four major classes: aminoquinolines, antifolates and artemisinin derivatives (Belete, 2020). These interact with only a small selection of parasitic drug targets, including heme detoxification, folate metabolism and the pathogen's mitochondria. Future therapies using drugs based on the known chemistry backbones may be ineffectual within an even shorter period of time due to the priming effect on resistance (Duffy & Avery, 2012).

Protozoa parasites have evolved multiple strategies to evade attacks from the host immune system, such as encoding and switching between dozens of genes that encode different cell surface proteins (Tediosi et al, 2017). Mutations of *P. falciparum* and *P. vivax* dihydrofolate reductase (*pfdhr* and *pvdhfr*) (de Pecoulas et al, 1998) and dihydropteroate synthase (*pfdhps* and *pvdhps*) (Korsinczky et al, 2004) genes have been linked with resistance to pyrimethamine and sulphadoxine respectively (Venkatesan et al, 2013). The resistance of *P. falciparum* strains to 4-aminoquinolines is affiliated with mutations in the chloroquine resistance transporter gene (PfCRT) (Sidhu et al, 2002) and the multidrug resistance gene 1 (*pfmdr1*). Mutations of the *P. vivax* multidrug resistance gene 1 (*pvmdr1*) (Brega et al, 2005) have been identified in some studies but not others (Barnadas et al, 2008). To enhance IPT efficacy, drug resistance markers must be considered a routine assessment (Venkatesan et al, 2013).

Formulating a highly effective malaria vaccine is the ultimate goal of many researchers, however, the absence of a sterilising immune response to a disease as prevalent as malaria illustrates how difficult it will be to achieve this (Doolan et al, 2009). It is noted that current candidate compounds provide a protective efficacy in the range of 30-50% (RTS Clinical Trials, 2014), whilst impressive, it is evident that there is some way to go before this ultimate goal can be achieved.

There is, however, a vaccine which has recently been shown to produce a substantial reduction (30%) in severe malaria cases and mortalities among young children living in regions with moderate to high *P. falciparum* transmission. The prospect of RTS, S/ AS01 vaccine introduction into these

endemic areas is highly promising for malaria control efforts (WHO, 2021). The active substance of the RTS, S vaccine represents viral-like particles comprised of the RTS (fusion protein of a portion of the *P. falciparum* circumsporozoite protein and the amino terminal end of the Hepatitis B virus S protein) and S proteins (hepatitis B surface antigen) co-expressed in genetically engineered *Saccharomyces cerevisiae* (RIX4397) yeast cells (Laurens, 2020). Although first created in 1984, the vaccine was first recommended by WHO until October 2021 due to the positive results from an ongoing pilot programme in Ghana, Kenya and Malawi. Across the three countries over 2.3 million doses have been administered in two years, despite the Covid-19 pandemic. The future rollout of the of the RTS, S/ AS01 vaccine will ultimately rely on the political engagement of endemic countries and funding (WHO, 2021).

1.5 Novel Compound Development & Stability Testing

Reports of drug-resistant *Plasmodium* species began over a century ago (Peters, 1982) and since then research has revealed the efficacies of most antimalarial agents to be compromised by the same predicament (Bloland, 2001). Pharmaceutical companies are therefore faced with a continual need to develop novel antimalarial drugs in the face of emerging resistance.

Once a lead compound has been identified, optimisation of the structure can begin (Belete, 2020). This largely involves investigation and multifactorial optimisation of potency, selectivity and biopharmaceutical properties such as absorption, distribution, metabolism, excretion and toxicity (Meanwell, 2011). Cell penetration by a compound is hampered by excessive polarity which is not compatible with lipophilic nature of biological membranes (Wanka et al, 2013). It is, therefore, critical to have a thorough understanding of the acid/base character of any compound (Manallack et al, 2013). The nature of any acidic or basic functional groups in physiological conditions will have a great impact on the compound's pharmacokinetic and biopharmaceutical properties and, ultimately affect the efficacy of the treatment (Wanka et al, 2013).

Lipinski (2000) initially determined the 'Rule of Five' which suggests that poor oral absorption and permeation are likely to be encountered if a compound meets two or more of the following criteria: molecular weight >500 g/mol, ClogP >5, H-bond donors >5 and H-bond acceptors >10. The ClogP value of a compound refers to the logarithm of its partition coefficient between n-octanol and water (Meanwell, 2011) and is a well-established measure of the compound's hydrophilicity and subsequent permeability (Manallack et al, 2013).

Accompanied by the gathering of biological data, significant efforts continue to be made to understanding the physiochemical properties of a compound (Wanka et al, 2013) and how they relate to its long-term viability (Meanwell et al, 2011). To this end, there are many approaches used to assess a compounds drug-like characteristics (Reddy & Zhang, 2013) and pharmaceutical

companies apply variations of these principles to assess their compounds (Manallack et al, 2013). Typically, prospective compounds are assessed using broad and easily calculated parameters including molecular weight, ClogP, polar surface area (PSA), the number of hydrogen bond donors/ acceptors, aromatic character and the number of rotatable bonds (Manallack et al, 2013).

Determination of stability in terms of pharmaceutical quality involves performing multiple chemical and physical tests described especially in Pharmacopoeias, recommendations and other industrial regulations (Rägo & Santoso, 2008). Stability is defined as the state in which the active pharmaceutical ingredient (API) is not degraded by exposure to specific factors (Waterman & Adami, 2005). The entire process of pharmaceutical evaluation related to the stability is composed of different tests, which aim to determine whether the API is chemically and physically stable (Rägo & Santoso, 2008). The influence of different factors on the API's chemistry and physical state (humidity, temperature, pH, the presence of oxidizing agents, as well as the influence of light) in the case of instability, results in the formation of degradation products (Jamrógiewicz & Pieńkowska, 2019).

Atovaquone, a hydroxy-1,4-naphthoquinone, breaks one of Lipinski's (2000) 'Rule of Five' rules; it has a ClogP value of 6.31. It has a molecular weight of 366.1 g/mol, a PSA of 54.37Å, 3 hydrogen bond acceptors, 1 hydrogen bond donor and 2 rotatable bonds (Hastings et al, 2016). Atovaquone is a yellow crystalline powder and is formulated with proguanil hydrochloride (Baggish & Hill, 2002). In its original packaging, tablets are described as having a shelf life ≤3 years when stored below 25°C and protected from moisture (EMC, 2017).

Chloroquine, a 4-aminoquinoline derivative breaks none of Lipinski's (2000) 'Rule of Five'. It has a molecular weight of 319.18 g/mol, a PSA of 28.16Å, a ClogP value of 4.27, 3 hydrogen bond acceptors, 1 hydrogen bond donor and 8 rotatable bonds (IPCS, 1994). Chloroquine is a white or slightly yellow, odourless powder with a bitter taste. Although most commonly dispensed as a phosphate, chloroquine can be formulated with as a sulfate or hydrochloride salt. In tablet form, chloroquine is described as having a shelf-life of ≤5 years when stored below 30°C and protected from light and moisture (IPCS, 1994).

On one hand, the often tropical climates of malaria endemic regions only escalate the need for stability studies and the subsequent identification of possible consequences due to long and unfavourable conditions during distribution and storage of drug products. On the contrary, if a compound was not intended for use in humans, a lack of such evaluations would be much less detrimental (Jamrógiewicz & Pieńkowska, 2019).

1.6 Integrated Vector Management

IVM involves a dynamic and continually evolving group of strategies aimed to limit or eradicate the vector insects as opposed to the *Plasmodium* in the human host. The methods used and where they are implemented are selected in a manner which achieves the greatest disease-control benefit in the most cost-effective way (Korenromp et al, 2016). These include environmental management, the use of biological controls, chemical methods and personal protection tools (WHO, 2014).

The habitat requirements of the mosquito larval stages (e.g. irrigation water, drainage ditches, floodplains etc.) affect the distribution pattern of the adult mosquitoes. By targeting the larvae found in standing water (Figure 3), larval source management (LSM) aims to kill the immature mosquitoes before the females develop into flying adults with the capacity to transmit disease (Fillinger & Lindsey, 2011). This can be done by permanently removing standing water sources or by killing the larvae (Tusting et al, 2013) with either chemical or biological larvicides (Benelli & Beier, 2017). LSM programmes have proven the most effective in areas where larval habitats are less extensive and thus WHO recommends LSM only in areas where the "larval habitats are few, fixed and findable" (WHO, 2019).

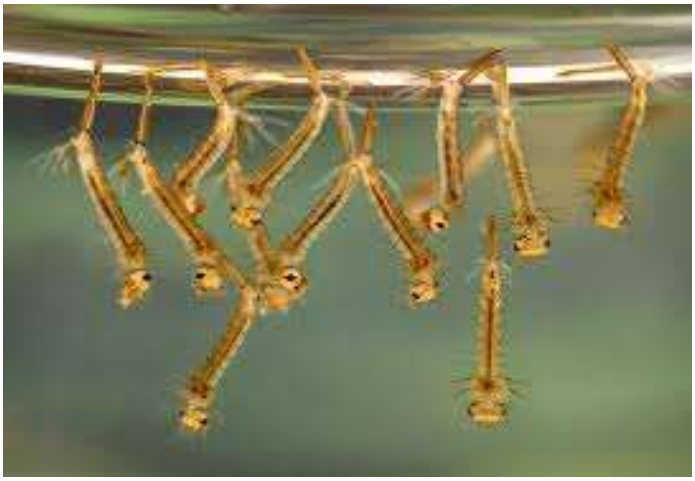

Figure 3. The larval stage of the mosquito lifecycle lasts approximately 2 weeks before they become non-feeding pupae (CDC, 2020).

There are a broad range of effective larvicides that have been developed for *Anopheles* larvae control (Benelli & Beier, 2017). The diverse formulations provide a wide range of MoAs against the larvae including insect growth regulators that inhibit the larvae from maturing, microbials that lyse the gut epithelium and toxins that directly impede the insects' metabolism (Fillinger & Lindsay, 2011). Unlike their predecessors, modern larvicides have been developed to have no or minimal effect on non-target invertebrate populations and aquatic ecosystems (Benelli & Beier, 2017).

LSM is a well-established strategy, with large-scale programmes *in situ* worldwide (Patterson, 2009). It is, however, not suitable as the primary tool selected in areas of hyper- or holoendemicity and any nation-wide applications would face considerable limitations. The efficacy of LSM programmes depends on the prevalent local eco-epidemiological conditions (Tesfazghi et al, 2016) and thus are difficult to accurately assess and compare. Nevertheless, LSM does reduce transmission in a synergistic fashion and has the potential to be integrated into local control programmes (Fillinger & Lindsey, 2011).

Methods of control that target adult vectors are intended to impact the mosquito density (Benelli & Beier, 2017). Adulticide deposits are either applied via indoor residual spraying (IRS) to treat a specific surface or space (Figure 4). The first inexpensive and relatively long-lasting insecticide, dichloro-diphenyl-trichloroethylene (DDT) was discovered in 1939 (Russell, 1946). DDT exerts its toxic effects in a mechanism common amongst axonic excitotoxins including pyrethroid (O'Reilly et al, 2006). The compounds prevent the closure of the voltage-gated sodium channels in the axonal membrane, propagating an action potential and leaving the axonal membrane permanently depolarised, rendering the organism paralysed (Soderlund, 2012). Other than DDT, there are currently eleven insecticides available against mosquitoes. These belong to three distinct chemical groups (six pyrethroids, three organophosphates and two carbamates) (WHO, 2015).

Residual contact insecticides are also used in the production of insecticide-treated nets (ITNs) which provide an effective physical and chemical barrier between the mosquito and sleeping human (Benelli & Beier, 2017) (Figure 5). Long-lasting insecticidal nets (LLINs) have also been produced and have largely replaced the previously popular ITNs. Unlike ITNs, LLINs are typically treated with pyrethroid insecticide at the manufacturing unit and retain their efficacy against mosquitoes for a minimum of 3 years or 20 standard washes (Churcher et al, 2015). Where implemented, they have proven to an effective control intervention for malaria endemic countries (Giardina et al, 2014).

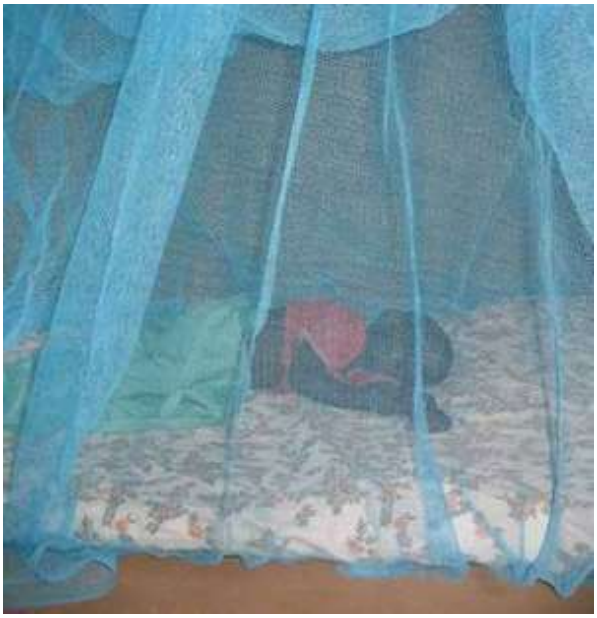

Figure 4. An insecticide-treated bednet (CDC, 2019b).

The use of LLINs has been shown to be a highly cost-effective strategy for malaria prevention (Benelli & Beier, 2017) and has contributed to a significant reduction is the disease morbidity and mortality figures (WHO, 2015). Studies on LLIN coverage are widespread in the African continent (Mboma et al, 2018, Leonard et al, 2014) with many trials reaching the same conclusion as to their limited ongoing success. IRS uses the same inventory of insecticides. This method describes the application of insecticide products to the interior surfaces of

Figure 5. Indoor residual spraying (CDC, 2019b).

households, public buildings or animal dwellings in areas where malaria is endemic. The process must be repeated every 3-9 months, depending on the given setting (WHO, 2015). Such programmes have been widely used in malaria control globally since the 1940s (Mabaso et al, 2004). The successful elimination of malaria from more than two dozen countries in Europe, the Americas and Asia during the Global Malaria Eradication Campaign (1955-1969) has largely been attributed to extensive IRS campaigns using DDT (Najera, 2011).

1.7 Limitations of Current Intervention Strategies

Regarding the use of chemical insecticides, there is substantial evidence of the development and spread of resistance to DDT and other insecticides within local anopheline populations. Pyrethroid resistant *Anopheles gambiae* have been reported in many African countries (Smith et al, 2016) including Tanzania (Kabula et al, 2012), Kenya (Vulule et al, 1999), Nigeria (Awola et al, 2002) and Cameroon (Etang et al, 2003). Furthermore, multiple insecticide resistant *An. gambiae* and *Culex Quinquefasciatus* have been reported in Benin (Corbel et al, 2008). There have been multiple complex mechanisms implicated in the development of insecticide resistance including mutations in coding sequence within a gene, gene overexpression and amplification (Liu, 2015).

The rate of resistance development depends on several factors: the proportion of resistant individuals originally present, degree of isolation of the population from neighbouring populations not exposed to insecticide, alongside the reproductive rate and generation time of the local mosquito vectors (Corbel & N'Guessan, 2013). Resistance develops as a consequence of mutations resulting in target-site insensitivity, a lower penetration or sequestration of the insecticide (Liu, 2015), or an increased biodegradation of the insecticide due to enhanced metabolic detoxification activities (Muller et al, 2008). Knockdown resistance, for example, describes resistance to DDT and pyrethroid insecticides due to the target-site insensitivity of sodium channels (Liu, 2015). The overexpression or conformational changes of the enzymes involved in metabolic resistance have been identified in *Ae. aegypti* populations from multiple geographic locations (Smith et al, 2016, Bisset et al, 2014). The main enzymes identified to confer such resistance include esterase, CYP monooxygenases, glutathione S-transferases (Smith et al, 2016).

New chemical insecticides with novel MoAs are expected to enter the market over the next decade but, if used individually, will likely be similarly vulnerable to selection for physiological resistance (Hemingway et al, 2016). With our current level of understanding, management strategies such as MoA rotations (Seixas et al, 2017) and alternatives to chemical-based vector control interventions are widely accepted as key to achieving global malaria eradication.

Despite the emergence of insecticide resistant mosquitoes, ITNs and IRS have been highly effective interventions in LMICs; they account for an estimated three quarters of clinical malaria

cases averted (Bhatti et al, 2015). In sub-Saharan Africa the proportion of children under 5 years of age sleeping under ITNs has increased from <2% in the year 2000 to an estimated 68% in 2015, although the estimates vary widely between countries (WHO, 2015). Many studies have reached the same conclusions as to the fundamental limitations of both strategies (Mnzava et al, 2015), including the inefficiency in the distribution of, ownership and actual use of ITNs (Nkumama et al, 2016). ITN possession in Africa is linked to a multitude of factors such as proximity to distribution sites, cost, socioeconomic status and the method of distribution used (Olapeju et al, 2019). As a result, only 31% of African households have sufficient ITNs, and global IRS coverage have shrunk to only 3.5% of the world's at-risk population (Killeen et al, 2017).

The impact of any IVM programme is biologically limited to its reliance on strong vector behavioural preferences (Benelli & Beier, 2017). As indoor focused strategies are notably most effective on endophagic and endophilic species (Killeen et al, 2017), mosquito species that do not fit such criteria are likely to contribute to residual transmission: a limiting factor in the maximum effectiveness of IVMs (Benelli & Beier, 2017). Accordingly, in areas where mosquitoes are exophilic, exophagic or can enter houses but rapidly exit again; malaria transmission is likely to persist despite a scale-up of current IVM strategies (Killeen et al, 2016). The issue may also be compounded by shifts in vector behaviour (Sougoufara et al, 2020).

It is evident that in areas of self-sustaining levels of residual transmission, elimination of malaria cannot be achieved by the use of ITN and IRS alone, even if such were to be applied universally against a fully insecticide-susceptible vector population (Killeen et al, 2017).

1.8 Attractive Sugar Bait Stations

The application of insecticides on both walls and bednets inside houses to kill mosquitoes has reduced malaria transmission (Bhatti et al, 2015). While these methods have been successful at removing the majority of vectors in many settings, there remains to be residual transmission. New methods are needed that will protect the entire community continuously, won't contribute to insecticide-resistance and won't interfere with the tools already in use. Insect baiting to dissuade a behaviour or induce mortality is not a novel concept (Fiorenzano et al, 2017). Mosquitoes are liquid feeders and toxins incorporated into a feeding stimulant (Xue et al, 2006) and placed into environments where mosquitoes are resting such as larval habitats and foliage near host habitats have been successful at reducing the densities of targeted mosquito populations (Allan, 2011). The use of sugar bait stations to control malaria transmission have been largely unexplored and could be an important area of research in the future (Xue et al, 2006, Meza et al, 2020).

Mosquitoes, like other Diptera, must intermittently seek carbohydrates sources throughout their life. By mimicking the scent of naturally occurring sugars, it is possible to attract mosquitoes

into an insecticide-laden trap (Fiorenzano et al, 2017). Typically, these stations use attractive toxic sugar bait (ATSB) which includes a number of oral insecticides manufactured from readily available inexpensive ingredients in tropical and sub-tropical areas (Allan, 2011). The outdoor use of ATSB stations aims to reduce residual outdoor transmission without the need for indiscriminate insecticide spraying (Müller t al, 2010b) which has previously proven to have dire consequences for the surrounding environment (Reigart &Roberts, 1999). The use of ATSBs has therefore been suggested as a potential alternative to managing the development of insecticide-resistant mosquito species (Stewart et al, 2013). There have been countless studies using ATSBs with a variety of active ingredients: tolfenpyrad (Stewart et al, 2013) dinotefuran (Dethier, 1947), Eugenol (Qualls et al, 2014), Boric acid (Qualls et al, 2015, Xue et al, 2006) and Spinosad (Müller et al, 2010a). Both rudimentary ATSB and commercially available ATSB methods have shown significant success in controlling multiple genera of mosquitoes (Fiorenzano et al, 2017).

It was Lea who, in 1965 pioneered the ATSB methods in laboratory studies by integrating multiple concentrations of a toxicant, malathion into 20% sucrose solutions, which was fed to *Aedes aegypti* via treated paper. The mosquitoes readily ate the ATSB, resulting in over 85% mortality (Lea, 1965). Malathion is an irreversible cholinesterase inhibitor; its major metabolites are mono- and di-carboxylic acid derivatives, and malaoxon is a minor metabolite. Cholinesterases play an essential role in mediating neurotransmission in cholinergic synapses, binding to acetylcholine at the nerve junction, effectively ending the stimulation to the next neuron (Tchounwou et al, 2015). When bound to malaoxon (Fiorenzano et al, 2017), the acetyl cholinesterase (*AChE*) enzyme can no longer catalyse the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid (Reigart & Roberts, 1999). Acetylcholine accumulates at the nerve junction and results in overstimulation of the nervous system (Ramos et al, 2006).

ATSB for mosquito control was originally developed in Israel in an arid habitat with relatively little sugar source vegetation (Müller & Schlein, 2006). In the setting studied, it was feasible to cover a high proportion of the local sugar sources with ATSB. This method was hugely successful in Israel with two reports of *Anopheles sergenti* population being reduced by over 95% (Beier et al, 2012). Following this trial, this same concept of applying ATSB in a semi-arid area with low levels of sugar source vegetation was trialled in Mali. Again, the ATSB drastically reduced the local *An. gambiae s.l.* population by 90% (Müller et al, 2010b). Here too, the application of ATSB to a large percentage of the locally available sugar source vegetation was feasible due to the low levels of locally competing sugar sources.

Though ATSB stations were not as successful as spraying ATSB directly onto vegetation in these settings, ATSB stations (Figure 6) have been successful when placed in mosquito resting and breeding sites (Müller et al, 2010b) such as water cisterns in Israel and storm drain systems in Florida, USA (Müller & Schlein, 2008) and effectively take advantage of the fact that mosquitoes emerge to adulthood with critically low energy reserves and tend to a require a sugar meal within the first few hours (Foster, 1995).

Figure 6. The JennyPro ATSB station is designed to be easily placed on any surface (Westham Co., 2018).

The use of ATSB stations can bring about a dramatic decline in the population of local mosquitoes. In Israel, the ATSB stations placed in water cisterns caused a dramatic decline of *Anopheles claviger* reducing the number of human-landing mosquitoes in the area by more than 10-fold (Müller & Schlein, 2008). In Florida, 83.7% of the *Cx. Quinquefasciatus* females were reduced from the storm drains over an eight-day period (Müller et al, 2010c).

The 'attract and kill' method of insect control is not a recent innovation (Deither, 1947), but methods in which it can be utilised is still evolving. As the method stands now, ATSB stations are recommended for use in environments with low levels of competing sugar sources (Xue et al, 2006) and to areas with relatively few localised sources of mosquitoes such as in breeding and resting sites. Furthermore, ATSB use has been found to be more effect when used in combination with ITNs (Stone et al, 2012) which could restrict its effective application from most of malaria burdened sub-Saharan Africa and drastically reduce the effective range. Applied indiscriminately, the method would likely see little to no effect for mosquito control. Yet, when applied with appropriate entomological insight (Fiorenzano et al, 2017), it has proven to be a powerful tool for IVM and there is clearly considerable potential for future large-scale application.

1.9 Malaria in the Mosquito

The sexual reproduction of *Plasmodium* (Figure 7) occurs only within the mosquito's midgut lumen (Hajkazeman et al, 2020). Because sexual development is essential for transmission to the next human host and completion of the lifecycle, this stage can be considered a major focus for malaria control (Aly et al, 2009). During a blood meal (Boyd & Kitchen, 1939), the female mosquito ingests gametocytes (Al-Olayan et al, 2002). The stage V gametocytes (Carter & Miller, 1979) are activated by an increase in calcium concentration, a decrease in temperature and acidity and the presence of the mosquito-derived metabolic intermediate xanthurenic acid (Billker et al, 2004). The gametocytes mature into microgametocytes (male) and macrogametocytes (female). Once activated the microgamete undergoes three rounds of DNA synthesis and mitosis within 12 minutes to generate a cell with an 8n genomic complement (Gerald et al, 2011). The genome then separates to produce eight flagellated haploid microgametes on the surface of the microgametocyte. There are several factors considered to be important to the induction of flagellation including a rapid rise in intracellular calcium, activation of phospholipase C and guanyl cyclase which ultimately activate the cGMP-dependent protein kinases (PKG) (Sinden et al, 2010). During exflagellation, the microgamete detaches from the residual body and becomes freely motile. The macrogametocyte is also activated by exposure to mosquito factors resulting in a sudden onset of protein synthesis, but DNA replication does not occur. This gamete exits the erythrocyte, allowing a microgamete to attach, fuse and produce a diploid zygote (Al-Olayan et al, 2002).

