Novel insecticides and application strategies for malaria vector control

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September, 2015

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LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE

Thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy of the University of London

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Abstract

Targeting malaria vector mosquitoes outdoors has become a research priority to address residual malaria transmission. Mosquito larval source management provides an excellent and well established tool. However, there is a need to reduce the cost and effort of larviciding programmes by testing persistent larvicides that reduce the frequency of application and by exploring novel strategies of application. This thesis aimed to evaluate two larval control agents, with unique mode of actions: the self-spreading silicone-based film Aquatain Mosquito Formulation (AMF) and the pyriproxyfen-based insect growth regulator Sumilarv®.

Dose-response tests and standardized field tests were conducted to assess the susceptibility of *Anopheles gambiae sensu stricto* and *An. arabiensis* to the two insecticides and determine their residual activity. Adults that survived exposure to larvicide-treated water at the larval stages were assessed for possible effects on fecundity and fertility. Both species were highly susceptible to both control agents at very low doses. Both control agents provided residual control of up to six weeks. Adults that emerged from larvicide-treated water laid fewer eggs and had low egg hatching rates. Consequently, the impact of three-weekly operational application of pyriproxyfen to habitats in the western Kenya highlands was assessed by comparing adult vector emergences from aquatic habitats in intervention and non-intervention sites. Pyriproxyfen application led to over 80% adult vector emergence inhibition from treated aquatic habitats.

To assess if larvicide-treated water could serve as 'reproductive sinks' for gravid mosquitoes, the oviposition response of gravid *An. gambiae s.s.* to water treated with pyriproxyfen or surface film was tested under semi-field conditions using squares of electrocuting nets. Larvicide-treated water did not affect the pre-oviposition behaviour of gravid females. This study however did not demonstrate that 'attract and