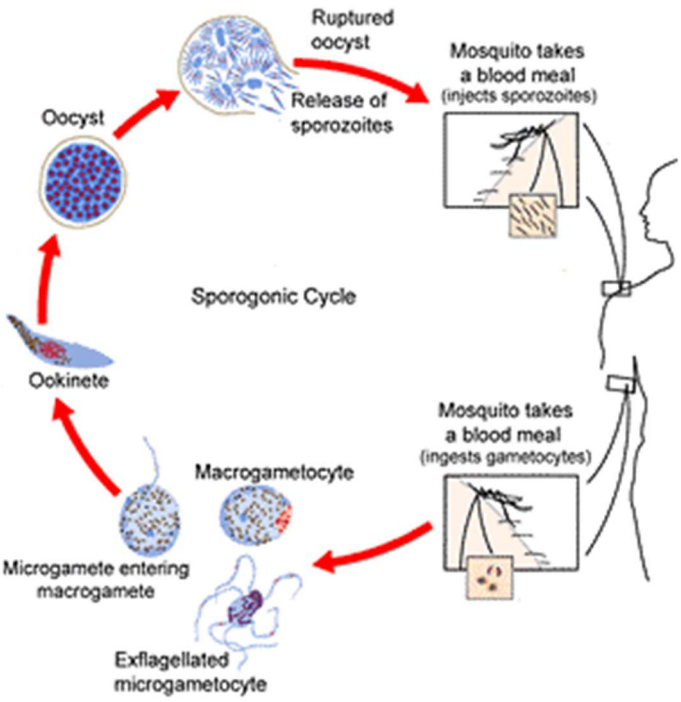

Figure 7. The mosquito stages of the Plasmodium lifecycle following a blood meal (adapted from CDC, 2017).

Nuclear fusion in the zygote is followed by approximately 3 hours of DNA replication and meiosis (Janse et al, 1986), with the zygote becoming tetraploid. The transformation into a motile ookinete is completed at 19-36 hours post blood feeding (Ngwa et al, 2016). These traverse the midgut epithelium to embed themselves in the basal lamina beneath the midgut epithelial wall and differentiate over 10-12 days into sessile oocysts (Beier, 1998).

Over the course of several days, an individual oocyst undergoes 10-11 rounds of DNA replication to create a sporoblast with thousands of nuclei (Rosenberg & Rungsiwongse, 1991). In an enormous cytokinesis event, thousands of haploid daughter sporozoites assemble from the surface of the mother cell (Gerald et al, 2011). The oocysts eventually rupture to release thousands of sporozoites into the mosquito haemolymph. The sporozoites then migrate in the haemolymph before attaching to the basal side of the salivary gland acinar cells. They traverse the acinar cells and enter the ducts on the salivary glands and can be subsequently transmitted when the mosquito takes another blood meal (Circimotich et al, 2010).

The sporogonic cycle represents the longest replicative process of the *Plasmodium* lifecycle and, depending on the species, lasts between 12-16 days (Hajkazemian et al, 2020). The female mosquito lives for roughly 30 days (McDonald, 1977), repeating the process of blood-feeding and oviposition every 3-4 days (Smith & Jacobs-Lorena, 2010). The parasite utilises the insect's cyclic feeding pattern for its transmission between vertebrate hosts. To achieve this transmission, plasmodia have evolved strategies to evade the mosquito's immune defences whilst maintaining a low virulence to their insect host (Hajkazeman et al, 2020). From the mosquito's perspective, it has been suggested that permissiveness to low numbers of the parasite is advantageous due to the high cost of mounting an immune defence (Niaré et al, 2002). There is contradictory evidence as to whether the *Plasmodium* reduce the lifespan of the mosquito (Dawes et al, 2009) or not (Hogg & Hurd, 1997), with some positing the greater virulence was observed due to studies using vector-parasite combinations that are not co-indigenous to one another (Dong et al, 2006). The results of studies observing mosquito fecundity as an indicator for *Plasmodium* virulence are much more consistent (Alout et al, 2016, Vézilier et al, 2012). Furthermore, while studies have confirmed a significant reduction in the number of eggs laid by infected mosquitoes, it is also hypothesised that infection may be detrimental to other indirect fitness determinants such as larval hatch rate (Hajkazemian et al, 2020), mating success and flight ability (Dao et al, 2010). There is evidence that, during oocyst proliferation, the parasite acquires energetic resources such as lipids, sugars and proteins (Atella et al, 2006, Ball & Chao, 1976) and thus, the mosquito may use subsequent feeding to refill their energy stores before producing eggs (Hurd et al, 1995).

Female hematophagous anopheline feed on sugar sources (Foster, 1995) to provide them enough energy to serve their physiological needs (Van Handel, 1984). Of the sporogonic cycle, oocyst development is relatively long and is the only extracellular stage of the *Plasmodium* lifecycle, making it a clear target for malaria control techniques (Aly et al, 2009). Sugar baits stations offer unique vehicles of insecticide delivery because the sugars trigger an automatic tactical feeding response causing the insect to ingest any integrated active ingredients (Fiorenzano et al, 2017).

There is a growing realisation that IRS and ITN are highly effective against some species but not as effective against others (Killeen et al, 2016). IRS has eliminated localised populations of

Anopheles funestus in Tanzania due to its habit of resting indoors after feeding (Bruce-Chwatt, 1973). The scale-up of ITNs has been highly effective at reducing the density of anthropophagic and endophagic disease vectors such as *An. gambiae* but less effective at controlling zoophagic and exophagic disease vectors such as *Anopheles arabiensis* (Russel et al, 2010). The unilateral targeting of vectors has likely been a contributing factor leading to a species shift to more zoophagic and exophagic disease vectors (Kitau et al, 2012). ATSBs would likely target an additive subset of vector species.

1.10 Compounds with Sporontocidal and Gametocidal Activity

Further testing to optimise feeding by wild populations of vector mosquito species is a key component for the future development of this method and should be of significant focus for future trials (Müller et al, 2010c). In conjunction with attractant optimisation studies, bait station design and positing have also been observed as playing significant roles in mortality optimisation with ATSBs and thus should not be overlooked. Antimalarial drugs in humans limit the disease by killing the *Plasmodium* parasite in their exo-erythrocytic and erythrocytic stages. There has been very little research done in which an antimalarial medication was introduced to mosquitoes through direct contact.

Currently, the antimalarial efficacy of compounds can be determined via several methods; *in vivo* therapeutic efficacy studies, *in vitro* tests, molecular marker studies and measurement of drug concentrations (Maji, 2018, Adjalley et al, 2011). Previously, studies (Butcher et al, 1989) used the standard membrane feeding assay (SFMA) to screen the transmission-blocking effect of the curative administration of antimalarial compounds. Mature stage gametocytes are incubated with the compound of interest for 24 hours and then fed to *Anopheles* mosquitoes through an artificial membrane. After a number of days, the mosquito midguts are examined and the parasitaemia is determined using light microscopy to count the number of oocysts. A similar approach was used by Terzian (et al, 1968) to test compounds for their antimalarial properties. This method is limited to primary drugs and is much less reliable than current screening methods. Terzian and colleagues (1968), however, did confirm the sporontocidal activities of cycloguanil (an active metabolite of proguanil) and pyrimethamine. The prime purpose for developing anti-plasmodial compounds is obviously to produce a drug to prevent or cure the infection in patients. It is suggested that the sporontocidal and gametocidal properties of these compounds could also be used to reduce the prevalence of the disease in the population by reducing the onward transmission of the parasite by the vector (Wadi et al, 2019). Sinden (et al, 2012) proposed that antimalarial drugs, or their metabolites, may be able to travel to the mosquito midgut where they can block the parasite either

by being directly toxic or by disturbing the fine-tuned mosquito physiological pathways that are essential for parasite development.

Quinine has only a limited effect on gametocytes, killing only the young forms of the parasite; patients may be infective for up to 21 days after treatment (Klein et al, 1993). Mature gametocytes of *P. falciparum* are effectively at a resting stage and are therefore less vulnerable to drugs such as chloroquine and other compounds which MoA affects haemoglobin digestion (Foote & Cowman, 1994). Drugs with the most impact on mature gametocytes (i.e. primaquine, artemisinin) have more general cytotoxic properties with mitochondria usually the main target (Butcher, 1997). Drugs targeting nucleotide metabolism (i.e. pyrimethamine, proguanil and atovaquone) have no effect on mature gametocytes (Fry & Pudney, 1992). Atovaquone prevents recruitments of young gametocytes of *P. falciparum in vitro* and has been shown to target dihydroorotate dehydrogenase of mitochondria (Ittarat et al, 1994).

Paton and colleagues (2019) coated a petri dish with atovaquone and allowed the mosquitoes to land on the treated surface for approximately six minutes. Following this, they were provided with a blood-meal containing plasmodia and a week later none of the treated insects harboured the parasite. Furthermore, the atovaquone had no notable effect on the mosquito, meaning there is no selection for them to develop resistance. Although the plasmodia may develop resistance, this outcome would be more manageable than insecticide resistance.

1.11 Novel Antimalarials

The emergence of new drug resistant strains of *Plasmodium* against currently available antimalarial agents is a key concern for those striving for malaria elimination and eradication (Butcher, 1997). There is a general consensus that monitoring the efficacy of different antimalarial drugs and identifying novel antimalarial agents is critical. Over the previous two decades there has been a heartening surge in antimalarial drug development with product development partnerships taking a lead role. Globally, there are many agents in clinical development, most of which are blood schizonticides for the treatment of uncomplicated falciparum malaria. To begin with, the objective of such research was to develop a single dose radical cure, however emphasis now rests of cell-based screening to identify phenotypic changes that occur following *Plasmodium* exposure to drug candidates. This method does not require prior knowledge of the drug target and enables the quick elimination of compound unable to permeate the cell membrane.

Gardner at colleagues (2002) were the first to sequence the *P. falciparum* genome using the 3D7 isolate of African origin. Comprising of 22.8 megabases distributed over 14 chromosomes, the genome was incredibly rich (>80%) in adenine/ thymine bases and encoded approximately 5,270 genes (Gardner et al, 2002). By visualising the *Plasmodium* genome and being able to compare it

with the human genome, researchers can discover parasite-specific, novel molecular targets for malaria therapy and prevention (Ntoumi et al, 2007). The Medicines for Malaria Venture (MMV) is a non-profit public-private partnership who aims is the discovery, development and launch of small molecules anti-malarial agents (MMV, 2020). Towards this end, MMV coordinated screenings of over 5 million compounds against *P. falciparum in vitro* and generated a list of 20,000-30,000 hits in the sub-micromolar range (Ullah et al, 2016). Cluster analysis (through the Pareto protocol) and commercial availability reduced this to a set of 400 confirmed blood-stage active antimalarial compounds with chemical diversity- the 'Malaria Box'. Typically, organisations are reluctant to publish the full hit sets produced in High Throughput Screening (HTS) meaning drug development is time-consuming and laborious. The welcome decision to share this data allowed new projects to start within many research communities globally (Spangenberg et al, 2013).

The application of HTS technologies to the differing stages of *Plasmodium* are receiving an increased interest as a necessary addition to efforts in antimalarial drug discovery. Innovation in technologies and novel MoAs become increasingly important in an area of emerging antimalarial resistance and the plateau in declining malaria incidence (Yahiya et al, 2019). A single dose of any new generation anti-malarial, much like the traditionally used anti-malarial compounds, when administered alone, will not fully clear sexual or asexual stage parasitaemia. Treatment with three or four drug combinations is becoming standard in other diseases such as Tuberculosis (Wallis et al, 2016)), HIV/ AIDS (Bhatti et al, 2016) and neonatal strep (Du Pont-Thibodeau et al, 2014).

Ideal candidates would have an immediate effect to rapidly eliminate the plasmodia, minimally as fast as chloroquine but ideally as fast as artesunate (Burrows et al, 2013). The additional challenges in antimalarial drug development include: the risk of haemolysis in patients with a G6PD deficiency, treatment for drug-resistant strains, paediatric dosing, serious drug-drug interactions, transmission blocking and relapse prevention. Within a relatively short period, MMV and its academic partners have established an impressive portfolio of antimalarial drug candidates, some of which are in the advanced stages of development (Fairlamb et al, 2005). Furthermore, open access to the Malaria Box has led to the discovery of newly identified interactions against various pathogens of human and livestock. MMV688934 (Tolfenpyrad) has displayed promising activity against helminths. Also, MMV688768 was shown to demonstrate inhibitory activity against *Candida albicans* biofilm formation (Patra et al, 2019).

The emergence and spread of drug-resistant pathogenic strains is a risk for any treatment. Potential anti-malarial candidates must be active against the greatest possible number of *Plasmodium* strains (Zani et al, 2014) without exhibiting any emerging resistance against laboratory *Plasmodium* species with known drug-resistance mutations or primary clinical isolates, such as those from geographical regions known for anti-malarial resistance. The risk of resistance selection must be thoroughly assessed *in vitro* to determine how often relevant genomic changes occur, how

easily they are selected, their fitness cost and transmissibility relative to the wild-type parasites (Burrows et al, 2017).

The Malaria Box includes 200 drug-like compounds which have 'Rule of Five' -compliant physiochemical properties as a starting point for oral drug discovery (Spangenberg et al, 2013). These compounds do not have highly selective activity profiles and thus, many elicit their actions through polypharmacology (Reddy & Zhang, 2013). These may be incorporated into future an orally absorbed drug. In addition, the Malaria Box includes 200 probe-like compounds which represent the broadest cross-section of structural diversity and are proposed to be useful as tools for probing biological mechanisms (Spangenberg et al, 2013).

From a drug discovery perspective, probe-like compounds are integral in the validation of new molecular targets for a therapeutic indication. Probe-like compounds do not meet the same requirements in terms of pharmacokinetics, pharmacodynamics and bioactivity as the drug-like compounds (Workman & Collins, 2010) and thus, are unlikely to be selected for use on humans.

Most of the Malaria Box portfolio was originally identified from screening against asexual blood schizonts and many compounds have exhibited activity which prevents the production of gametocytes as well as hepatic schizonts (Burrows et al, 2017). The compounds had varied activities against the different clinical isolates, suggesting that different parasitic proteins were being interacted with. There has been very little enquiry, however, into whether these compounds are active against the sporogonic *Plasmodium* form.

2 Aims

The aim of this study is to pose the question as to whether attractive sugar bait stations could be used to eliminate *Plasmodium* within the mosquito population. To do this, a compound would be required to have a nanomolar potency against both the asexual and sexual stages of *P. falciparum* while maintaining relative stability in field-like conditions. For such a method to be biologically safe, the compound used would have to have been identified as unlikely for use in humans. The format of the study was affected by limitations put onto travel and laboratory-based research which were brought about by the current Covid-19 pandemic. The project was, therefore, redesigned to include reviews of the current literature, as well as concurrent research carried out in collaboration with colleagues in Ifakara, Tanzania. The revised objectives were as follows:

- To evaluate the efficacy of the implementation of ATSB stations in the socioeconomic setting of Tanzania (pg.24).
- To assess the existing evidence of sporontocidal and gametocidal activities of compounds and gain an un understanding of the various methods used to understand these (pg.31)
- To identify a series of methods through which the viability of anti-plasmodial compounds can be investigated for their application into a mosquito bait station (pg.39).

3 Review of ATSB Trials in Tanzania

This systematic review will assess the existing evidence regarding the implementation of ATSB stations in Tanzania which was published prior to January 2021. A search of PubMed and WebofScience was performed independently using predetermined criteria. Further, abstracts from selected articles were screened, and reference scanning of the search results was performed. Only three of the studies met the selection criteria and were included in this review. The data collected in this review illustrates the limitations surrounding the current understanding of vector behaviour in Tanzania and the execution of ATSB programmes in resource poor communities. The review considers how ATSB stations could provide operationally simple, safe and cost-effective method for malaria vector control, particularly in LMICs such as those found in Tanzania.

Financial constraints form a major impediment in the implementation of malaria interventions in Tanzania. Thus, strategies such as ATSBs could be vital for mitigating the burden of malaria. Residual transmission occurs as a result of a combination of human and vector behaviours; LLINs and IRS cannot control mosquitoes that bite outdoors or that bite people before they go to sleep protected by a LLIN. The use of ATSB stations have proven to bring about a dramatic decline of local mosquito populations in studies (Müller and Schlein) and if optimised and used alongside current vector control methods, could prove useful in areas with mixed populations of susceptible and resistant mosquitoes.

In Tanzania and other parts of Eastern Africa, the dominant malarial parasite is *P. falciparum* and the main mosquito species are *An. gambiae*, *An. arabiensis* and *An. funestus*. Currently, *An. arabiensis* is regarded as the key vector of malaria due to its typically exophagic and exophilic habits limiting the efficacy of current vector control strategies. Furthermore, their affinity for urban environments and high degree of behavioural plasticity and rapidly increasing levels of insecticide resistance exacerbates their potential for continued survival. In view of this, ATSBs have been suggested as a means of introducing novel insecticides, in a fashion complementary to current techniques, with the aims of to managing resistance, preventing malaria resurgence and maintaining the drive towards malaria elimination.

Search Methods

A search of peer reviewed literature using ‘attractive toxic sugar baits’ and relevant synonyms in published in English before January 2021. Searches were then further screened to only include studies that had occurred in Tanzania. Searches were made using two of the largest databases for life sciences and technology, PubMed and WebofScience, thus enabling a comprehensive search. The three studies identified shown in Table 2.

Table 2. The studies selected for the review of ATSB trials in Tanzania.

Stewart et al, 2013	Indoor Application of Attractive Toxic Sugar Bait (ATSB) in Combination with Mosquito Nets for Control of Pyrethroid Resistant Mosquitoes.
Tenywa et al, 2017	The development of an ivermectin-based attractive toxic sugar bait (ATSB) to target Anopheles arabiensis.
Maia et al, 2018	Attractive toxic sugar baits for controlling mosquitoes: a qualitative study in Bagamoyo, Tanzania

Review Findings

Stewart et al (2013) conducted a bioassay in which to study the mortality rate as a result of the ingestion of three oral insecticides. Chlorfenapyr (pyrrole), tolfenpyrad (pyrazole) and boric acid (inorganic acid) are insecticides with different modes of action, identified for their activity against phytophagous pests and have not been used widely in public health. Three different mosquito strains were evaluated including the F_1 generation of *An. arabiensis*, collected from Lower Moshi, Tanzania. In the laboratory-based study, chlorfenapyr (0.5% v/v), tolfenpyrad (1% v/v) and boric acid (2% w/v) killed 91%, 83%, 89% respectively of the *An. arabiensis* within 24 hours compared to the 4% in the control group.

A similar bioassay was utilised by Tenywa et al (2017) to determine the mortality rate as a result of the ingestion of ivermectin at a range of concentrations. Ivermectin (endectocide) is a commonly used anthelmintic agent. The injectable form, Ivomec®, is widely available in rural Eastern Africa as communities as it is commonly used as a treatment for parasite infections such as gastrointestinal roundworms in cattle and other livestock. The drug does not lose its efficacy when stored ≤30°C which is of particular importance in Tanzania where regulated pharmaceutical storing facilities are sparse. Compared to the control group, significant mortality of *An. arabiensis* (Ifakara strain) was observed 24 hours post introduction of the sugar meal containing as little as 0.005% ivermectin. In the laboratory-based study, ivermectin (0.01%) killed approximately 50% of the *An. arabiensis* within 24 hours compared to the control (mortality value unprovided). Furthermore, ivermectin (0.01%) resulted in 95% mortality within 48 hours post feeding.

Although slower in producing the mortality figures produced by the compounds studied by Stewart et al (2013), ivermectin is considered less toxic than chlorfenapyr, tolfenpyrad and boric acid. Ivermectin has a proven safety record in humans and is approved for use in humans, whereas there is a variety of toxic effects noted for chlorfenapyr, tolfenpyrad and boric acid.

The safety concerns of ATSB were raised by community members involved in the qualitative study conducted by Maia et al (2018) in Bagamoyo, Tanzania. In this study, participants expressed

they had safety concerns regarding toxicity of the bait solution and what effect it could have on their children should accidental ingestion occur. Although many of the individuals involved in the study pro-actively addressed this concern by hanging the ATSB stations out of the children's reach, product development must choose a toxicant with very low or negligible mammalian toxicity.

Natural sugars sources are vital to mosquito survival; they are an essential source of energy for females and the only food source for male mosquitoes. In order to be successful, ATSB methods must be as attractive, if not more so, than the available natural plant sugar sources to the local mosquito populations. Although the host-seeking behaviour of *An. arabiensis* is well characterised, its sugar feeding behaviour is poorly documented. In general, mosquitoes are selective in their attraction to the sugar sources i.e. flowers, fruit, seedpods of indigenous plant species. Similar to methods of blood-meal location, the selection of a sugar source is thought to depend on numerous factors including visual and olfactory stimuli. Stewart et al (2013) did not compare different attractants; the study used a solution made up of 35% v/v guava juice and 10% w/v brown sugar.

In Bagamoyo, Tenywa et al (2017) designed an experiment in which to identify the most attractive sugar source to *An. arabiensis* using semi-field conditions. The mixtures were prepared using 10% sucrose solution added to the pulp of the following locally sourced fruits: papaya, banana, tomato, mango, orange and watermelon. Non-locally sourced guava juice was also included in the study. *An. arabiensis* preferred to feed on orange (*Citrus sinensis*) to the other fruit juice, resulting in the highest number of sugar fed mosquitoes, however it was not significantly more attractive than the control solution (10% sucrose solution with no fruit) (Figure 8). The high level of attraction to the control solution is a compelling result, as fermentation of the fruit pulps could result in a limited lifespan for ATSB. Additional, being able to produce ATSB without the new of adding fruit juices will reduce the cost and may increase community compliance to ATSB uses.

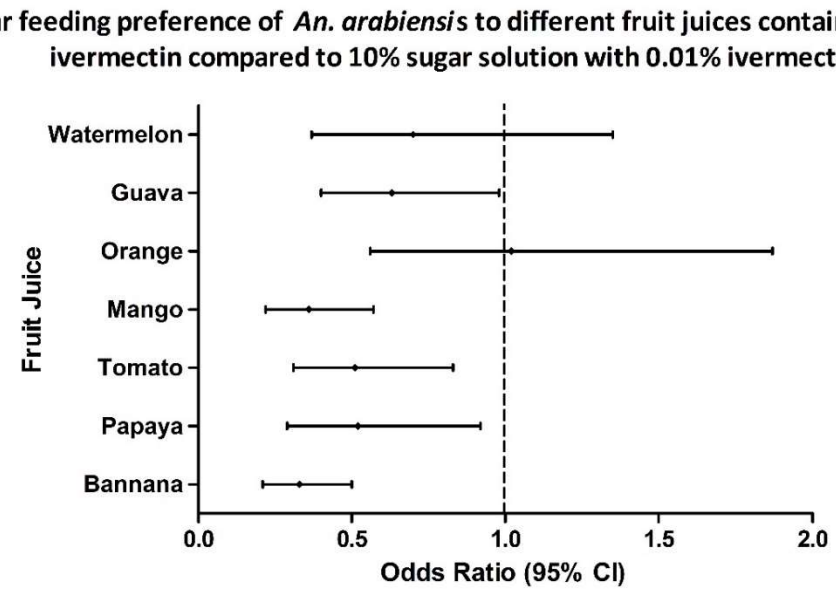

Figure 8. Investigating the most attractive sugar source to *An. arabiensis* mosquitoes in semi-field conditions (data adapted from Tenywa et al, 2017).

The experimental hut studies carried out by Stewart et al (2013) used bait stations in which cotton towels were positioned over plastic drip trays that served as reservoirs for re-absorption of solution by the towel wicks. The other studies in this review used bait stations made using basic materials commonly found in household waste reducing the associated costs and making their implementation more pragmatic for LMICs. Materials used included: different sized plastic water bottles, sponge from old mattresses, cloth and string.

Tenywa et al (2017) made prototypes with the aim of identifying which was most successful in attracting *An. arabiensis* to the sugary meal. Three different prototypes, denominated A, B and C were designed and assigned to pairs of experimental cages and tested using the orange juice concoction determined as the most successful in the previous objective. The prototypes significantly different in their ability to attract mosquitoes to sugar feed. Prototype B was 3-fold more likely to attract *An. arabiensis.* This prototype was made by cutting a 12L bottle in half, fitting a piece of sponge onto the bottom, and lining it with black cloth (Figure 9). Mosquitoes were drawn to rest on the dark and damp walls of the bucket-shaped bait, where they could be easily tempted to feed on the sugar source available.

Figure 9. Prototype B design (Tenywa et al, 2017).

Maia et al (2018) demonstrated the production of the bait station to the study's participants. The majority were happy with the design of the baits and liked the fact that they were made with locally available materials and did not require officials to distribute them. There was, however, a consensus that the station required a logo that would delineate with mosquito control. The need for identification on the stations was requisite to their implementation in the more rural communities where the baits were linked to witchcraft.

Female mosquitoes are known to quest for sugar before and after obtaining a bloodmeal so to maintain their fitness throughout their life. To exploit this behaviour, an ATSB must be strongly compete with the natural sugar resources available. The placement of the ATSB stations is therefore key to the number of mosquitoes effected by the control strategy. Each of the studies in this review used field trials to determine the optimum positioning of ATSBs at different peridomestic locations.

Stewart et al (2013) investigated the indoor application of ATSBs in combination with mosquito nets using four experimental huts in the Kilimanjaro Region, Tanzania. Previous studies in this area had confirmed the *An. arabiensis* population to be resistant to pyrethroid insecticides, which is more than likely due to local ITN and IRS campaigns. If ATSB stations could be distributed using underutilised insecticides (chlorfenapyr, tolfenpyrad and boric acid) they present an important new method to be used alongside older methods for controlling populations of resistant mosquitoes inside the home. In the study, four ATSB stations were hung from the ceiling at the corners of an

untreated bednet (Figure 10) and another 3 were positioned near each window, with one in the centre and two on either side. An adult volunteer slept in each hut under an undamaged untreated mosquito net from dusk until dawn for sixteen nights and each morning the number of dead and live mosquitoes was counted.

Figure 10. Bait station layout used to assess the indoor application of ATSBs alongside ITN use (Stewart et al, 2013).

The difference between the control group (no insecticide added to stations) and that of the groups exposed to the ATSB was significant for all three insecticides implemented. The control mortality of *An. arabiensis* was 18%, while the mortalities for the exposed groups were 48%, 45% and 41% for chlorfenapyr (0.5% v/v), tolfenpyrad (1% v/v) and boric acid (2% w/v) respectively. Although none of the treatments were able to kill over half of overall mosquitoes in trial, the overall level of mortality observed is still considerable. Furthermore, Stewart et al (2013) hypothesised that as with other forms of indoor vector control, there will be a cumulative effect of the insecticide as the individual female mosquito returns to feed after each gonotrophic cycle. ATSBs could therefore have significant impact on the lifespan of the mosquito population, which is an essential factor in malaria transmission.

Tenywa et al (2017) used similar experimental huts in Bagamoyo Region with the addition to a biodome. The biodome provided a control environment while maintaining similar climatic conditions as outdoors. This semi-field system enabled the investigation of 24 potential ATSB deployment locations both within the home and the surrounding area. Due to the design of the experimental huts, once inside, the mosquitoes could not leave other than through exit traps. The ATSB used in each station position was dyed a different colour to enable the identification of where each of the mosquitoes had fed. Laboratory raised *An. arabiensis* (Ifakara strain) were released into the biodomes each night for sixteen nights and two adult volunteers slept in each hut under either an intact LLIN or an undamaged untreated bednet.

Of the 55% of mosquitoes that were recaptured (e.g. in exit traps, resting on surfaces both inside and outside), 49% of the recaptured mosquitoes had taken a sugar meal. The majority (51%) of the recaptured sugar-fed *An. arabiensis* fed on baits deployed outdoors close to the vegetation (*Ricinus communis*) and a 66% were observed to feed (all outdoor ATSBs) before being attracted to enter the hut in search of a blood meal. This suggests that despite the presence of a nearby blood meal, the mosquitoes often chose to sugar-feed first.

While inspecting the presence and colour of the food dye in the mosquito midgut, Tenywa et al (2017) also investigated whether the mosquito had half or fully sugar fed. Interestingly, most of

the mosquitoes that fed indoors were observed to be fully engorged on the sugar solution, while a higher proportion of the outdoor-feeding individuals only took half a sugar meal. Considering the amount of sugar meal taken by mosquitoes with respect to bait deployment locations; mosquitoes that fed outdoors may have still intended to take a blood meal, thus energy was only required for the flight in search of this and did not fully feed sugar in order to maintain enough space in the midgut for blood.

Although not statistically significant, fewer mosquitoes were caught in the huts with LLINs compared to those with untreated bednets. The insecticides used on LLINs repel mosquitoes, reducing the number in the hut. Without this deterrent, it was suggested that mosquitoes were more likely to enter the hut without first obtaining a sugar meal. The availability and surmised convenience of the meal sources is known to play a great role in mosquito feeding choices. Upon nearing the inaccessible blood-meal and depleted of energy from the flight, the mosquitoes were forced to opted for the convenience of the sugar meal. With the alternative meal source protected by the bednet, the mosquito fully engorges on the sugar solution. This suggests that, although not as successful as outdoor placement of ATSBs, they can still be effective indoors if used in combination with a bednet.

Following the study carried out by Tenywa et al (2017), Maia et al (2018) encouraged their participants to place their ATSB stations outdoors amidst vegetation. Despite the explanation behind this placement, participants were sceptical about placing the baits outside their homes, as there was little trust that this placement would kill the mosquitoes responsible for biting them. Following the two-week experiment, over half of the participants affirmed they had brought the ATSB inside their homes, where they claimed the bait would be more efficient as that was where they were primarily bitten. Further to this, participants asserted that they placed ATSBs inside for multiple reasons; a few said they have preferred to place it inside to avoid accusations from neighbours mistaking it for witchcraft, others said due to living in rented houses with other tenants and thus had no control over the yard area. Additionally, those living in the more urbanised areas feared the ATSBs would be stolen or vandalised if left outside.

ATSBs were not tested in the absence of mosquito nets in any of the studies. It is doubtful whether the hematophagous mosquitoes would be diverted from a host to the ATSB stations were the physical barrier not in place. It seems unlikely that indoor ATSB would provide protection in the absence of a net, but this has yet to be demonstrated and would be unethical to do with human volunteers. It is more than likely that mosquitoes become diverted to the sugar source after expending energy in an unsuccessful attempt to source a blood meal from the human host.

Review Discussion

The ATSB constitutes an underutilised mode of insecticide delivery that offers the prospect of harnessing the use of novel classes of insecticide with the ultimate aim of malaria control and elimination. The use of recycled materials, locally produced attractants, and the local availability of Ivomec® suggest ATSBs could provide a cost-effective strategy for targeting the most important vector of malaria in Tanzania and other resource poor settings. The behaviour and responses of *An. arabiensis* demonstrated in the studies has significant implications for the deployment of ATSBs; however, these behaviours still need to be studied in both sugar rich and sugar poor environments as competing sugars will likely influence the effectiveness of any intervention intended to kill mosquitoes by exploiting their sugar feeding habits. Moreover, identifying such techniques are futile if the communities do not perceive a benefit from them. As opposed to LLINs and IRS, ATSBs do not directly protect users from mosquito bites and do not trap the mosquitoes, thus it would be appropriate to brief communities on mosquito behaviour prior to further trials and explain why the ATSBs are designed as they are.

4 Review of Compounds with Gametocidal or Sporontocidal Activity

This systematic review will assess the existing evidence investigating the sporontocidal and gametocidal activity of antimalarial compounds published prior to January 2021. A search of PubMed and WebofScience was performed independently using predetermined criteria. Further, abstracts from selected articles were screened, and reference scanning of the search results was performed. In total, seven of the studies met the selection criteria and were considered for the review. The data collected in this review illustrates the limitations surrounding the current understanding of the molecular processes that elicit the sexual differentiation and development of *P. falciparum.* The review does, however, highlight novel techniques aimed at reducing these gaps in knowledge. The research reviewed collectively makes significant movement towards the identification of compounds with gametocytocidal activities.

The treatment of malaria is based on the therapeutic efficacy against the asexual stages of *Plasmodium*. To achieve malaria eradication, chemotherapy is required to not only treat symptomatic infection but also to reduce transmission potential by reducing the carriage of gametocytes by the mosquito vector.

The sexual differentiation and development of *Plasmodium* is marked by a series of morphological changes and coincides with a distinct pattern of expression of sexual stage specific proteins. Biological studies of *P. falciparum* during the sporogonic cycle are very limited since they

cannot be produced easily in culture. This review poses the question, could antimalarial compounds be used to target the sexual stages of the *Plasmodium* life cycle?

Search Methods

A search of the peer-reviewed literature using the terms 'sporontocidal' OR 'gametocidal' AND 'falciparum' in science and technology published in English between 1st January 2000 and 1st January 2021. Searches were made using two of the largest databases for life sciences and technology, PubMed and WebofScience, thus enabling a comprehensive search. The searches were undertaken without restrictions regarding the compounds investigated and using keywords qualified by titles/ abstracts.

The following inclusion criteria were applied: peer-reviewed original observation studies describing the activity of compounds against the sexual stages of *P. falciparum*. Once screened, the included articles were classified by the class of compound being explored. The seven studies identified shown in Table 3.

Table 3. The studies selected for the review of compounds with gametocidal or sporontocidal activity.

Enosse et al, 2000	The mosquito transmission of malaria: the effects of atovaquone-proguanil (Malarone) and chloroquine	pg.33
Mockenhaupt et al, 2005	A randomised, placebo-controlled, double-blind trial on sulphadoxine-pyrimethamine alone or combined with artesunate or amodiaquine in uncomplicated malaria.	pg.34
Agomo et al,2008	Efficacy, safety and tolerability of artesunate-mefloquine in the treatment of uncomplicated Plasmodium falciparum malaria in four geographic zones of Nigeria.	pg.34
Panwar et al, 2020	Lead Optimisation of Dehydroemetine for Repositioned Use in Malaria.	pg.35
Singh et al, 2019	Computational and experimental elucidation of Plasmodium falciparum phosphoethanolamine methyltransferase inhibitors: Pivotal drug target.	pg.36
Moyo et al, 2020	Napthylisoquinoline alkaloids, validated as hit multistage antiplasmodial natural products.	pg.37
Ridgeway et al, 2020	Novel method for the separation of male and female gametocytes of malaria parasite plasmodium falciparum that enables biological and drug discovery	pg.38

Review Findings

Enosse et al (2000) conducted a study to contrast the impact of atovaquone-proguanil and chloroquine on the transmission of *P. falciparum*. Blood samples were taken from 65 infected adults before they were randomly allocated to treatment with either chloroquine (600mg followed by 300mg at 6 hr, 300mg on days one and two) or atovaquone- proguanil (1000mg/ day atovaquone and 4000mg/day proguanil for 3 days). At each time point, blood samples were obtained, centrifuged and the cells were resuspended with the patient's own plasma or a compatible control serum to 33% haematocrit. These sample were presented to *An. arabiensis* with the use of membrane feeders and 8 days after feeding, the mosquitoes were dissected for oocyst counts.

To pool the observations from different patients, mean gametocyte counts post-treatment were expressed as a percentage of those present pre-treatment. Similarly, the mean oocyst count of each batch of mosquitoes was expressed as a percentage of the equivalent count for day zero.

The gametocyte numbers in atovaquone-proguanil treated subjects declined to 68% by day 4, 31% on the end of the first week and 4% by the end of the second and to zero after 3 weeks. Further to this by day seven, no oocysts were observed in mosquitoes fed gametocytes with patient's plasma and only 6% of those fed with gametocytes in control serum. The prevalence of infection in mosquitoes fell from a mean of 19-20% in control and patient's plasma on day zero to about 1% for both groups on day 4. However, by day 14, the prevalence rose for both groups. It was hypothesised that the plasma concentrations of the constituent drugs had fallen below 'transmission-blocking' levels within 2 weeks.

The gametocyte numbers in chloroquine treated subjects declined to 75% by the end of the first week and 17% at 3 weeks. The prevalence of infection in mosquitoes declined on day 4, however by day 7 this number rose to over 140%. After 3 weeks, mosquitoes fed gametocytes in patient plasma has more oocysts that those fed control serum 72% and 17% respectively.

Enosse et al (2000) concluded that, chloroquine caused a marked enhancement of *P. falciparum* infection after three weeks and suggested this may be significant in the spread of chloroquine-resistant strains. In comparison, atovaquone-proguanil reduced gametocyte numbers more rapidly (half-life of 5.4 days compared to 11.1 days). Although atovaquone-proguanil reduced infectivity to the low level initially, the persistent 'drug-insensitive' gametocytes increased noticeably. Thus, whilst atovaquone-proguanil does not kill mature gametocytes, it is sporontocidal for *P. falciparum* at least for a week. The drug's efficacy, however, is compromised by the short duration of the effective drug concentration (7 days as presently formulated) compared to the long survival time of the infectious gametocyte (<14 days in this study).

Mockenhaupt et al (2005) conducted a study to contrast the impact of sulphadoxine-pyrimethamine (SP) alone and in combination with artesunate or amodiaquine on the transmission

of *P. falciparum*. Blood samples were taken from 438 children (aged 6-59 months) before they were randomly allocated to treatment with SP (25mg/kg sulphadoxine and 1.25 mg/kg pyrimethamine as a single dose), SP with artesunate (4 mg/kg/day artesunate for 3 days) or SP with amodiaquine (10 mg/kg/day for 3 days).

After a week, individuals receiving SP alone had the highest gametocyte prevalence (63.5%) and density (105.9%). This result was almost over two-fold of the gametocyte density of both the other alternate treatments. The reduced gametocyte numbers in the combination treatments reflected the effect of the additional drug on gametocyte survival and development. The gametocyte prevalence after a week of receiving treatment with SP and artesunate (14.7%) was lower than that of those receiving SP with amodiaquine (33.9%). Mockenhaupt et al (2005) concluded that the lower gametocyte numbers reflected the additional drugs' activity on gametocyte survival and density. Furthermore, while artesunate is suspected to have a gametocidal effect, the effect of amodiaquine on gametocytes is limited to the young stages.

Agomo et al (2008) conducted a study of artesunate-mefloquine treatment on the transmission of malaria. The trial was conducted across four geographical zones of Nigeria, all of which were recognised to be homogenous and hyperendemic for *P. falciparum* infection. Blood samples were taken from volunteers, and participants who met the studies criteria were separated into treatment group 1 (T1) made up of adults and treatment group 2 (T2) being children weighing less than 30kg. The two groups were allocated the appropriate three-day treatment regime of artesunate (4 mg/kg body weight/ day) and a single dose of mefloquine (25 mg/ kg body weight). Over the next four weeks, participants returned on set screening days to provide further blood samples. Thick and thin blood films were performed to investigate the prevalence and density of gametocytes in each of the samples.

The gametocyte clearance time was determine using the data gathered from both groups. The times were calculated as 42.0 hours and 45.6 hours in T1 and T2 respectively. Agomo and colleagues (2008) stated that these results validated artesunate-mefloquine treatment as a gametocidal treatment as it was successful in clearing gametocytes from peripheral blood.

The treatments currently used in the treatment of malaria are often regarded as inadequate due to sub-optimal safety profiles and the detection of drug-resistant *Plasmodium* strains. There is also further potential for the development of cross-resistance, in which resistance mutations against one drug confers resistance to any alternative drugs that have a similar structure or that share the same MoA. There is, therefore, substantial need for the discovery of new anti-malarial compounds and/ or the repurposing of drug previously unexplored for anti-plasmodial activity.

Drug repurposing involves the investigation of existing licensed drugs for new therapeutic purposes. This strategy provides an effective alternative to *de novo* drug design and would enable patients to receive new treatments quicker. The antiprotozoal compound, emetine dihydrochloride

has been previously recognised as a potent inhibitor of the multidrug-resistant strain (K1) of *P. falciparum*, however its use is limited by its toxicity. Panwar et al (2020) investigated the gametocidal activities two diastereomers of emetine, in a bid to determine the potential of these compounds as transmission-blocking drugs. Emetine blocks protein synthesis in eukaryotic cells by binding to the 40S subunit of the eukaryotic ribosome. By defining and understanding the target binding site of emetine, a structure-based approach can be used to predict emetine's efficacy against pathogens.

Panwar and colleagues (2020) first used molecular modelling techniques to predict the activity and potency values of the emetine diastereomers against the multidrug-resistant *P. falciparum* K1 strain. They used molecular docking to predict how the compounds were interact with the emetine binding region of the *P. falciparum* 40S ribosomal subunit (*Pf*40S) *in silico*. The docking scores of emetine was calculated as -7.2 kcal/mol and the scores of two diastereomers (R/S and S/S) were -7.3 kcal/mol and -6.5 kcal/mol respectively. The results suggested that the docked emetine geometry maps well not the binding site and that the R-S-dehydroemetine mimicked this alignment better than the bound pose of emetine more closely S-S dehydroisoemetine.

Panwar used an SYBR green-based assay to confirm the predictions made using the computational methods. Ring-stage *P. falciparum* (K1) cultures were treated with either of the two stereoisomers at a range of concentrations and dose-response curves were determined. These results were then used to calculate the concentration at which the two isomers inhibited 50% of growth *in vitro* (IC_{50}). As expected, R-S-dehydroemetine was much more potent than the S-S-dehydroisoemetine, resulting in an IC_{50} value over 29x smaller 72 hours post exposure.

Panwar et al (2020) performed a series of assays using synchronous cultures of *P. falciparum* (K1) at different stages of their asexual development. The concentration-dependent growth of schizonts and rings were recorded after 24-hour incubation with either of the two isomers. Consistent with the hypothesised target of the compounds, both were found to be more active in the late trophozoite/schizont stages. Furthermore, the transmission-blocking potential of isomers was assessed using *P. falciparum* (NF54 strain) by estimating their ability to inhibit gametocyte activation and maturation. The maturation of male gametocytes was evaluated by the process of exflagellation (detected via movement of surrounding erythrocytes) whereas the activation of female gametocytes was evaluation based on the specific expression of the *P. falciparum* s25 (*Pfs25*) protein. R, S-dehydroemetine displayed significant potency against both male and female gametes.

In a similar series of experiments to those carried out by Panwar et al (2020), Singh et al (2019) tested the *in vitro* schizonticidal, gametocidal and cytotoxicity of compounds identified using computational modelling. Firstly, *P. falciparum* (strain 3D7) genomic DNA was isolated from blood samples and two primers were designed based on the nucleotide phosphoethanolamine methyl transferase (PMT). Phosphatidylcholine (PC) is the most abundant phospholipid in the membranes

of *P. falciparum*. The parasite has two different pathways by which it can synthesize PC: the CDP-choline pathway and the serine decarboxylase-phosphoethanolamine methyltransferase (SDPM) pathway which utilise the host's choline and serine molecules respectively. The *P. falciparum* PMT (*PfPMT*) is critical to the synthesis of PC via the SDPM pathway. Singh and colleagues (2019) compared the *PfPMT* sequence of the 3D7 strain with that of an Indian isolate strain and observed both the functional and structural conservation.

The *PfPMT* protein is expressed throughout the asexual and sexual phases of the parasite's life cycle and the absence of its human orthologues has led to the suggestion that it may be used as a target for the development of novel antimalarials. Singh et al (2019) identified *in silico* the potential well-conserved active sites of the *PfPMT* protein.

Singh et al (2019) first prefiltered their compound library through the Lipinski 'Rule of Five' and disregarded any found to be carcinogenic or toxic. They then used molecular modelling techniques to predict the activity and potency values of the remaining library. Three hundred hits were found binding with amino acids within the *PfPMT* binding pocket. The compounds responsible for the hits were analysed and organised into seven clusters of compounds containing conserved features. The most druglike compound of each group was taken further for analysis *in vitro.*

Through the *in vitro* analysis of the reduced compound library using synchronous *P. falciparum,* Singh et al (2019) identified three compounds as showing good schizonticidal activities after 26-40 hours of incubation. Next, gametes of *P. falciparum* (strain RKL-9) were produced *in vitro* using hypoxanthine supplemented media. Cultures were treated with the three remaining compounds and dose-response curves were determined. These results were then used to calculate the IC_{50} values of the compounds on the gametes. Two compounds resulted in IC_{50} values <5uM. The two compounds, 6-[Amino-2-(4-fluorophenyl)-ethyl]-pyridin-2-yl-1H-pyrimidin-4-one (compound A) and N-[2-(3,4-Dimethoxyphenyl)-ethyl]-3-3[(tetrahydrofuran-2-ylmethyl)-amino)-acetamide (compound B), both contained heterocyclic furan, pyridine and pyrimidine chemotypes and were, through further testing, found to be non-toxic to human embryonic kidney cells 293 (HEK293).

Singh et al (2019) then went onto carry out in *vivo* studies using the compounds to treat *Plasmodium berghei* infected male albino mice of 12-16 months. The first dose was administered two hours post infection and three further doses were given at 24-hour intervals. On the fifth day blood samples were acquired and the percentage parasitaemia was checked using microscopy. The test compounds both displayed good inhibition activity when used both intravenously and orally. This finding reiterates the druglike and interaction properties of the compounds found *in silico.*

Moyo et al, (2020) studied a total of thirty naphthylisoquinoline (NIQ) alkaloids and their synthetic analogues as multistage antimalarial drug candidates. NIQ alkaloids are secondary metabolites exclusively produced by the tropical plant families, *Ancistrocladaceaea and Dioncophyllaceae*. These plants produce many organic chemicals as self-defence and based on their

MoA are predicted to possess antibacterial and antiprotozoal activity. The *in vitro* activity of NIQs and their synthetic analogues were first assessed against the synchronous asexual stages of drug-sensitive *P. falciparum* (NF54 strain) using SYBR Green I fluorescence assay. The 17 compounds that demonstrated anti-plasmodial inhibition were further screened against several multidrug-resistant strains and clinical isolates. A total of 15 of the compounds retained potency across all strains and clinical isolates. Further assays were used to evaluate the cytotoxicity of these remaining compounds on human hepatocellular liver carcinoma cell line (HepG2) *in vitro* and all of them demonstrated minimal to no cytotoxicity.

Driven by the goal to identify NIQs that were active against different stages of *Plasmodium* development, Moyo et al (2020) assayed the 15 remaining compounds using both early and late-stage *P. falciparum* gametocytes *in vitro.* To facilitate these assays, gametocytogenesis was induced from synchronised asexual *P. falciparum* parasites of the transgenic luciferase-expressing strain NF54$^{\text{-Pfs16-GFP-Luc}}$. The tested compounds displayed significant activity against both early- and late-stage gametocytes, whilst additionally inhibiting male gamete exflagellation.

The results of the assays conducted by Moyo et al (2020) collectively suggest that structurally unique dimeric NIQs, were active across all three of the distinct stages studied (asexual stages, gametocytes and male gametes). Alternatively, the monomeric NIQs and simplified analogues were distinctively selective against asexual parasites and male gametes, with moderate activity against gametocytes.

Moyo and colleagues recognised the compound, dioncophylline C, as a front runner from their studies, as it displayed all of the determined requirements of a novel transmission-blocking antimalarial compound: potency against both asexual and sexual *P. falciparum* life stages, no notable cross-resistance to chloroquine, good solubility and microsomal stability. This is a significant finding as a compound with inhibitory activity against all stages of the malarial parasite found in the human host could potentially be applied as a multistage-active antimalarial drug with the ability to stop the transmission of *P. falciparum* to the invertebrate host.

Most gametocyte-killing drug screens are not sex specific and overlook the fact that killing one sex of gametocyte would be sufficient to sterilise the parasite and block transmission. With this understanding, Ridgeway and colleagues (2020) designed a novel technique with the ultimate aim of separating and collecting cocultured *P. falciparum* gametocytes based on sex. Once sorted, the gametocyte populations could then be used to identify novel sex-specific markers using quantitative PCR and thus indicate compounds that would target gametocytes in a sex-specific manner.

A *P. falciparum* 3D7 strain that expresses a green fluorescent protein (GFP)-tagged gametocyte ATP-binding cassette transporter family member 2 (gABCG2) protein was used throughout by Ridgway et al (2020). The gABCG2 protein is female-specific and is locate in a singular round structure in the cytoplasm of gametocytes. Asexual parasites were cultured and induced to form

gametocytes with modifications to reduce asexual parasite proliferation. Gametocytes were incubated with Hoechst 33342 which selectively stains parasitized erythrocytes; hence females are positive for both GFP and Hoechst, while males are Hoechst positive but GFP-negative.

Following staining, Ridgeway et al (2020) were able to sort the cultured cells using a flow cytometry technique called fluorescence-activated cell sorting which separated the two groups based on forward-scatter, side-scatter and fluorescent signal data. This method identified all gametocytes based on the presence of Hoescht staining and identified the female gametocytes via an additional GFP signal. The success of this approach was evaluated by visually inspecting the sorted populations by microscopy and the purity of the sorted cells was assessed by subjecting the resulting populations to another round of cell sorting while maintaining the same gating strategy. The male and female gametocyte populations were re-gated as 99% and 97% respectively, indicating the purity and reproducibility of this approach.

RNA was extracted from collected male and female gametocytes and saponin-isolated trophozoites and used to validate novel sex-specific markers using quantitative reverse transcription (qRT-) PCR. A reference gene and potential were selected based on RNA-sequence profiles for each of the populations. For sex-specific gametocyte identification, the male-specific cysteine protein (P230) and the female-specific ookinete surface protein 25 (p25) had been previously described. Ridgeway and colleagues (2020) were able to identify two novel sex-specific markers, the putative male marker, *Pf3D71477700*, and the putative female marker, *Pf3D71447600*. The transcripts for these markers were >10-fold and >3-fold greater respectively than those currently used. The high sensitivity and improved specificity of these marker, in comparison to those currently used, provide a reliable means for more accurately measuring a gametocyte population's density and sex ratio. The method could therefore provide an alternative to current techniques of separating gametocyte populations that are expensive and labour-intensive.

Ridgeway et al (2020) further implemented this novel method to determine the sex-specific effect of eight MMV Malaria Box compounds (MMV006389, MMV007127, MMV019918, MMV396749, MMV645672, MMV665972, MMV666026 and MMV667491) on gametocytes. Gametocytes at six days post-commitment were exposed to 10uM concentrations of each compound and the sex-specific viability was measured by flow cytometry. Of the eight compounds, two (MMV667491 and MMV019918) killed >80% of gametocytes and neither of which showed significant difference in sex specific IC_{50}. This novel method for drug screening could be useful as an effective drug development strategy against *P. falciparum* involving the rational design of compounds targeted to essential, parasite-specific functions and in particular those involved in the transition from human host to the mosquito and the corresponding sexual development of the parasite.

Review Discussion

The ability to continuously culture and study the *Plasmodium* intraerythrocytic stages *in vitro* has provided great insight into their metabolism. Both the erythrocytic stages and young gametocytes rely primarily on glucose uptake and glycolysis for ATP synthesis and survival. During these stages, the parasite utilises the erythrocyte haemoglobin as a major amino acid source. They are sensitive to chloroquine and other quinoline containing compounds, as they inhibit the detoxification of haem, a bioproduct of the *Plasmodium* haemoglobin degradation and cause it to accumulate in the digestive vacuole. As gametocytes mature, they undergo numerous genetic changes, resulting in their quiescence. These changes have a significant effect on drug efficacy, with primaquine being the only antimalarial drug indicated for the treatment of *P. falciparum* gametocytaemia.

There are currently very few available agents with identified activity against gametocytes. To this point, however, the increasing interest in the activity of drugs against these stages, along with the advancement of techniques required for such studies, is paving the way towards the identification and optimisation of new gametocytocidal compounds.

5 Materials and Methods

5.1 Materials

5.1.1 Research Governance and Ethics

In vitro cell culture used depleted leukocyte type O+ human blood provided by the National Blood and Transfusion Service. All blood was maintained and stored in fridges at 4°C and used within 4 weeks within the Haldane Laboratories under the provisions of the Human Tissue Act (HTA) and as described within the Code of Practice for Category III laboratory for all laboratory work including tissue culture.

5.1.2 Anti-plasmodial Compounds

Chloroquine and atovaquone were provided by Sigma Aldrich. The MMV Malaria Box is a collection of diverse chemical compounds with proven anti-plasmodial activity (MMV, 2020).

Chloroquine, MMV011895 and MMV666060 were diluted to 10mM using deionised water. Atovaquone was diluted to 1mM using deionised water. Diluted compounds were stored at 4°C until needed.

5.1.3 Culture

Assays were performed using culture-adapted *P. falciparum* ($Dd2^{luc}$) which express luciferase under the control of *Pfpcna* flanking sequences to produce a strong peak of temporal reporter expression during S-phase in trophozoite stage parasites (Wong et al, 2011). The parasites were maintained daily using standard continuous culture conditions in complete medium (RPMI1640 medium supplemented with 18.75ml 1mM HEPES (Sigma), 2ml 45% D-glucose, 2.5ml of 1mM NaOH, 5ml 200mM L-glutamine, 0.5ml 0.1M hypoxanthine, 1.25ml gentamycin (10mg/ml), 2.5% v/v albumax-II (Gibco) and 2.5% v/v pooled human serum) at 4% haematocrit (hct) in an atmosphere of 1% O_2, 3% CO_2 and 96% N_2 at 37°C.

Staging and parasitaemia were determined by light microscopy of Giemsa-stained thin blood smears. Parasite cultures were always maintained at <2% parasitaemia. Stage synchronisation was attained using sequential sorbitol (5% w/v) lysis treatment as described by Lambros & Vanderberg (1979).

5.1.4 Climatic Chambers

Two climatic chambers (Thermofisher, USA) were set up to simulate the temperature of the Tanzanian and Malian environments. The 24-hour repeating temperature cycles, shown in Table 4, were set up using the average hourly temperatures of Ifakara (Tanzania) and Bamako (Mali) on the 1st of September taken from historical record (NOAA, 2020).

Table 4. The bihourly average temperatures of Ifakara, Tanzania and Bamako, Mali (NOAA, 2020) used in climate chamber set-up.

Time	00:00	02:00	04:00	06:00	08:00	10:00	12:00	14:00	16:00	18:00	20:00	22:00
Tanzania Temp (°C)	24.4	23.2	22.4	22.0	23.8	27.2	29.8	31.8	32.0	30.4	27.0	25.6
Mali Temp (°C)	24.4	23.6	23.2	23.0	24.0	26.6	29.0	29.8	29.8	28.4	26.0	25.0

5.2 Methods

5.2.1 Measuring EC_{50}

Trophozoite-stage cultures of *P. falciparum* (100µl, 2% parasitaemia, 4% hct) were added to 96-multiwell plates containing 100µl of pre-dosed (two-fold serial dilution series) complete medium. On each of the plates (see Figure 11 for template), three wells containing 200µl of 2% parasitaemia cell culture (2% hct) in the absence of drugs served as the positive control (100% growth), whereas the same culture mix in the presence of a 10µM supralethal dose of chloroquine served as a negative growth control (0% growth). The outermost wells on each plate contained 200µl of incomplete medium (complete medium lacking human serum and albumax II) to minimize edge effects from evaporation. The plates were then stored for 48hrs in a gassed (1% O_2, 3% CO_2 and 96% N_2) chamber incubated at 37°C.

Following incubation, fluorescent signals were measured using a standard Malaria Sybr Green I Fluorescence (MSF) assay (Smilkstein et al, 2004) which directly monitors DNA content. An equal volume of MSF lysis buffer (100µl of 20mM Tris (pH7.5), 5mM EDTA, 0.008% (w/v) saponin and 0.08% (v/v) Triton X-100) containing SYBR green I (1 x final concentration, from 5000x stock supplied by Invitrogen, UK) was added to 100µl of *P. falciparum* culture aliquoted onto a black 96-multiwell plate (Greiner, UK). Well contents were homogenised by repeated pipetting and incubated for one hour in the dark at room temperature. The fluorescent signal, in relative fluorescence units (RFU),

was measured using the blue fluorescence module (excitation 490 nm: emission 510-570nm) of a Glomax Multi Detection System (Promega, UK).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		2000	1000	500	250	125	62.5	31.3	15.6	7.8	+ve control	
C		2000	1000	500	250	125	62.5	31.3	15.6	7.8		
D		2000	1000	500	250	125	62.5	31.3	15.6	7.8		
E		100	50	25	12.5	6.3	3.1	1.6	0.8	0.4	-ve control	
F		100	50	25	12.5	6.3	3.1	1.6	0.8	0.4		
G		100	50	25	12.5	6.3	3.1	1.6	0.8	0.4		
H												

Figure 11 Example EC_{50} plate layout: rows B, C, D (columns 2-10) contain a two-fold dilution series of chloroquine across columns 2-10 (nM stated), rows E, F and G (columns 2-10) contain a two-fold dilution series of atovaquone across columns 2-10 (nM stated), '+ve control' refers to 100% growth control and '-ve control' refers to 0% growth control.

In the fluorescent assays, the percentage growth was calculated as follows: $100 \times \left[\frac{\mu_{(S)}-\mu_{(-)}}{\mu_{(+)}-\mu_{(-)}}\right]$: where $\mu_{(S)}$, $\mu_{(+)}$ and $\mu_{(-)}$ represent the means for the sample in question and 100% and 0% growth controls respectively. The percentage growth was plotted against log_{10}- transformed drug concentration and the EC_{50} determined using a nonlinear regression (sigmoidal dose-response/ variable slope equation) in GraphPad v5.0 (GraphPad Software, Inc, San Diego, CA). The EC_{50} values calculated were used to determine the optimum drug concentrations to be used in the subsequent luminescence assays.

5.2.2 Experiment A

This study was designed to investigate the effect of the Ifakara temperature cycle on the stability of the compounds over a prolonged period, when stored in either neutral or acidic conditions. To this end, compounds were diluted to 1000x their EC_{50} values into either a neutral PBS solution (pH7) or PBS solution adjusted to pH5 using concentrated HCl. Samples were then exposed to the Ifakara temperature cycle for a total of 45 days. During their exposure, samples were taken on days: 0, 12, 24, 32 and 45. Samples were stored at -20°C until collection was complete, and luciferase assays could be used to measure any changes that had occurred in the potency of the compounds.

5.2.3 Experiment B

This study was designed to investigate changes in the stability of the compounds when diluted in either neutral or acidic conditions without extended incubation. To this end, compounds were diluted to 4000x, 1000x and 250x their EC_{50} values into either a neutral PBS solution (pH7) or PBS

solution adjusted to pH5 using concentrated HCl. Immediately after their production, the samples underwent luciferase assays to understand any changes to potency caused by pH conditions only.

The effect of pH in the absence of the anti-plasmodial compounds was also investigated. To this aim, four different solutions were prepared to produce 4 different pH values as opposed to adjusting the pre-made PBS solutions using HCl. Solutions were made using sodium chloride (NaCl), potassium chloride (KCl), dibasic anhydrous sodium phosphate (Na_2HPO_4) and monobasic anhydrous sodium phosphate (NaH_2PO_4) (see Table 5 for the quantities used). This was done to establish whether the method of pH adjustment had any influence on the solutions' potency.

Table 5. The quantities of NaCl, KCl, dibasic sodium phosphate and monobasic sodium phosphate used to attain PBS solutions of differing pH values, calculated using the equation devised by Debye & Hückel (1923).

pH	NaCl (mg/L)	KCl (mg/L)	Na_2HPO_4 (mg/L)	NaH_2PO_4 (mg/L)
4.5	8006.3	201.3	2.6	1379.0
6.0	8006.3	201.3	362.6	1193.0
7.25	8006.3	201.3	1972.0	364.5
8.0	8006.3	201.3	2520.0	82.4

5.2.4 Experiment C

This study was designed to investigate the effect of the Bamako temperature cycle on the stability of the compounds over an elongated period, when stored in either neutral or acidic conditions. To this end, compounds were diluted to 1000x their EC_{50} values into either a neutral PBS solution (pH7) or PBS solution adjusted to pH5 using concentrated HCl. Samples were then exposed to the Bamako temperature cycle for a total of 28 days. During their exposure, samples were taken on days: 0,7, 14 and 28. Samples were stored at -20°C until collection was complete, and luciferase assays could be used to measure any changes that had occurred in the potency of the compounds.

5.2.6 Luciferase Assays

The transgenic *P. falciparum* $Dd2^{luc}$ strain expresses luciferase in the trophozoite stage (Hasenkamp et al, 2012). Once converted to a lysate, the greater the bioluminescent output, the higher the content of the luciferase enzyme and thus the greater the content of *P. falciparum* in the

transfected cells. Luciferase assays were performed using *P. falciparu*m Dd2^{luc} in to evaluate the inhibitory efficacy of the compounds after 48 hours continual exposure. After environmental exposure, trophozoite-stage cultures of *P. falciparum* (100µl, 2% parasitaemia, 4% hct) were added to 96-multiwell plates containing 100ul of pre-dosed (4 two-fold serial dilution series beginning at 10xEC_{50} values previously determined) complete medium.

Each sample was processed as two technical repeats on the same plate. On each assay plate (as seen on Figures 12-13), six wells containing 200µl of 2% parasitaemia cell culture (2% hct) in the absence of drugs served as the positive control (100% growth). The outermost wells on each plate contained 200µl of incomplete medium to minimize edge effects from evaporation during 48 hr incubation in a gassed (1% O_2, 3% CO_2 and 96% N_2) chamber at 37°C.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		40x	20x	10x	5x	10x	5x	2.5x	1.25x	+ve control		
C												
D		2.5x	1.25x	0.625x	0.3125x	40x	20x	10x	5x			
E												
F		10x	5x	2.5x	1.25x	2.5x	1.25x	0.625x	0.3125x			
G												
H												

Figure 12. Example luciferase plate layout of samples collected in experiments A & C: each sample underwent two technical repeats (e.g. rows B & C) across a two-fold dilution series i.e. columns 2-5 and columns 6-9. Values are stated as a multiplication of the compounds EC_{50} value. There are six samples tested per plate, '+ve control' refers to 100% growth control.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		10x	5x	2.5x	1.25x	10x	5x	2.5x	1.25x	+ve control		
C												
D		10x	5x	2.5x	1.25x	10x	5x	2.5x	1.25x			
E												
F		10x	5x	2.5x	1.25x	10x	5x	2.5x	1.25x			
G												
H												

Figure 13. Example luciferase plate layout of samples collected in experiment B: each sample underwent two technical repeats (e.g. rows B & C) across a two-fold dilution series i.e. columns 2-5 and columns 6-9. Values are stated as a multiplication of the compounds EC_{50} value. There are six samples tested per plate, '+ve control' refers to 100% growth control.

Samples of 40µl cultured 2% trophozoite-stage compound-exposed *P. falciparum* were transferred onto a white 96 multiwell plate (Greiner, UK) and 10µl passive lysis buffer (Promega, UK) was added and homogenised using a pipette. An equal volume, 50µl, of luminogenic substrate was mixed with the lysed parasites and the bioluminescence in relative light units (RLU) was measured for 2 sec in a Glomax Multi Detection System (Promega, UK).

5.3 Proposal for Field-based Studies in Ifakara, Tanzania

Due to the ongoing Covid-19 pandemic, travel to Tanzania was not possible. The four compounds and their respective EC_{50} values were sent to a Mgeni Mohamed and colleagues based at the St. Francis University College of Health and Allied Sciences in Ifakara, Tanzania. A study was carried out to demonstrate whether an antimalarial compound delivered by sugar baits could block the development of *P. falciparum* in *Anopheles* mosquitoes.

Following microscopic screening, 200µl infected blood was drawn from participants presumptive to be gametocyte-positive and RNA was extracted using the RNeasy® plus mini kit protocol for spin column followed by on-column DNAase digestion (Qiagen, Germany). The RNA was processed using a quantitative PCR (qPCR) assay that uses *P. falciparum pfs25* transcripts. These transcripts encode a 25-kDA protein expressed on the surface of mature gametocytes. Once it was confirmed that the participants were positive for gametocytes, 5ml of venous blood was collected and standard local artemisinin combination therapy was given.

Recently emerged (1-2 days) and unfed female *An. gambiae s.s.* were supplied with 10% sucrose solution before being starved for 6 hours on day 3-4 and provided with a blood meal using a membrane feeding assay (MFA). Each of the feeders were supplied with either infectious or heat-inactivated (43°C/12 minutes) non-infectious blood (negative control). After feeding, mosquitoes were kept in standard conditions (27±2 C°, 75±5% relative humidity with a 12-hr light/ dark cycle) and stored in a sealed container within a sealed contained (additional safety measure).

The compounds at $10xEC_{50}$ in 10% sucrose solution were provided to the potentially infected mosquitoes, 48 hours following the MFA. For each of the 5 conditions (control, atovaquone, chloroquine, MMV011895, MMV666060), ≥190 mosquitoes were used in each of the two technical repeats done for either biological repeat (blood provided by two different volunteers) (n=4). A third of the mosquitoes in each condition group were dissected on day 8 for microscopy-based oocyst appraisal and the remaining underwent qPCR for sporozoite detection on day 14. The proportion of *An. gambiae* presenting with oocysts or sporozoites were reported.

5.4 Data Analysis

From the luciferase assays, the normalised growth (%) was calculated as follows: $100 \times \left[\frac{\mu_{(S)}}{\mu_{(+)}}\right]$ where $\mu_{(S)}$ and $\mu_{(+)}$ represent the means for the sample in question and 100% growth control respectively. The normalised growth (%) values calculated for the compounds in the different conditions were plotted against multiplicative EC_{50} values in GraphPad v5.0 (GraphPad Software, Inc, San Diego, CA).

Microsoft Excel was used to work out the percentage normalised growth of parasites by dividing the assay readings of the compounds by that of the positive control. Microsoft Excel was then used to work out the average and standard deviations where appropriate (n=6 for Experiment A, n=4 for Experiment B and n=4 for Experiment C) for biological analysis. Using the programme GraphPad prism v5.0 allowed production of graphs and statistical analysis.

For the field-based studies conducted in Tanzania, the proportion of mosquitoes (%) infected with plasmodia on days 8 (oocysts) and 14 (sporozoites), were calculated as follows: $100 \times \left[\frac{N_{(i)}}{N_{(T)}}\right]$ where $N_{(i)}$ and $N_{(T)}$ represent the number of infected mosquitoes and the total number of mosquitoes examined respectively.

6 Results

6.1 Selection of Anti-plasmodial Compounds

The compounds selected for comparison within the study were atovaquone and chloroquine (Figure 14). Atovaquone has previously been identified as exhibiting potency during the parasites' sporogonic cycle when fed directly to the mosquito (Paton et al, 2019); it has been used here to provide a positive control. Chloroquine, with no known action against the sexual stages of plasmodia development has been used as a negative control. The two control compounds used could thus be used in ongoing field-based studies.

The ideal candidate for real-world application of an anti-plasmodial incorporated into a sugar bait would need to be commercially available, possess activity against both the asexual and sexual stages of *P. falciparum* and, to carry out all the tests, a minimum of 10mg of the compounds was required. Additionally, the compounds must have been found to be unsuitable for use within humans as the feeding of the compound to mosquitoes could lead to the emergence of resistant plasmodia strains. In this regard, the pipeline for drugs used on humans needs to be actively protected. The MMV library was shortlisted using the following criteria:

- Compounds of known pathways were removed.
- Compounds that were drug-like were removed.
- Compounds with an identified IC_{50} >1000µM were removed.
- Compounds with a principal component 1 outside the range of -170-30 were removed.

This reduced list was sent to a professional contact within the MMV who then sent samples of MMV011895 and MMV666060 (Figure 14) for use within the study.

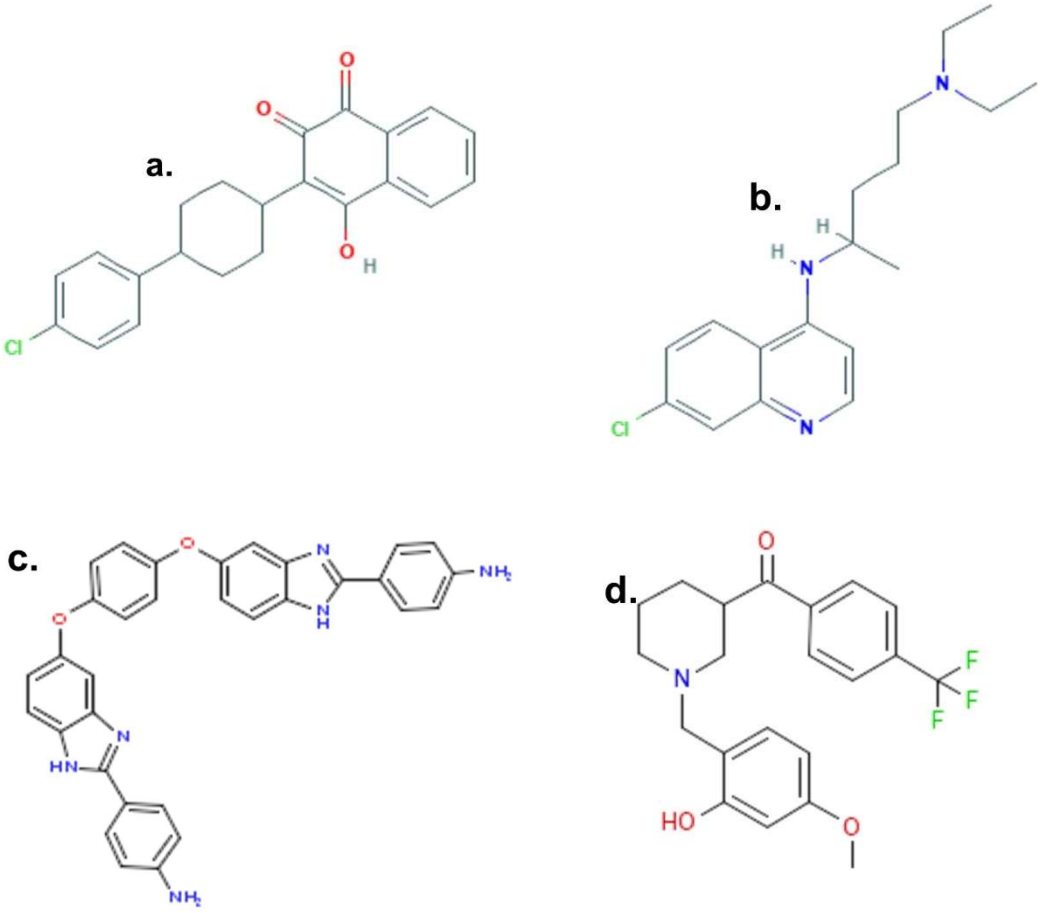

Figure 14. The 2D structures of (a) atovaquone (NIH, 2019a), (b) chloroquine (NIH, 2019b), (c) MMV01189 (NIH, 2019c) 5 and (d) MMV666060 (NIH, 2019d).

The simplified molecular input line entry system (SMILES) of the compounds is presented in Table 6 along with the alternate ID names used and the molecular weights. This data elucidates specifies the chemical structures and physiochemical properties for cheminformatics purposes.

Table 6. The structure of Malaria Box compounds (adapted from Van Voorhis et al, 2016).

Compound	SMILES	Alternate compound ID	CHEMBL ID	Molecular weight (g/mol)
MMV011895	Nc1ccc(cc1)c2nc3cc(Oc4ccc(Oc5ccc6[nH]c(nc6c5)c7ccc(N)cc7)cc4)ccc3[nH]2	GNF-Pf-3264	CHEMBL582072	524.6
MMV666060	COc1ccc(CN2CCCC(C2)C(=O)c3ccc(cc3)C(F)(F)F)c(O)c1	TCMDC-124631	CHEMBL529968	393.4

As both compounds were denoted as 'probe-like' they are unlikely to be used on humans in the future. The potency of the compounds against the *P. falciparum* asexual stages enables the viability screening to be carried out using *in vitro* assays, whereas the compound's activity against the sexual stages is vital to stopping the parasite's development within the mosquito.

Van Voorhis et al (2016) describes a collection of >290 assays depicting the many activities of the Malaria Box compounds from >50 research laboratories. The activities of MMV011895 and MMV666060 against the mature stage (IV-V) of *P. falciparum* gametocytes were quantified by six laboratories using an array of different strains and different experimental methods (Table 7).

Table 7. Compound inhibition (%) of *P. falciparum* late stage (IV-V) gametocytes with incubation at varying concentrations as reported by [a] Duffy & Avery, 2013, [b,] Taramelli et al, undated (from Van Voorhis et al, 2016), [d] Lucantoni et al, 2016, [e] Bowman et al, 2014, [f] Mancama et al, undated (from Van Voorhis et al, 2016) and [g] Sanders et al, 2014.

Concentration (µM)	0.5[a]	3.7[b]	5.0[a]	5.0[d]	5.0[e]	5.0[f]	10.0[g]
MMV011895	13.4	25.8	32.9	5.9	38.1	82.2	66.2
MMV666060	14.8	27.1	16.7	28.8	93.6	44.5	57.2

Duffy & Avery (2013) conducted a mature gametocyte assay using the transgenic *P. falciparum* NF54^{Pfs16} strain. In brief, gametocytes (12 days post gametocytogenesis induction) were incubated with the compounds for 72 hours before MitoTracker Red CM-H2XRos (MTR) was added and confocal microscopy used. GFP fluorescence allowed for the identification of the gametocyte and MTR fluorescence indicated viable, respiring gametocytes. Taramelli and colleagues (data presented in Van Voorhis et al, 2016) measured gametocyte viability using the parasite lactate dehydrogenase (pLDH) assay, a 72-hour incubation period and *P. falciparum* (3D7$^{elo1\text{-}pfs16\text{-}CBG99}$ strain) gametocytes. Lucantoni et al (2016) used the transgenic *P. falciparum* NF54^{Pfs16} strain. This study incubated gametocytes (8 days post gametocytogenesis induction) with the compound for 72 hours before a luciferase assay was used to assess the action of the compounds. Bowman and colleagues (2014) used *P. falciparum* NF54 strain gametocytes (at day 13 of gametocyte culture) to evaluate the compound's antimalarial activity. Following 72 hours of incubation, the alamarBlue viability assay was applied in this study. Mancama and colleagues (data presented in Van Voorhis et al, 2016) measured gametocytocidal activity using the resazurin-based dye assay. *P. falciparum* (NF54 strain) gametocytes were incubated for 72 hours before the inhibition caused by the compounds was measured using the resazurin-based dye assay. Sanders et al (2014) employed the SYBR Green I assay technique, a 48-hour incubation period and the *P. falciparum* NF54 strain gametocytes.

Further to this, the activities of MMV011895 and MMV666060 against ookinetes (Table 8) were quantified by a single laboratory group (Ruecker et al, 2014) using *P. berghei*-infected Murine blood. After a 22-hour incubation with the compounds, individual ookinetes were counted by fluorescence microscopy.

This same laboratory used a similar method, differing only in the data capture method to quantify the action of known and marketed antimalarial drugs. The inhibition of atovaquone and chloroquine were recorded at 114.24%±4.96% and -4.00%±7.31% respectively (Delves et al, 2012).

Table 8. Compound inhibition (%) of *P. berghei* (CTRPp. GFP reporter strain) ookinetes with incubation over 24 hours as reported by Ruecker et al, 2014 (from Van Voorhis et al, 2016).

Concentration (µM)	1.0	10.0
MMV011895	13.1	26.2
MMV666060	-17.5	-0.8

The activities of MMV011895 and MMV666060 against the asexual, erythrocytic stage of *P. falciparum* was confirmed by three laboratories on different strains (Table 9).

Avery and colleagues (data presented in Van Voorhis et al, 2016) used the HCI assay to determine the EC_{50} values against asexual P. falciparum (3D7 and K1 strains). In brief, the compounds are incubated in the presence of 2-3% parasitaemia (0.3% hct), for 72 hours at 37°C. After incubation, the plates are stained with the DNA-intercalating dye, DAPI (4', 6', -diamidino-2-phenylindole), in the presence of saponin and Triton X-100 and incubated at room temperature for a further 5 hours in the dark before imaging. Horrocks and colleagues (data presented in Van Voorhis et al, 2016) used the MSF assay as described in 4.2.1 to determine the EC_{50} value against the asexual *P. falciparum* Dd2 strain. Ayong and colleagues (data presented in Van Voorhis et al, 2016) used a novel image acquisition and data mining technique (Moon et al, 2013) to determine the EC50 values against asexual *P. falciparum* (3D7, HB3, Dd2, W2, K1 and FCR3 strains). In this series of assays, compounds were incubated in the presence of parasite infected erythrocyte cultures for 72 hours at 37°C.

Table 9. The activity of Malaria Box compounds against the asexual blood stages of different strains of *P. falciparum* as determined by *Avery et al, undated, **Horrocks et al, undated and *** Ayong et al, undated (adapted from Van Voorhis et al, 2016).

Compound	3D7 EC_{50} nM *	K1 EC_{50} nM *	Dd2 EC_{50} nM **	3D7 EC_{50} nM ***	HB3 EC_{50} nM ***	Dd2 EC_{50} nM ***	W2 EC_{50} nM ***	K1 EC_{50} nM ***	FCR3 EC_{50} nM ***
MMV011895	842		315	161	142	61	162	150	121
MMV666060		728	635	500	375	316	82	64	201

6.2 EC_{50} Determined via Luciferase Assays

As indicated in Table 7, a compound's EC_{50} value varies across different laboratories. The length of incubation and the assaying methods are clearly important to the EC_{50} value obtained. Here, a 48-hour standard MSF assay (Smilkstein et al, 2004) will be used as originally described in section 4.2.1. The normalised growth (%) (determined from RLU readings) was plotted against the log_{10} transformed drug concentration (Figures 15-16). These assays were carried out to provide the baseline values for the exact strain and conditions used during the subsequent evaluations.

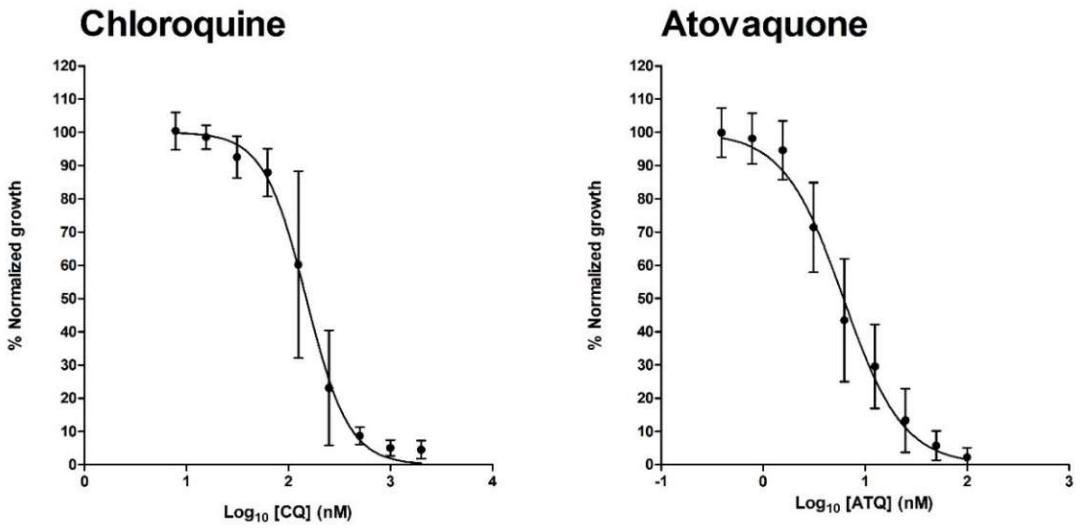

Figure 15. Dose-response curves of *P. falciparum* $Dd2^{luc}$ to 48hr incubation with chloroquine or atovaquone obtained via MSF assay.

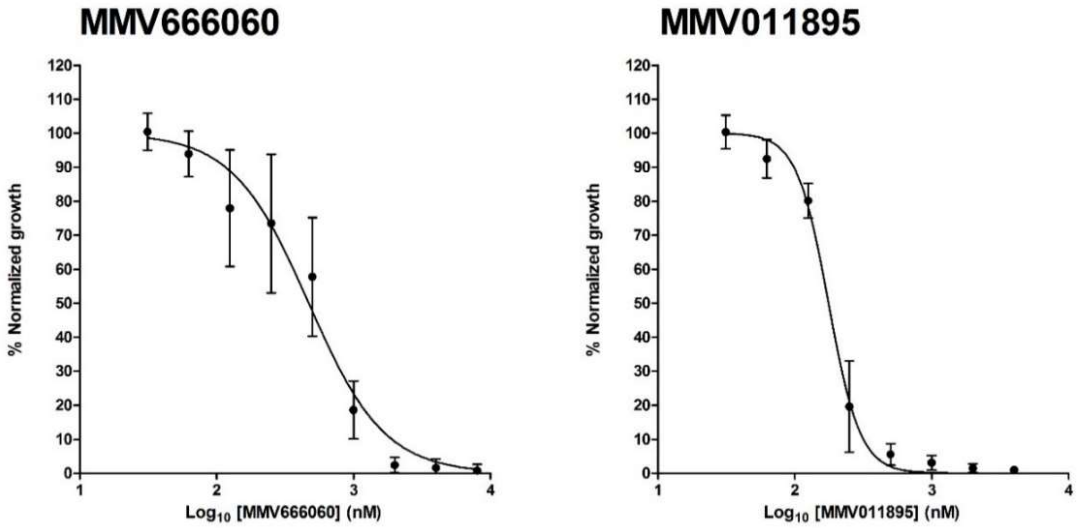

Figure 16. Dose-response curves of *P. falciparum* $Dd2^{luc}$ to 48hr incubation with MMV666060 and MMV011895 obtained via MSF assay.

Non-linear regression of Figures 15-16 (sigmoidal concentration-response/ variable slope equation), as described in section 4.2.1, yielded the EC_{50} (the concentration that causes 50% reduction in parasite growth) values in Table 10.

Table 10. Compound EC_{50} values of *P. falciparum* (Dd2) as determined from analysis of the curves in Figures 15-16.

Concentration (μM)	EC_{50} (nM)	95% CI (nM)
Atovaquone	6.1	5.3-7.2
Chloroquine	149.6	136.4-174.1
MMV011895	176.1	163.0-190.4
MMV666060	469.7	364.9-606.1

Ullah et al (2017) previously determined the EC_{50} values of atovaquone and chloroquine as 2.6nM (95% CI 1.7-3.7) and 209nM (95% CI 156-232). Similar studies presented in Van Voorhis et al (2016) determined the EC_{50} values of MMV011895 and MMV666060 as 315nM and 635nM respectively. Like the variation in Table 7, there are small relative variations in the EC_{50} values determined by different research teams using the different assays, importantly however, here the relative ranking order of atovaquone> chloroquine> MMV011895>MMV666060 remains the same.

6.3 Fruit Juice pH Evaluation

Typically, ATSBs employ locally sourced fruits as to attract the mosquitoes to feed. The pulps are then diluted with either water or sucrose solution (Tenywa et al, 2017). Being able to identify an attractive solution without using fruit juices is of particular significance as it would negate to need to regularly replace the solutions due to fermentation. Recently studies (Meza et al, 2020) have put forward the possibility of creating a synthetic long-lasting bait solution. With the aim of replacing the insecticide with an anti-plasmodial compound, it is important to recognise that the conditions within the bait solution may determine how stable the compounds are over a long period.

Fruit juice (orange, apple, grapefruit and pineapple) samples were purchased, and their pH values were determined (Table 9) using with a conventional pH probe.

Figure 17. Days on which the samples were taken, following setup and incubation of day zero.

From these results, it was determined that the stability of the anti-plasmodial compounds would be tested in very strongly acidic (4.5-5.0), moderately acidic (6.0), neutral conditions (7.0-7.25) and moderately alkaline (8.0) conditions. This range enables a basic understanding of the optimum conditions for the anti-plasmodial compounds and whether a diluted fruit juice would be effective or whether a synthetic solution is required.

Table 9. The pH of fruit juices.

Fruit juice	pH
Orange	4.2
Apple	4.0
Grapefruit	3.3
Pineapple	4.5

6.4 Assessing the Impact of Ifakara Conditions

To assess the stability of the compounds in field-like conditions, this trial evaluated the compounds on extended exposure to a temperature cycle attributed to Tanzania in both acidic and neutral conditions. Samples were taken over a 45-day period on specific days (Figure 17) and analysed all at once. For each compound, biological replicate samples underwent two technical repeats.

The data is presented as individual biological repeats as there are an insufficient number of technical replicates to group together and to provide consistency in the way the data is presented. For each compound, the data has been presented as averages of the biological replicates in the appendix.

Chloroquine has been used across this study to provide a negative control. Three biological repeats were carried out using chloroquine. The results of the luciferase assays conducted with chloroquine samples are presented in Figure 18. Although there are internal variations across the biological repeats, there consistently appears to be no significant effect of either pH or time. Chloroquine appears to remain stable and active throughout extended incubation in either pH.

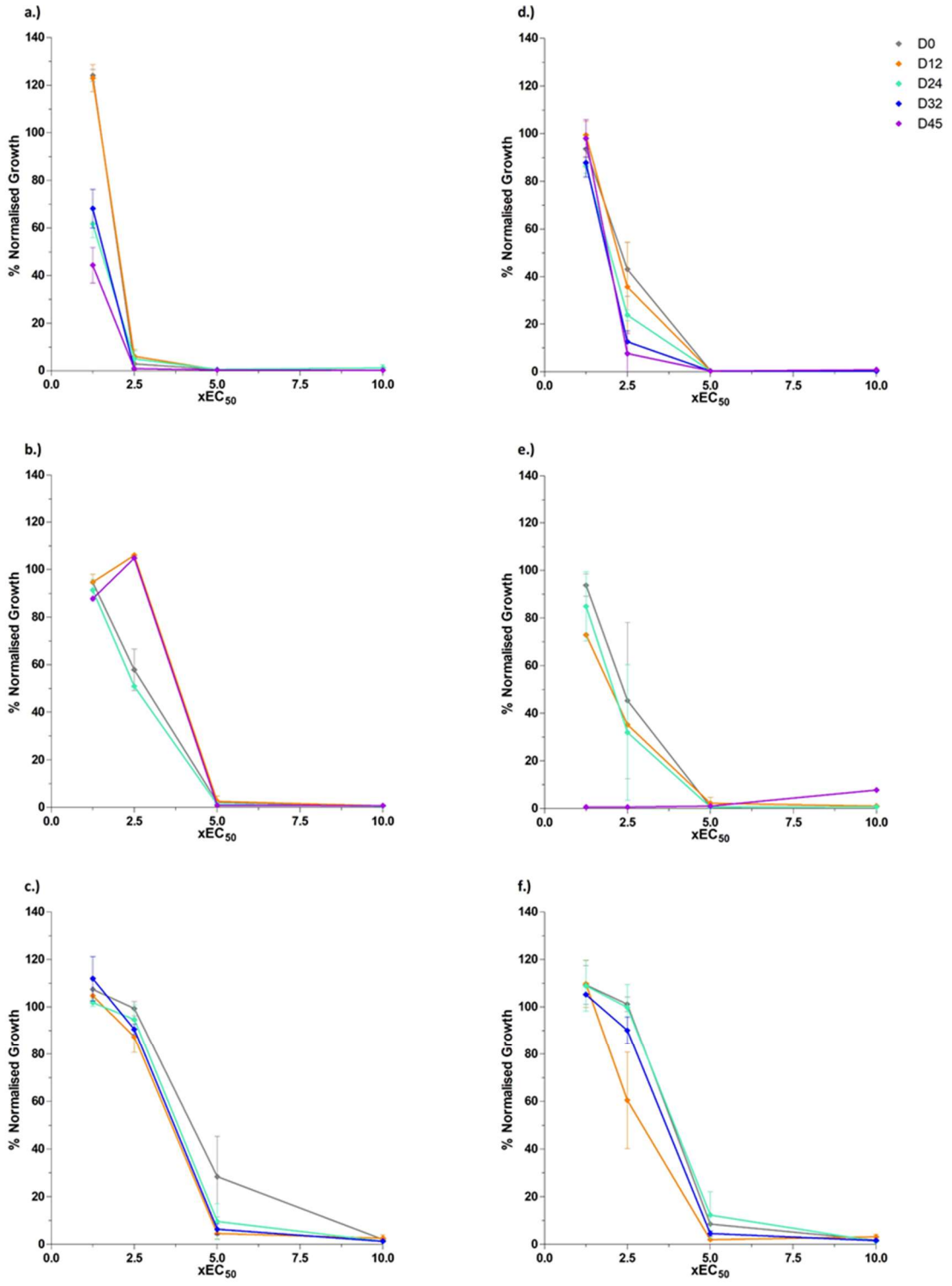

Figure 18. Solution containing chloroquine was exposed to Ifakara-like temperatures and samples were taken on days: 0 (D0), 12 (D12), 24 (D24), 32 (D32) and 45 (D45). Each row of graphs represents a biological repeat. The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a-c) pH7 and (d-f) pH5 (n=2).

Atovaquone has been used across this study to provide a positive control. Figure 19 displays the rapid and complete loss of potency of atovaquone when incubated in either pH5 or pH7. This effect was seen in both biological repeats carried out. Note that this loss of activity is apparent even in the samples taken on day 0. There are no time-dependent variations in either condition.

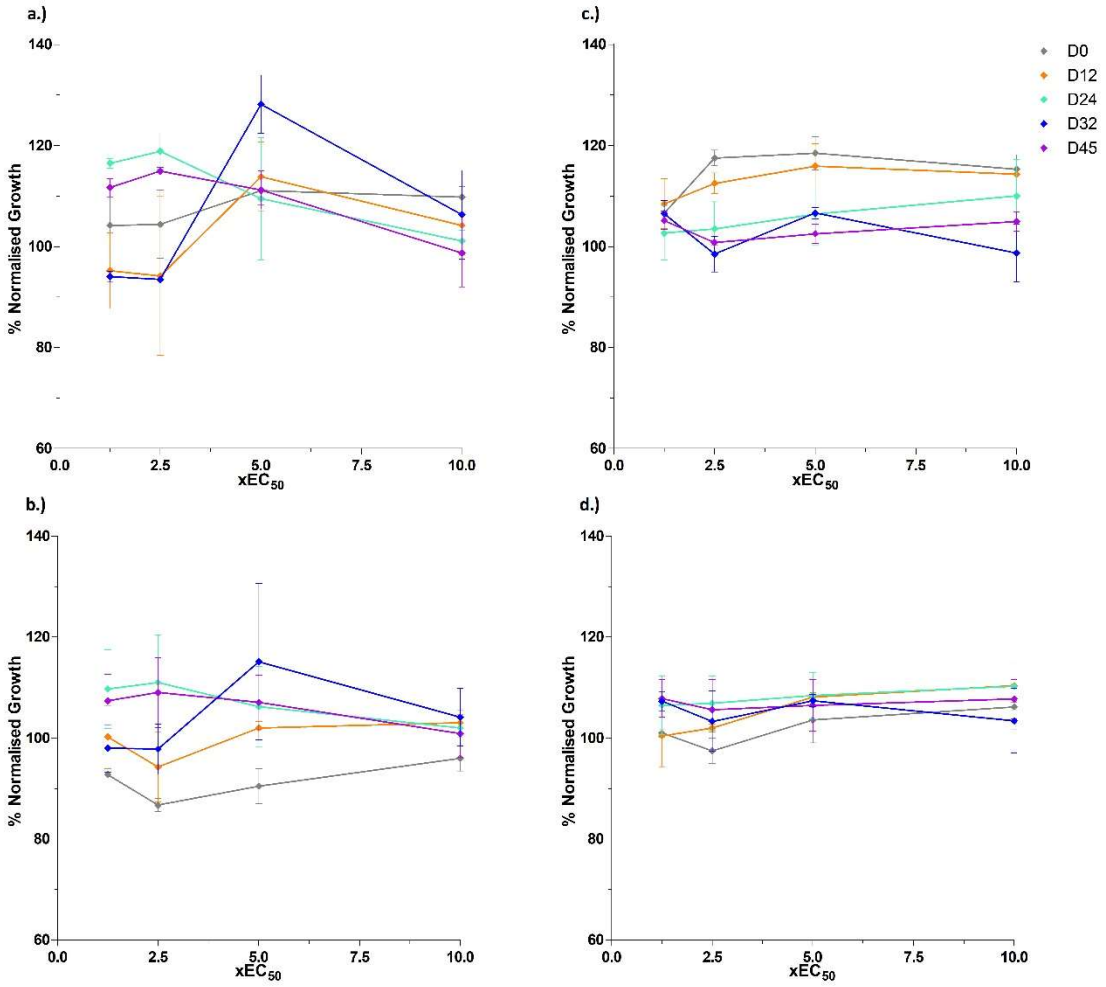

Figure 19. Solution containing atovaquone was exposed to Ifakara-like temperatures and samples were taken on days: 0 (D0), 12 (D12), 24 (D24), 32 (D32) and 45 (D45). Each row of graphs represents a biological repeat. The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a-b) pH7 and (c-d) pH5 (n=2).

The results of the assays conducted with MMV011895 samples are presented in Figure 20. Although there are internal variations across the two biological repeats, there consistently appears to be no significant effect of either pH or time.

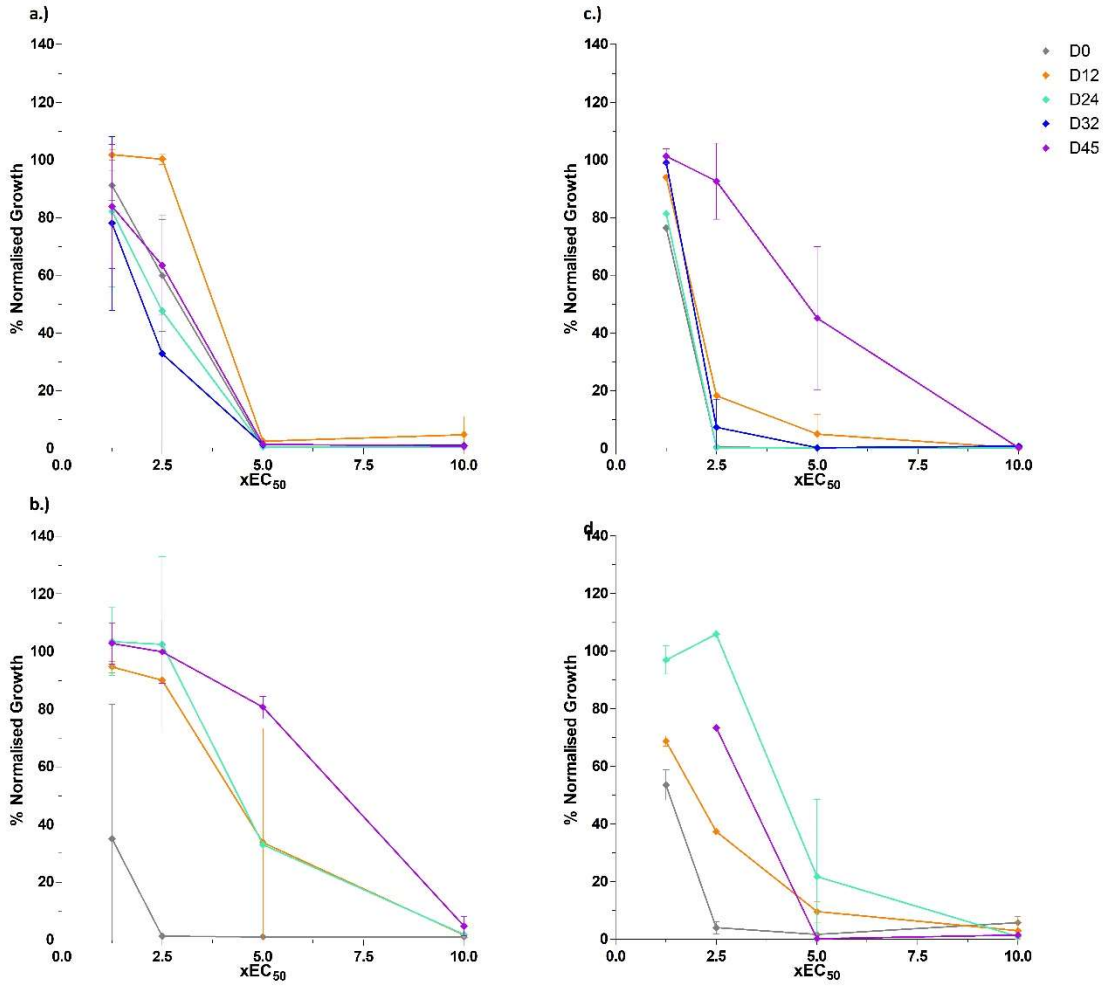

Figure 20. Solution containing MMV011895 was exposed to Ifakara-like temperatures and samples were taken on days: 0 (D0), 12 (D12), 24 (D24), 32 (D32) and 45 (D45). Each row of graphs represents a biological repeat. The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a-b) pH7 and (c-d) pH5 (n=2).

The results of the assays conducted with MMV666060 samples are presented in Figure 21. Although there are internal variations across the two biological repeats, there consistently appears to be no significant effect of time. There is, however, a clear difference in the stability of the compound at different pH values. Incubated in pH7 solution, the compound appears to completely and rapidly lose its potency, whereas incubation in pH5 solution, the compound maintains a high potency over time. From this data, one could assume that MMV666060 maintains its potency better when stored in acidic solution as opposed to neutral conditions. This finding is key to optimising the method by which the anti-plasmodial compound may be delivered to mosquitoes.

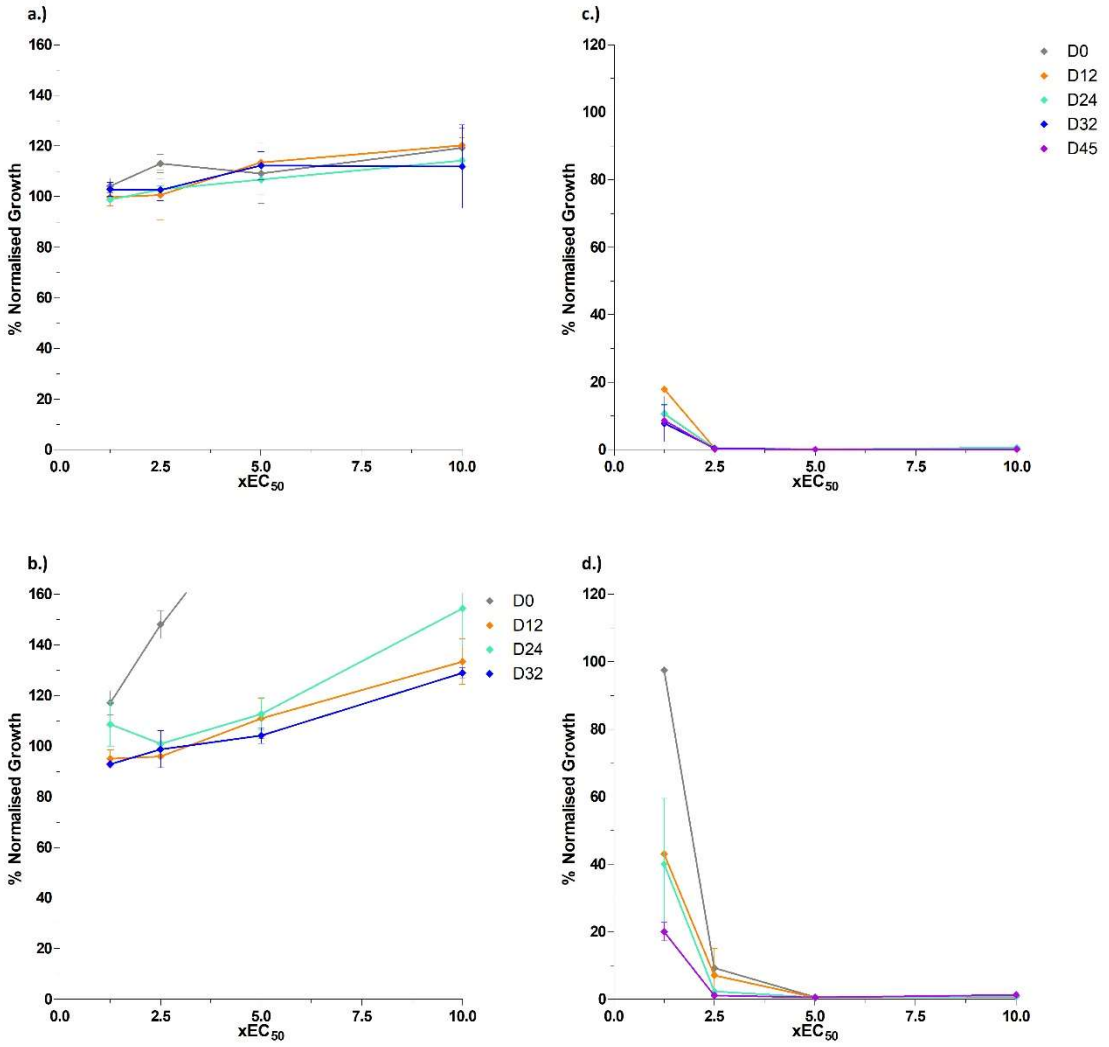

Figure 21. Solution containing MMV666060 was exposed to Ifakara-like temperatures and samples were taken on days: 0 (D0), 12 (D12), 24 (D24), 32 (D32) and 45 (D45). Each row of graphs represents a biological repeat. The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a-b) pH7 and (c-d) pH5 (n=2).

6.5 Validating the Differences Caused by pH

After the results of the trial assessing the impact of Ifakara conditions, the different potency of the compound MMV666060 within the two different pH conditions and the apparent loss of atovaquone's activity piqued interest. To further assess the stability of these two compounds, this next trial evaluated the compounds in both acidic and neutral conditions upon immediate exposure.

First, to assess the effect of pH on plasmodia growth in the absence of the compounds, solutions were made up to produce 4 different pH values as shown in Table 3. Five biological replicates were undertaken. Figure 22 (n=10) suggests acidic solutions affect intraerythrocytic growth more so than when using the more basic PBS solution, although this difference does not appear to be apparent at ≤5xEC_{50} equivalent.

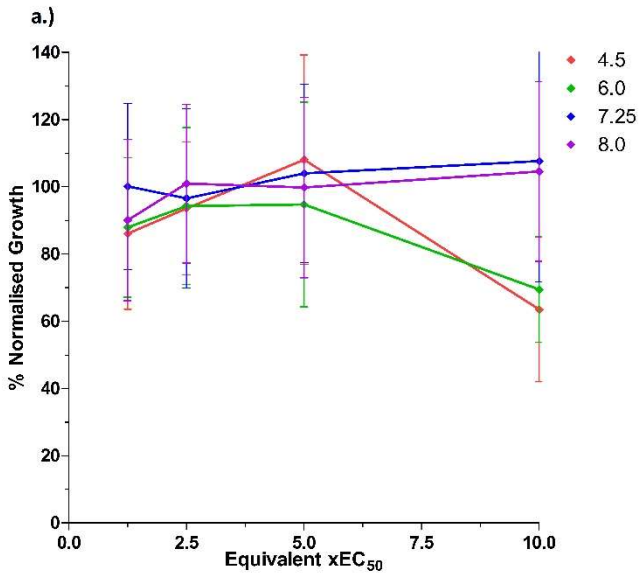

Figure 22. The normalised growth (%) of *P. falciparum* Dd2^{luc} following 48hr incubation with PBS only (measurements in table 8) at a range of pH levels (n=10) (individual replicates in the appendix, figure 35).

The buffer composition seems to influence the outcome of the assays and therefore should be assessed going forward. It is imperative to understand how different buffers (e.g., MES, ADA, HEPES) effect the anti-plasmodial compounds to reach an understanding of the ideal solution for use in real world application.

Next, samples of atovaquone and MMV666060 were made representing 40xEC_{50}, 10xEC_{50} and 2.5xEC_{50} at both pH5 and pH7 and tested immediately for their anti-plasmodial effect in a standard 48hr assay.

The results shown in Figure 23 indicate a pH dependent effect on anti-plasmodial activity. The potencies of both atovaquone and MMV666060 were enhanced in pH5 solution compared to pH7 solution. Figure 23 (a) shows neither of the atovaquone samples reaching ≤46% normalised growth. This may be because of atovaquone's slow onset of action and the only 48-hour incubation with the *P. falciparum* infected red blood cells. This likely reflects the known lag in anti-plasmodial activity against asexual parasites, a result commonly seen in compounds that affect the mitochondria function. MMV666060 in Figure 23 (b), on the other hand, appears to have different and much quicker mode of action, as it is able to attain almost 100% kill over the 48-hour time course. The mode of action by which MMV666060 eliminates the plasmodia is unlikely to involve the mitochondria.

Atovaquone's slow-acting nature, could also explain the lack of efficacy found in the previous setup. It is more likely, however, that human error was a greater factor in the limited results of the Ifakara experiment.

A key finding of this study is that both compounds display a pH dependent effect in the assay used. This is especially interesting as it suggests that acidic conditions may be preferable for maintaining the potency of anti-plasmodial compounds as opposed to neutral conditions. Going forward, this effect has important implications as to how compounds are assessed and the design of the final product.

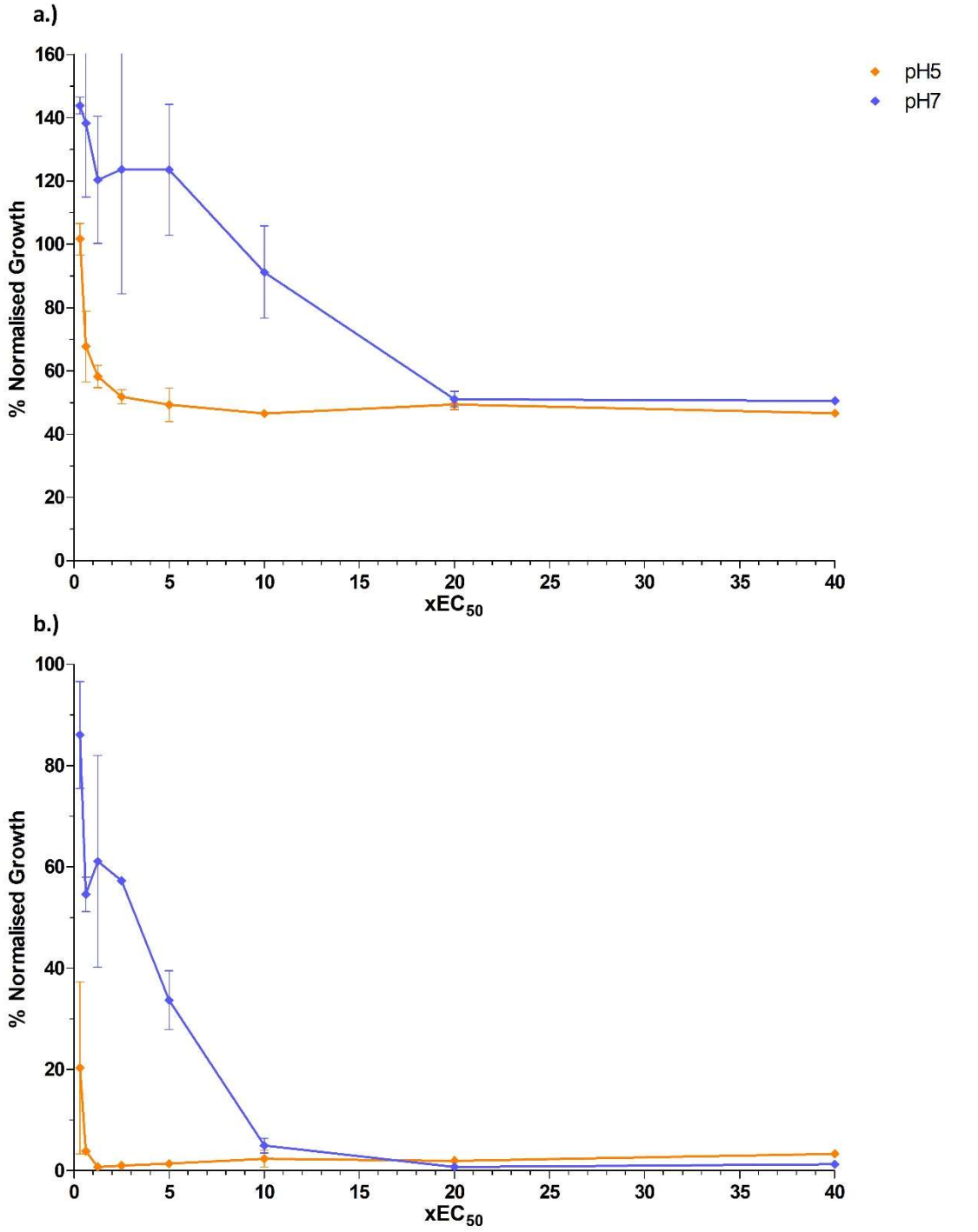

Figure 23. The normalised growth (%) of *P. falciparum* Dd2^{luc} following 48hr incubation with (a) atovaquone and (b) MMV666060 at both pH7 and pH5. Graphs show the combined results of three two-fold dilution series with starting concentrations: 40xEC_{50}, 10xEC_{50} and 2.5xEC_{50} (n≥2) (see appendix for individual graphs, figure 36).

6.6 Assessing the Impact of Bamako Conditions

To further assess the stability of the compounds in field-like conditions, this trial evaluated the compounds on extended exposure to a temperature cycle attributed to Mali in both acidic and neutral conditions. Samples were taken over a 28-day period on specific days (Figure 24) and analysed all at once. For each compound, two biological repeats were conducted. In this section, there is much less variation across the biological repeats compared to those of part 5.4. This is most likely due to a more efficient processing time and more experience with the plating methods used.

Figure 24. Days on which the samples were taken, following setup and incubation of day zero.

The results of the assays conducted with chloroquine, the negative control, are presented in Figure 25. As has been previously found, there appears to be no clear effect of temperature or light on the sample. There does, however, appear to be some effect of the pH value of the conditions, with pH5 seemingly maintaining the potency of chloroquine to a lower concentration. There is not enough data here to fully understand if the pH effect is significant.

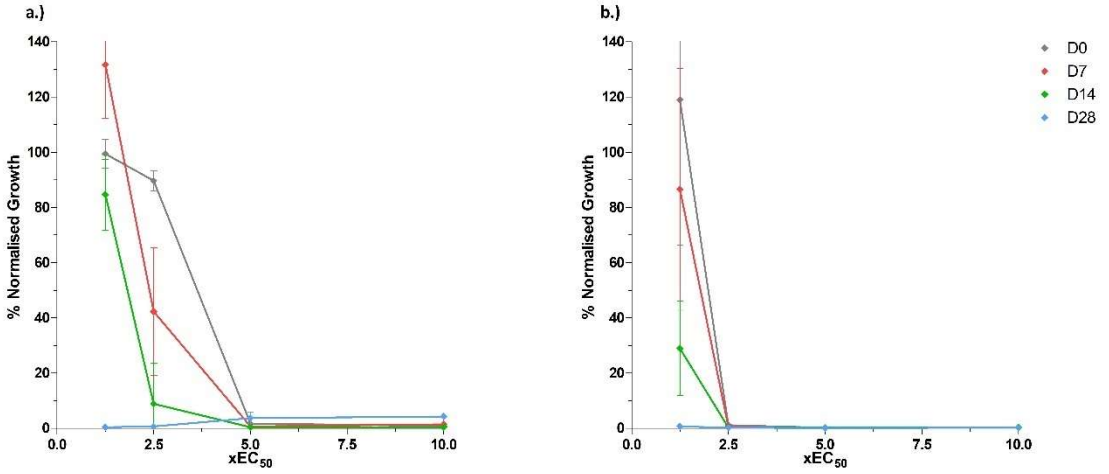

Figure 25. Solution containing chloroquine was exposed to Bamako-like temperatures and samples were taken on days: 0 (D0), 7 (D7), 14 (D14) and 28 (D28). The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a) pH7 and (b) pH5 (n=4) (see appendix for individual replicates).

In Figure 26, atovaquone appears to display a complete and rapid loss of activity when incubated in pH7. This result is concurrent with that seen in Figure 23 (a) and shows the pH effect on the compound accompanied with its storage in the temperature/ light cycle. This effect also appears to occur in the atovaquone sample in pH5 solution, however, the activity loss seems to be

more gradual as the day 0 sample shows concentration dependent activity. Although the slow onset of atovaquone's action may explain the limited results displayed in the previous sections, the activity of the day 0 sample in Figure 26 (b) suggests that there is a time-dependent loss in the compound's activity at a neutral pH. The limited use of the luciferase assay prior to the testing of the Ifakara incubated compounds may therefore have also contributed to a reduced accuracy in the previous results (in Figure 19).

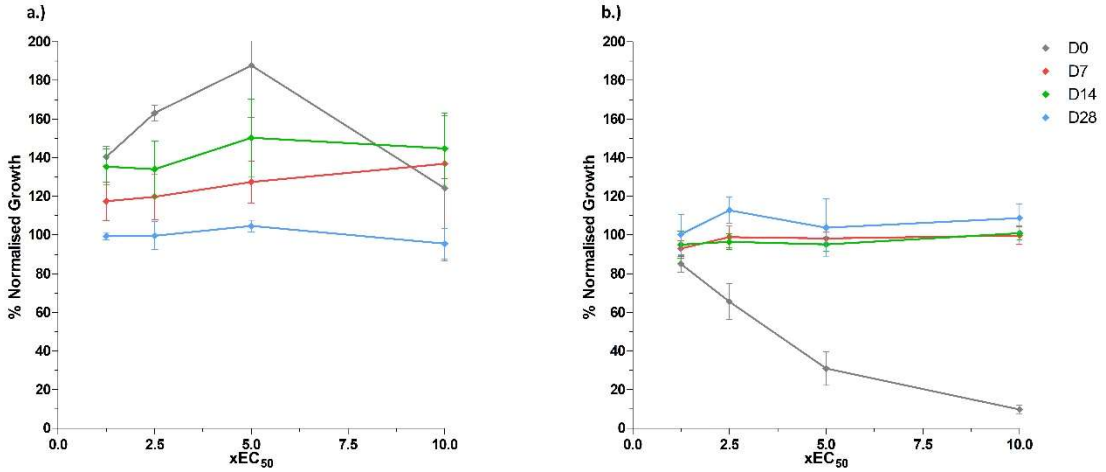

Figure 26. Solution containing atovaquone was exposed to Bamako-like temperatures and samples were taken on days: 0 (D0), 7 (D7), 14 (D14) and 28 (D28). The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a) pH7 and (b) pH5 (n=4) (see appendix for individual replicates).

The results of the luciferase assays conducted with MMV011895 samples are presented in Figure 27. Unlike the results of the Ifakara-incubated samples (Figure 20), there does appear to be consistent effect of both pH and time. It is hypothesised that the different results are due to improved handling and processing of the samples in the Bamako setup compared to the Ifakara setup. When incubated in pH7 conditions, the compound displays a loss of anti-plasmodial activity dependent on the period in which it is stored in the termperature/light cycle. This same effect is seen, although much less apparent, in the compound incubated in pH5 conditions.

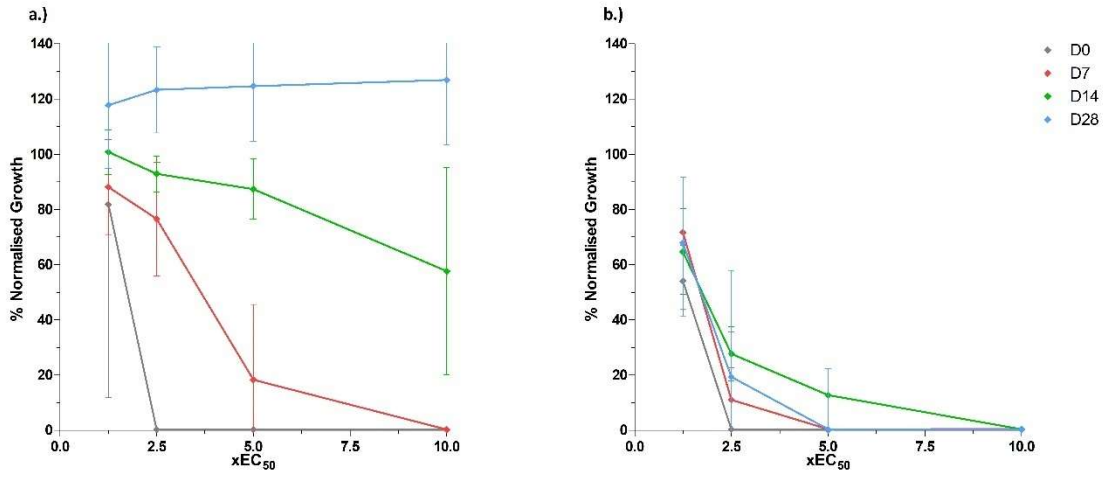

Figure 27. Solution containing MMV011895 was exposed to Bamako-like temperatures and samples were taken on days: 0 (D0), 7 (D7), 14 (D14) and 28 (D28). The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a) pH7 and (b) pH5 (n=4) (see appendix for individual replicates).

The results of the assays conducted with MMV666060 samples (Figure 28) remain consistent across the different experimental set ups, including those seen in Figure 23 (b). At pH7, there is a rapid and profound loss of anti-plasmodial activity. Similarly, to the results seen with atovaquone, MMV666060 consistently appears to be rendered non-active in pH7 conditions. The key difference in the findings of the two compounds, however, appears to be that MMV666060 is unaffected by storage in the temperature/ light cycle.

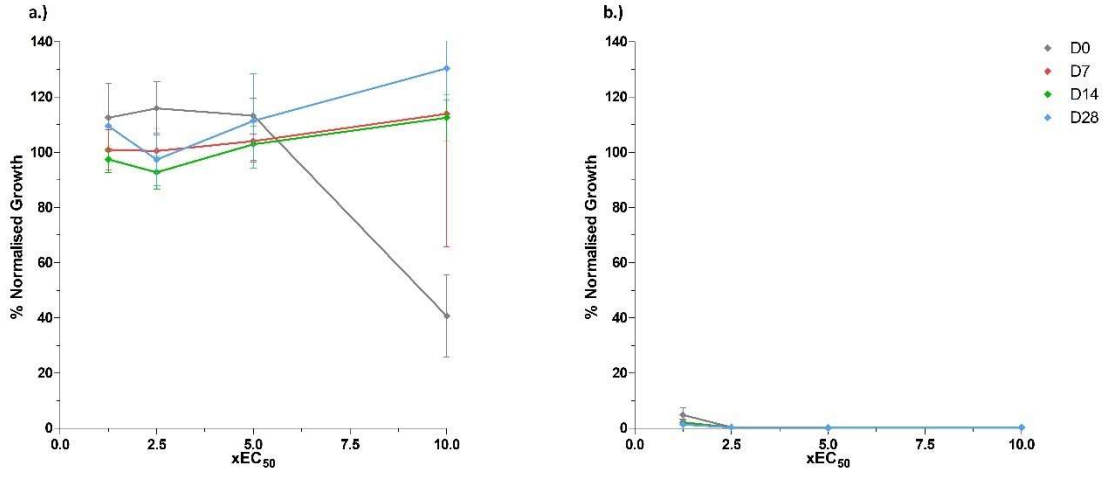

Figure 28. Solution containing MMV666060 was exposed to Bamako-like temperatures and samples were taken on days: 0 (D0), 7 (D7), 14 (D14) and 28 (D28). The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a) pH7 and (b) pH5 (n=4) (see appendix for individual replicates).

6.7 Ifakara, Tanzania based Studies

The aim of this study was to establish whether the feeding of anti-plasmodial compounds via sugar baits could be used to eliminate *Plasmodium* from infected *An. gambiae.* The format of the field-based study was affected by limitations put onto travel brought about by the current Covid-19 pandemic. Therefore, the field data was collected in collaboration with colleagues in Ifakara, Tanzania. The results, shown in Figure 29, are the first known trials in which anti-plasmodial compounds were fed to the mosquito within a sugar bait.

In Ifakara, *An. gambiae* mosquitoes were experimentally infected through a membrane system with blood from human patients carrying *P. falciparum* gametocytes. After 48 hours, the anti-plasmodial compounds (at 10xEC_{50}) were made available to groups of ≥190 mosquitoes through sugar water feeders. A sugar bait without anti-plasmodial compounds was provided to a group of mosquitoes as a control.

Figure 29 (a) shows the number of mosquitoes infected as measured by oocyst count on day 8. The number of mosquitoes infected following exposure to atovaquone sugar solution was significantly lower than that of the control group. Similar findings are presented in Figure 29 (b); this graph displays the number of mosquitoes infected as measured by sporozoite infection on day 14. The number of mosquitoes infected following exposure to atovaquone sugar solution was significantly lower than that of the control group.

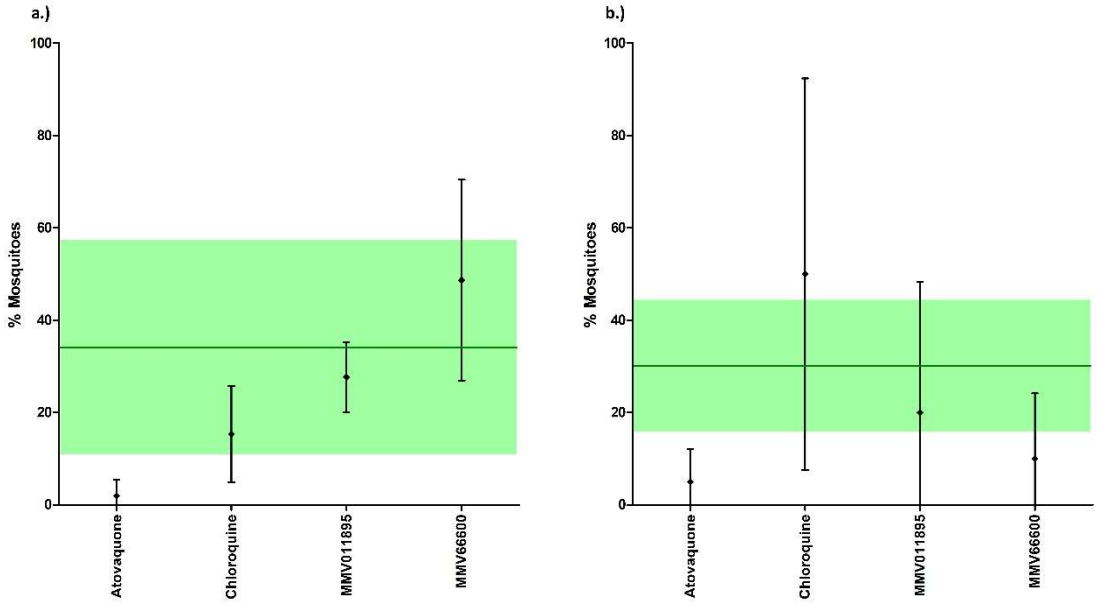

Figure 29. The amount of *An. gambiae* (%) infected with (a) oocysts on day 8 (n=3) and (b) sporozoites on day 14 (n=2) following feedings with either of the anti-plasmodial compounds. The amount of *An. gambiae* (%) infected following feeding from the control solution (mean shown by dark green line, SD shown by green square).

Atovaquone was the most potent of the compounds tested against both the sexual *Plasmodium* stages. Despite this, the use of this compound on wild mosquito populations would be injudicious as it may escalate the development of drug-resistant *Plasmodium* strains.

7 Discussion

The reduction in the rate of malaria is often attributed to the use of vector control methods such as IRS and ITNs (Bhatti et al, 2015). Despite successful IVM programmes, malaria tragically contributes to more deaths in the present day than in years prior. The reasons for this re-emergence include but are not limited to, the development of insecticide-resistant mosquito strains, residual transmission and changes in mosquito behaviour (Mnzava et al, 2015, Nkumama et al, 2016, Benelli & Beier, 2017, Sougoufara et al, 2020). Recent studies indicate that this trajectory is likely to continue despite the scaling up of established methods (Killeen et al, 2016) and the use of alternative insecticides. Therefore, it is vital to gain an understanding of how new and potentially more effective methods, can be utilised alongside existing methods. Such methods must be affordable (Nkumama et al, 2016, Olapeju et al, 2018) and easily operable to avoid any financial limitations.

The use of attractive antimalarial sugar baits (AASBs), proposed for the first time here, is predicted to be effective irrespective of the feeding and resting behaviours of the local mosquito population. Whilst the impact of current IVM programmes is biologically limited to their reliance on the vector's strong endophilic behavioural preferences (Benelli & Beier, 2017), stations can be placed either inside and/or outside the home. Entomological monitoring into the species composition of mosquito populations can be used to determine the appropriate placement of stations and avoid any loss of efficacy resulting from shifts in vector behaviours. Once an appropriate drug or combination of such, is identified and the baits are optimised, AASB stations have the potential to address the shortfalls of current IVM campaigns.

In this study, the aim was to investigate whether a novel compound could be used to eliminate *Plasmodium* within the mosquito population. To do this, a compound would be required to maintain its activity in the field conditions. These such conditions would include the pH value of the bait solution used to dilute the compound. Traditionally, compounds with anti-plasmodial activity are considered for human use and therefore must carry out their action at the slightly basic pH level of human serum (7.35-7.45) (Waught & Grant, 2007). For AASB application, however, the compounds would be required to carry out their action inside the adult female mosquito following ingestion (Aly et al, 2009, Al-Olayan et al, 2002). Corena-McLeod et al (2005) found that the posterior midgut pH in the adult female *An. gambiae* is between 8.0-9.5. A compound that will enact its activity at this pH is therefore essential to the AASB method. This, alongside the use of acidic fruit juices as an

attractant, means that a compound may be required to be stable over a large range of pH values if it is to be successful.

From the experiments, it is evident that the pH of the solution influences the final activity of the anti-plasmodial compounds. It was suggested that the differences between the results of the compounds may have been due a reaction with the hydrochloric acid used to prepare the PBS solution (see Figure 30). While this series of experiments used HCl adapted PBS, it would be prudent to acknowledge that different buffers may produce different results. Moving forward, different buffer solutions should be used to identify the compounds' optimal solute, whether it be organic or synthetic.

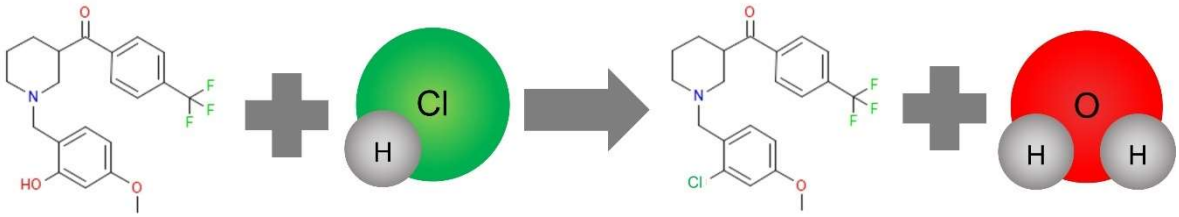

Figure 30. The possible condensation reaction occurring between MMV666060 and HCl. Typically, the (-OH) alcohol goup (such as that on MMV666060) will react with a hydrogen halide (in this case HCl) to produce an akyl halide group and water.

With the increase of interest into the use of ATSBs (Xue et al, 2006, Meza et al, 2020) and AASBs, there has been much research into the best attractant solution to use. In order to utilise the sugar sourcing behaviours of mosquitoes the baits need to be as attractive, if not more attractive, than the available natural plant sugar sources to the local mosquito populations. In their semi-field experiments, Tenywa and colleagues (2017) found no significant difference between the number of mosquitoes that chose to feed on the fruit juices compared to the control solution made up of solely sucrose solution. Other groups (Meza et al, 2020) have suggested the need for identifying a semiochemical attractant, which would negate the need for regular replacement due to fermentation. Regarding AASB implementation, the use of a synthetic solute would provide the further benefit of not needing to investigate how a fruit juice would affect the stability of any novel antimalarials and thus only the compound's optimum pH range would need to be identified.

Further to establishing the efficacy of AASB use, the stability of the compounds would need to be ensured over an extended period in the relevant temperatures. By using temperatures attributed to Tanzania and Mali, the method analysed the stability of the compounds at temperatures typical of endemic regions. While some of the compounds seemed to be unaffected over time, atovaquone appeared to break down almost immediately and MMV011895 appeared to break down dependent on other factors. This need for compound stability is essential as it would mean that the bait solution would not need to be changed and thus making the method more cost-effective and practical for communities that may be otherwise hesitant (Maia et al, 2018).

As a slow-acting anti-plasmodial agent with an established mode of action (Kessl et al, 2003, Birth et al, 2014), atovaquone was used in the study for the purpose of comparison. Additionally, the compound's acknowledged activity against sporontocidal *Plasmodium* (Enosse et al, 2000, Paton et al, 2019) meant it could be utilised as a positive control through to the field-based stages of the study. Within their original packaging, atovaquone tablets are described as having a shelf life ≤3 years when stored below 25°C and protected from moisture (EMC, 2017). For this study, however, the stability of the compound was tested under a range of atypical environmental pressures.

Upon a 45-day exposure to a Tanzania-simulated temperature cycle, within both acidic and neutral conditions, atovaquone appeared to lose its activity completely and rapidly. This effect was seen even with the samples taken on day zero. The lack of consistency across the technical repeats of this setup and the lack of prior execution of the technique means the results must be read with some reservation. As to substantiate the findings of this initial trial, atovaquone was exposed to both the acidic and neutral conditions and samples were immediately plated as to limit the effect of temperature and time. The result here confirmed the slow nature of atovaquone's action, whilst also suggesting the compound was more effective in acidic conditions over neutral conditions. It was also suggested that the acidic solution was able to maintain the activity of atovaquone over the short period of plate processing, whereas the neutral solution did not. The results of 28-day incubation of the compound in a Malian-simulated temperature cycle seem to corroborate this; the sample taken from the acidic conditions on day zero remained active against the erythrocytic *P. falciparum*, whereas the sample exposed to neutral conditions did not.

With a widely accepted mode of action affecting the haemoglobin digestion of asexual *Plasmodium* and efficacy against non-resistant strains, chloroquine is often used as an ideal for the means of identifying a favourable antimalarial drug. The compound's ability to quickly clear plasmodia in the human stages (Harraiz et al, 2019) and limited activity against the sexual forms of the parasites (Enosse et al, 2000) presented an interesting contrast to atovaquone in the study. Of particular interest was chloroquine's diprotic base structure (Browning, 2014), how this would be affected by the different conditions tested and ultimately, the changes in the compound's efficacy against *Plasmodium* as a result. As a tablet, chloroquine is described as having a shelf-life of ≤5 years when stored below 30°C and protected from light and moisture (IPCS, 1994). For this study, however, the stability of the compound was tested under a range of atypical environmental pressures.

In the initial trial, within both acidic and neutral conditions, chloroquine appeared to consistently maintain its activity. The length of incubation also appeared to have no effect when the compound underwent extended exposures to either of the temperature cycles. After incubation at

the Malian temperature cycle, however, the compound did display some loss of activity in the neutral conditions as opposed to the acidic conditions.

In this study, the aim was to investigate whether a novel compound could be used to eliminate *Plasmodium* within the mosquito population. To ensure the safety regarding the use of the compound to a mosquito population, there must be no likelihood of the compound's use on humans. A compound with the same or similar structure to an anti-malarial drug prescribed to humans would be inadvisable as this could contribute to the development of resistant *Plasmodium* strains. This is the reasoning behind using atovaquone and chloroquine as controls only, they would be unsuitable for any real-world application of AASBs. For this purpose the compounds MMV011895 and MMV666060, were selected from the 'probe-like section' of the Malaria Box library.

To implement this method, a novel compound (or group of compounds) must be identified. One that can withstand the field-conditions necessary. Following a 45-day exposure to a Tanzania-simulated temperature cycle, within both acidic and neutral conditions, MMV011895 displayed no significant variation over time. The results suggest that the pH of the solutions had no effect on the efficacy of MMV011895. During the latter 28-day exposure to a Malian-simulated temperature cycle, however, MMV011895 appeared to react differently when in the acidic and neutral conditions. Here, the compound appeared to lose its efficacy quicker in the neutral solution than in acidic solution. The other novel compound, MMV666060, continually proved to be more potent in the acidic conditions than the neutral conditions. Following storage in neutral conditions, the compound underwent a rapid and profound loss of anti-plasmodial activity. The change within the structure of the compound responsible for this appears to be almost instantaneous. There was seemingly no loss in MMV666060's activity when stored in the acidic solution.

Although the results of assays using *P. falciparum* trophozoite stages do not necessarily translate to the gametocidal outcomes of the compounds tested, the *in vitro* method does allow for a simpler method in which the viability of compounds can be tested. The use of the climatic chamber allowed for the reproducibility of the studies and although the incubation process represents the longest stage within the experiments, large numbers of compounds can be processed simultaneously. Through this, candidate compounds can be screened for any loss of activity following their incubation using *in vitro* assays. Compounds that do remain stable may then be prioritised for more costly and labour-intensive testing methods.

In the field-like stages of the trial, the compounds were fed to recently emerged and unfed mosquitoes, meaning there was little need for an attractant to be used within the sucrose solution. Despite them actively feeding on the solution, the compounds in the study did not display the necessary activity against oocysts and sporozoites. While MMV011895 displayed limited activity against the oocyst stage, it was far less effective against the sporozoite stage. The opposite was true

for MMV666060. As was expected, atovaquone displayed significant activity against both the oocyst and sporozoite numbers (Paton et al, 2019), whereas chloroquine did not. The results identified here are consistent with those presented by Van Voorhis and colleagues (2016), in which both compounds displayed activity against the *P. falciparum* (NF54) gametocytes, however, the activity of MMV666060 falls as the gametocytes enter the later stages of their development (Plouffe et al, 2016). MMV011895 results in lower numbers of ookinete infected mosquitoes than MMV666060; with both compounds' sexual stage activity ultimately being insufficient (Ruecker et al, 2014).

The ideal compound would possess pan-activity across the sexual stages and potentially the asexual stages of the *Plasmodium* lifecycle. Activity against the asexual stages enables viability screening to occur using *in vitro* assays. On the other hand, should a compound only possess activity against the sexual stages, the biological assays could be supplemented with the use of single ion mass spectrometry (MS). The complexity of *Plasmodium* lifecycle and the dearth of knowledge of the sexual stages of its development require much further investigation. By gathering the results of a wide variety of assays, such as that carried out by Van Voorhis and colleagues (2016) using the MMV's 'Malaria Box' library, candidate compounds can be identified and gauged for their suitability for an indeterminate number of future applications.

In a similar fashion to which the MMV denoted half of the Malaria Box library as 'probe-like' and thus unlikely to be prescribed to humans, candidate compounds could be attained from the repurposing of drugs previously unexplored for anti-plasmodial activity. Pharmaceutical companies typically abandon any candidate compounds once they are found to not fulfil the necessary pharmacological and safety properties (e.g. potential cardiac toxicity using assays of hERG channel function). These discarded compounds could therefore be assessed for their effect on the sexual stages of the *Plasmodium* within the mosquito without concern for contributing to the development of drug-resistant strains.

There are many compound libraries which would be compelling to investigate for their use in AASBs. One such library is the Tres Cantos Antimalarial Set (TCAMS) (Cabrera, 2019), of which 70 commercially available compounds were identified as having potential transmission-blocking and intraerythrocytic activity using the high throughput *P. berghei* ookinete development assay (PbODA) (Delves et al, 2019). With libraries such as TCAMs available, intraerythrocytic EC_{50} assays are the first step in evaluating the environmental stability of the compounds and optimising the parameters of environmental exposure. Compounds that are confirmed of relative stability during environmental exposure can then be prioritised to undergo mosquito feeding assays, such as those in section 5.7.

Once potential candidate compounds are identified, lower-throughput investigations can begin in field. This would include studies to validate the effectiveness of the anti-plasmodial

compounds against numerous field-strains and a range of mosquito populations. Following this, explorations into effective delivery strategies within bait stations can begin.

The repurposing of drugs from other areas of research also has the advantage of minimising expenditure during the execution of the pipeline. The price of the anti-plasmodial compound is of major significance to AASB application. Although the two drugs used in this study, MMV011895 and MMV666060, were provided from MMV without cost for the purposes of the trial, under normal circumstances, they must be synthesised and bought from chemical vendors. Upon request, the two compounds were quoted at £1,942g^{-1} (AKos GmbH, Germany) and £1,185g^{-1} (ChemBridge, USA) respectively. With the amount of the drug that would be required for a roll-out of any future AASB campaign, the relative costs would decrease substantially. Despite this, the ease of which a candidate compound can be synthesised has implications for its production costs which should be considered moving forward.

Historically, IVM often relies on financial aid and infrastructure enabled campaigns to accumulate the materials needed and distribute them appropriately (Korenromp et al, 2016). Unlike IRS and LLINs, however, bait stations can be locally manufactured using recycled and easily obtained materials (Stewart et al, 2013, Tenywa et al, 2017, Maia et al, 2018). This is of particular benefit to LMICs and rural populations. The foremost factor, therefore, in the successful implementation of AASBs, appears to be the existing level of community awareness and participation (Sexton, 2011). As opposed to LLINs and IRS, bait stations do not directly protect users from mosquito bites and do not trap the mosquitoes, thus it would be appropriate to educate communities on the rationale behind the method and explain how the stations can provide an operationally simple, safe and cost-effective method for malaria vector control (Maia et al, 2018).

An important aspect of safe vector control practices is to ensure the methods employed are environmentally sensitive. The mode of action of most insecticides currently used in malaria control is to block important biological pathways within the target insect. The binding site of such compounds, however, are often those with homology to non-target insects such as *Coleoptera*, *Diptera*, *Hymenoptera* and *Lepidoptera* (Qualls et al, 2014). These insects provide vital pollination and thus are integral to the maintenance of natural ecosystems. In comparison with ATSB stations and other methods which employ insecticides, AASBs could offer a strategy that altogether avoids impact on non-target insects. By identifying a compound that is specific to the target parasite, AASBs have the potential to offer a long-term ecologically safe solution towards the reduction of malaria transmission.

By not killing the mosquitoes outright, the strategy has the potential benefit of maintaining the ecological balance. It is thought that mosquitoes are permissive to low numbers of the parasite (Hajkazeman et al, 2020) despite it being of detriment to the insects' health, due to the high cost of mounting an immune defence (Niaré et al, 2002). It has been suggested that if an AASB could be

developed that had no detrimental effect on the target insect (Table 10), there is potential for mosquitoes to mount an outright immune response and thus lead to the complete elimination of the parasites.

Table 10. Compound killing rate (%) of *A. aegypti* following 48 hours of incubation (from Van Voorhis et al, 2016).

Concentration (µM)	10.0
MMV011895	14
MMV666060	35

Whilst this study was unable to identify a novel compound with the desired gametocidal activity, the proof of principle does stand. Going forward, as more compounds are progressed through the pipeline, it is hoped that such will contribute to the overall body of knowledge in the area and in doing so help ensure the optimum implementation of the AASB method. The methods used here, stand as the beginning of a credible series in which additional techniques can be easily inserted into as is necessary.

8 Conclusion

Current vector control strategies, such as IRS and bednets, have proven invaluable for the control of malaria. The efficacy of such methods, however, is declining and researchers have suggested a multitude of reasons for this, with the reasons suggested varying from the biological to socioeconomic. Additional tools are, therefore, required to complement such interventions.

One method suggested as able to fulfil this need are ATSBs which take advantage of the mosquito's attraction to sugar sources as a route of insecticide delivery. They promise an inexpensive tool deployable both indoors and outdoors. Despite its advantages, the method still requires the use of toxic compounds to kill the vector insect and as such could pose a threat to non-target organisms attracted by the sugar bait. Furthermore, ATSBs are vulnerable to the evolution of mosquito physiological resistance to toxic compounds or behavioural avoidance. By replacing the pesticide with a mosquito-stage anti-plasmodial, the novel concept of AASBs is formed. This innovation aims to address the current unsustainable approach of complete reliance on pesticides for the control of vector-borne diseases.

This study proposed the idea of AASBs as a more sustainable method to ATSBs in terms of management of pesticide resistance and limiting ecological impact. Attractive sugar baits laced with anti-plasmodial drugs that clear the malaria parasite stages found within mosquitoes, present an opportunity through which the transmission of parasites to humans is blocked. The successful

implementation of AASBs requires not only a compound that kills the sexual stages of the parasites' lifecycle, but also that displays relative stability over an extended period within a bait station.

In this study the aim was to identify a series of methods through which the viability of anti-plasmodial compounds can be studied for their application into an AASB. To do so, the activities of two known sexual-stage active compounds were evaluated, alongside two control compounds, before and after their prolonged exposure to the environmental conditions of an attractive bait station in sub-Saharan Africa. This assessment demonstrated that the effect of temperature, light and pH parameters on compound stability can be readily measured in *P. falciparum* intraerythrocytic stages expressing a bioluminescent reporter. In Ifakara, the same set of compounds were fed to mosquitoes experimentally infected with *P. falciparum* thereby demonstrating that antimalarial compounds that are active against ookinete and/or sporozoites can be characterised using the pipeline of bioassays prior to field evaluation.

Interestingly, while a decline in the different compounds' activities over time was expected, it was surprising that the acidic solutions appeared to maintain their relative stabilities more effectively than the neutral solutions. To this point, the successful execution of the AASB method will require a suitable solute to which the mosquitoes are attracted and that maintains the stability of the anti-plasmodial compound. Furthermore, of the novel compounds assessed within the study, neither displayed the desired activity against ookinete and/ or sporozoites. The methods used here, stand as the beginning of a credible series of bioassays through which compounds can be assessed for their suitability for AASB use. The significant advantages that AASBs pose are invaluable to the control of malaria and should, therefore, continue to be strived towards.

This innovation represents an important shift in strategy in the field of vector biology and control: targeting the aetiological agent of disease during transmission in insect vectors, rather than targeting the vectors themselves. The continuing development of antimalarial compounds has provided numbers of compounds that are effective, affordable and safe for human use. Compounds that meet the first two of such criteria and fail the latter are discarded continually throughout this process. By delivering transmission-blocking anti-plasmodial compounds directly to the mosquitoes, AASBs offers a significant route to repurposing what would otherwise be potentially dangerous to use in humans. The inclusion of new compound classes, against different parts of the human-vector transmission cycle offers an attractive tool in complementing our current antimalarial pharmacopeia. AASBs, thus, hold the potential for a significant and easily implementable step closer to the eradication of malaria.

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10 Appendix

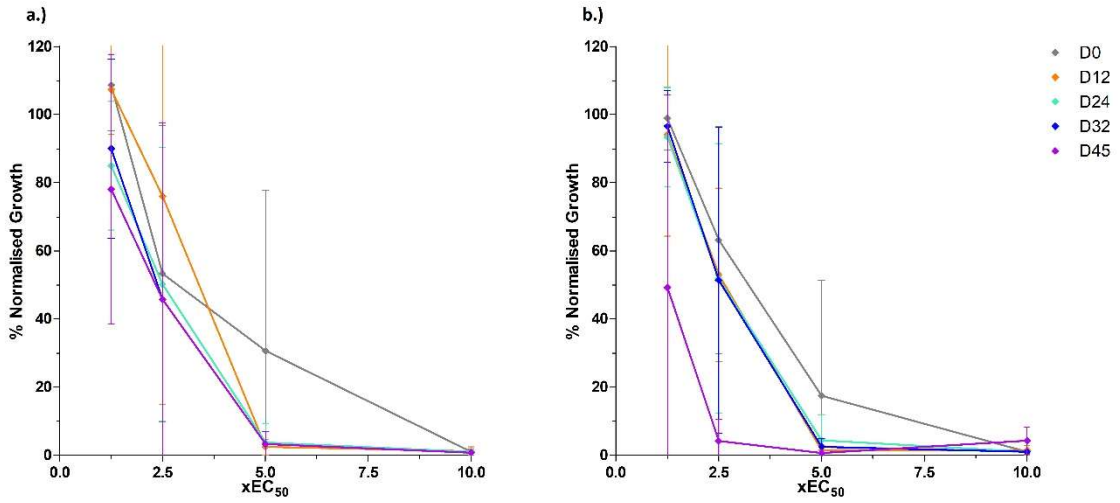

Figure 31. Solution containing chloroquine was exposed to Ifakara-like temperatures and samples were taken on days: 0 (D0), 12 (D12), 24 (D24), 32 (D32) and 45 (D45). Combined data of the replicates presented in Figure 18. The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a) pH7 and (b) pH5 (n=6).

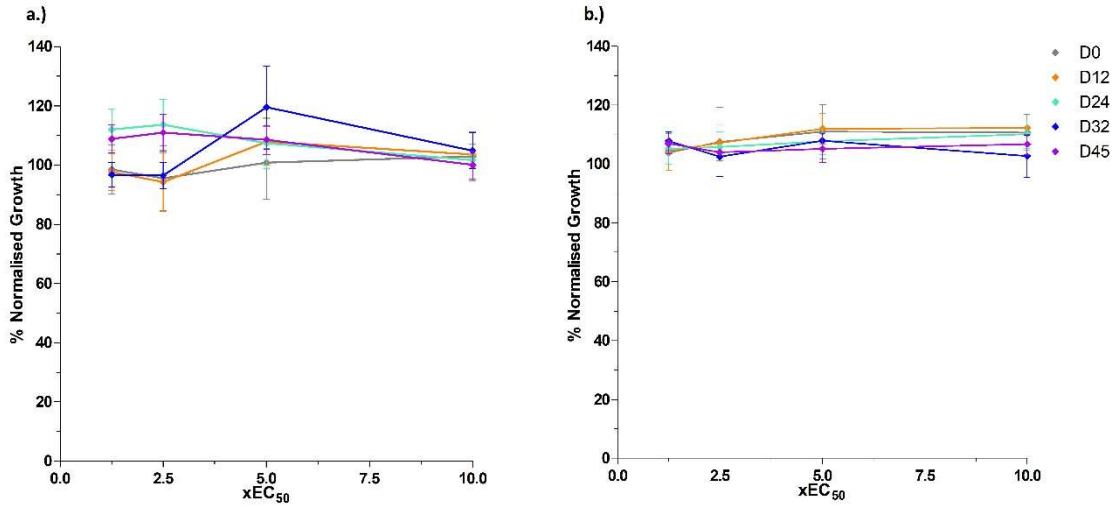

Figure 32. Solution containing atovaquone was exposed to Ifakara-like temperatures and samples were taken on days: 0 (D0), 12 (D12), 24 (D24), 32 (D32) and 45 (D45). Combined data of the replicates presented in Figure 19. The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a) pH7 and (b) pH5 (n=4).

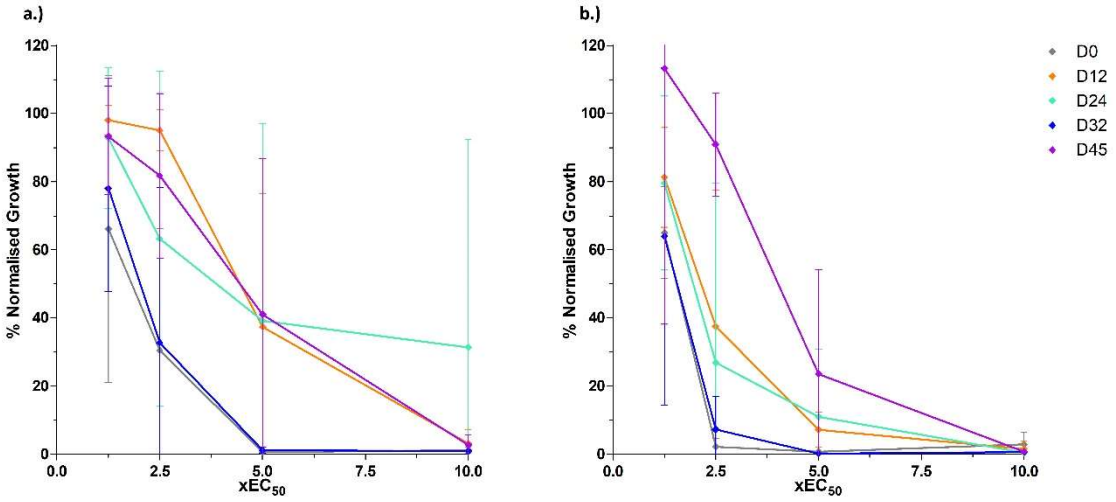

Figure 33. Solution containing MMV011895 was exposed to Ifakara-like temperatures and samples were taken on days: 0 (D0), 12 (D12), 24 (D24), 32 (D32) and 45 (D45). Combined data of the replicates presented in Figure 20. The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a) pH7 and (b) pH5 (n=4).

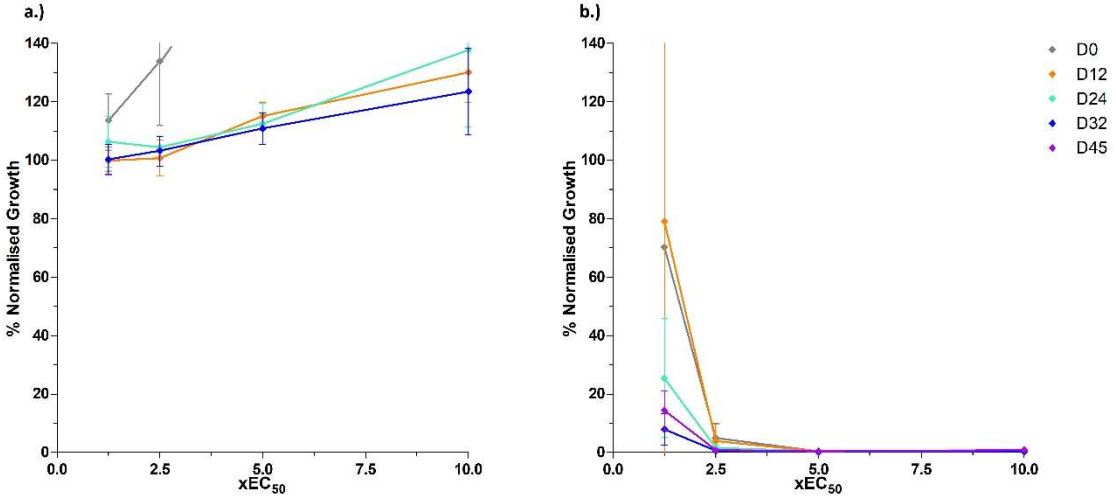

Figure 34. Solution containing MMV666060 was exposed to Ifakara-like temperatures and samples were taken on days: 0 (D0), 12 (D12), 24 (D24), 32 (D32) and 45 (D45). Combined data of the replicates presented in Figure 21. The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a) pH7 and (b) pH5 (n=4).

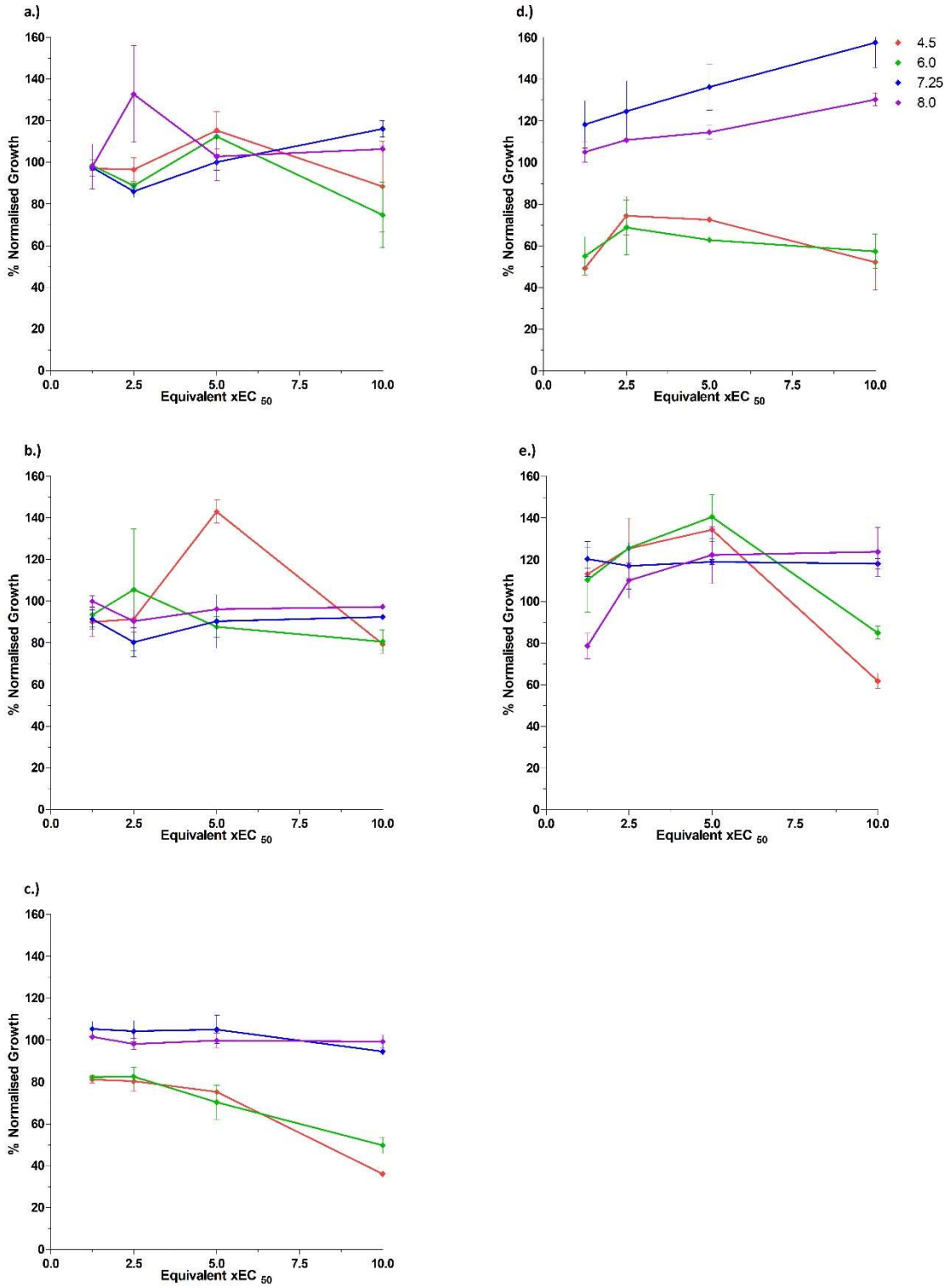

Figure 35. The normalised growth (%) of *P. falciparum* Dd2^{luc} following 48hr incubation with PBS only at pH levels: 4.5, 6.0, 7.25 and 8.0. Each graph represents one of five biological repeats, combined data of the replicates presented in Figure 22 (n=2).

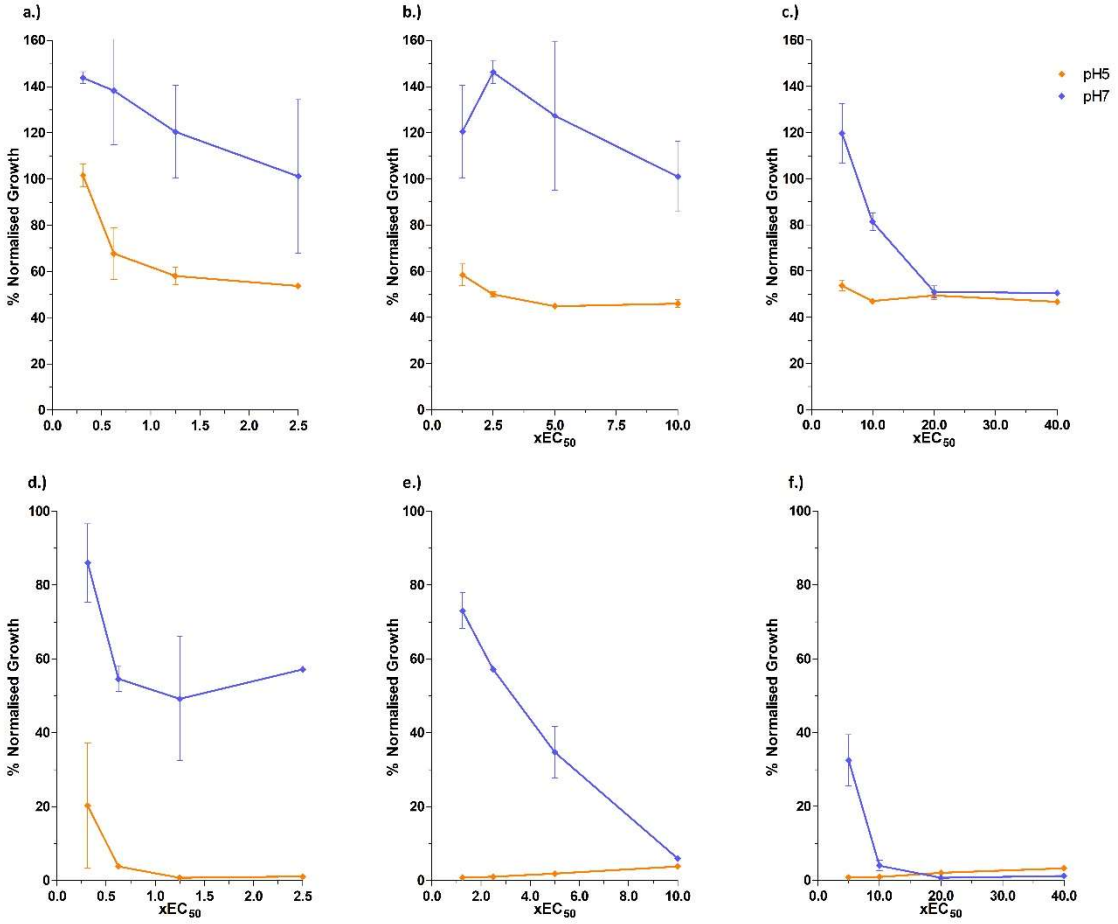

Figure 36. The normalised growth (%) of *P. falciparum* Dd2^{luc} following 48hr incubation with (a-c) atovaquone and (d-f) MMV666060 at both pH7 and pH5. Graphs show the results of three two-fold dilution series with starting concentrations: 40xEC50, 10xEC50 and 2.5xEC_{50} (n=2). Data combined in Figure 23.

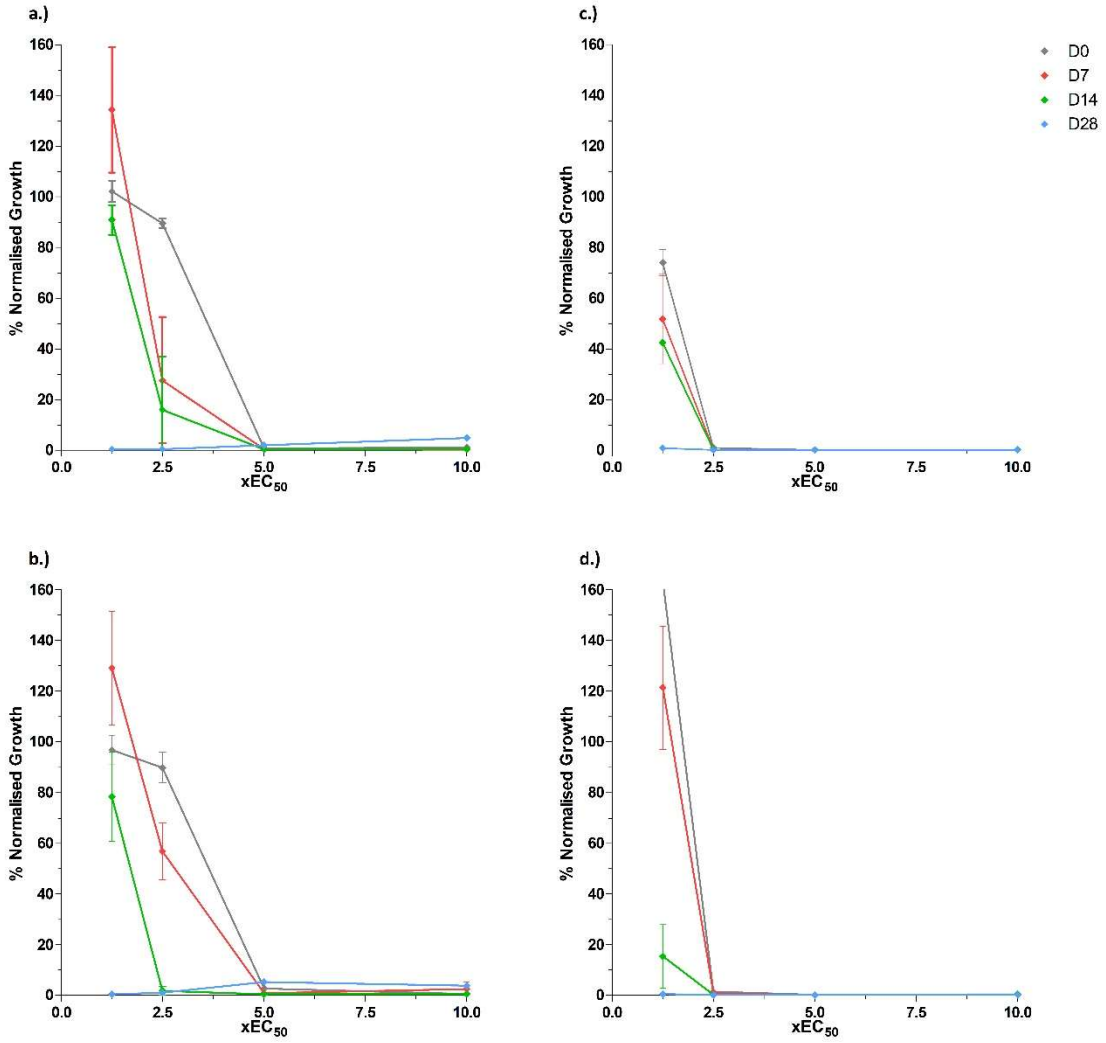

Figure 37. Solution containing chloroquine was exposed to Bamako-like temperatures and samples were taken on days: 0 (D0), 7 (D7), 14 (D14) and 28 (D28). Each row of graphs represents a biological repeat (combined data presented in Figure 25). The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a-b) pH7 and (c-d) pH5 (n=2).

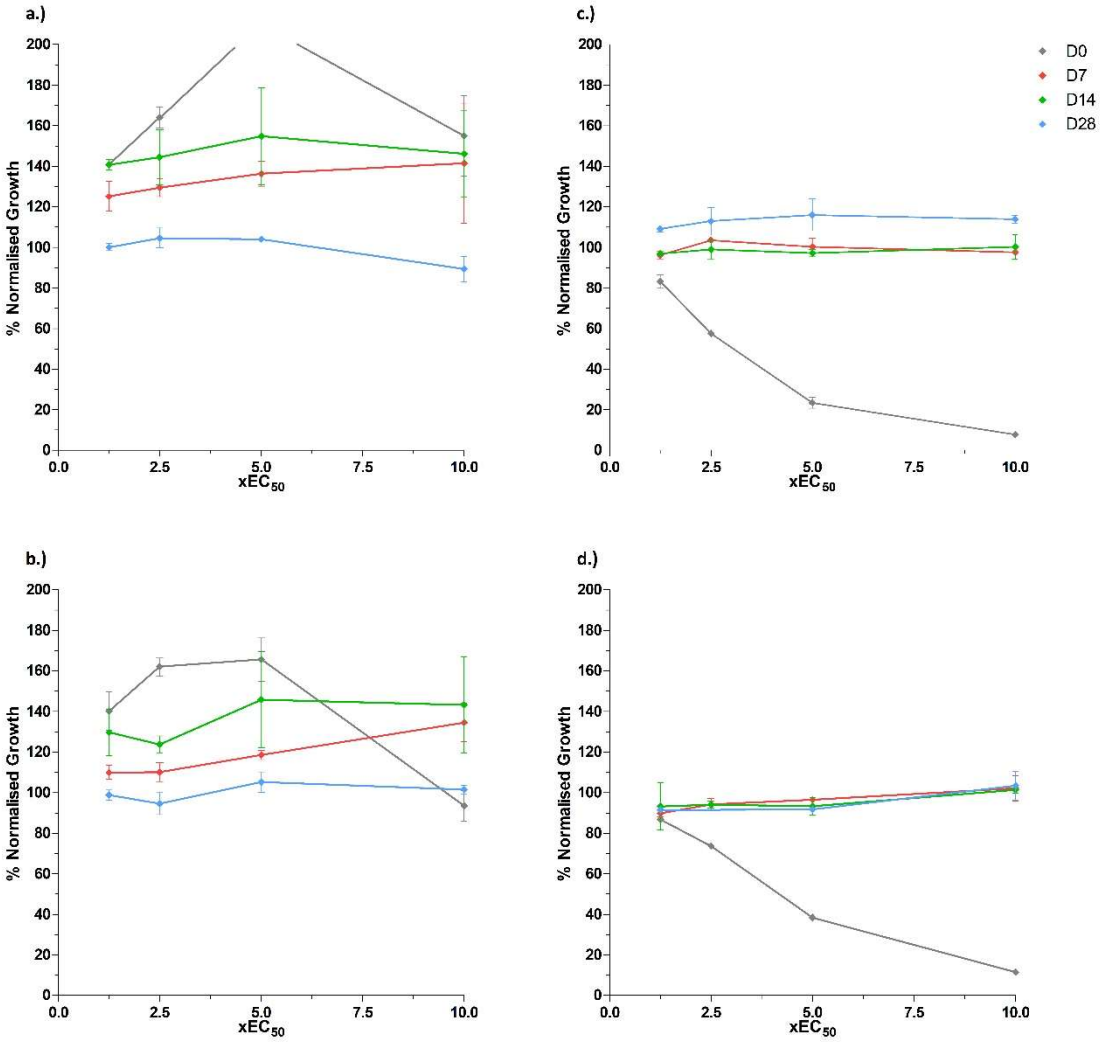

Figure 38. Solution containing atovaquone was exposed to Bamako-like temperatures and samples were taken on days: 0 (D0), 7 (D7), 14 (D14) and 28 (D28). Each row of graphs represents a biological repeat (combined data presented in Figure 26). The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a-b) pH7 and (c-d) pH5 (n=2).

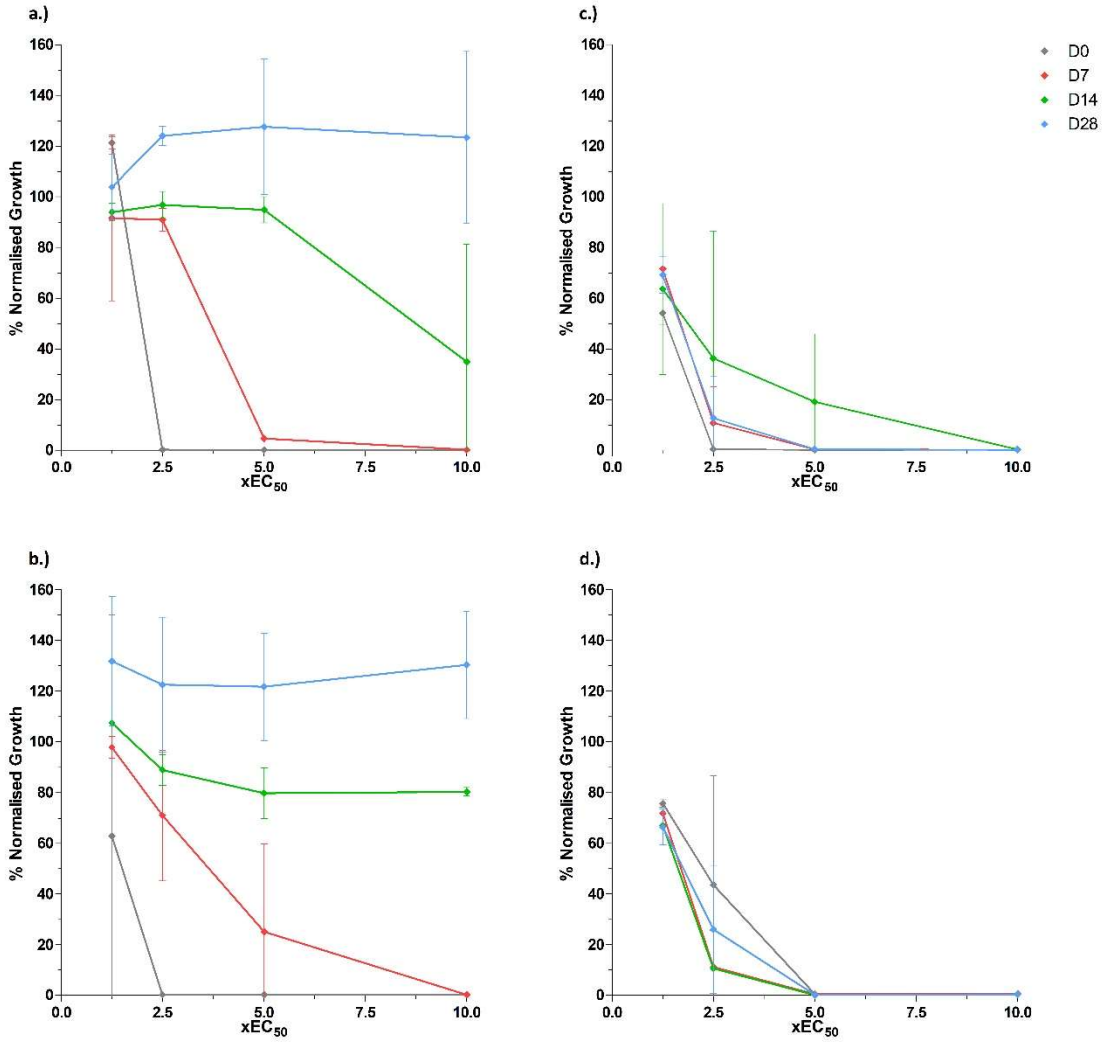

Figure 39. Solution containing a MMV011895 was exposed to Bamako-like temperatures and samples were taken on days: 0 (D0), 7 (D7), 14 (D14) and 28 (D28). Each row of graphs represents a biological repeat (combined data presented in Figure 27). The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a-b) pH7 and (c-d) pH5 (n=2).

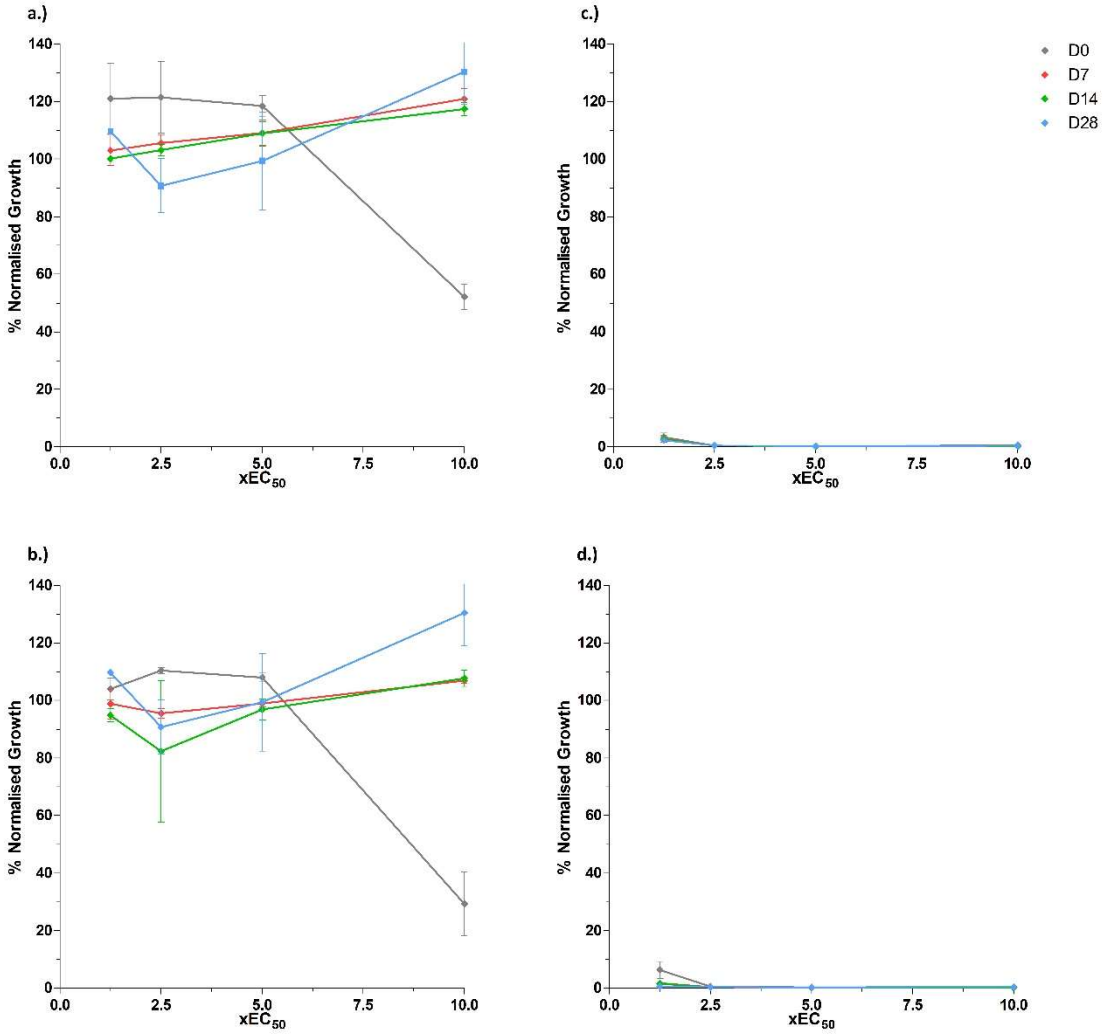

Figure 40. Solution containing a MMV666060 was exposed to Bamako-like temperatures and samples were taken on days: 0 (D0), 7 (D7), 14 (D14) and 28 (D28). Each row of graphs represents a biological repeat (combined data presented in Figure 28). The normalised growth (%) of *P. falciparum* Dd2^{luc} established using 48hr luciferase assays with samples at (a-b) pH7 and (c-d) pH5 (n=2).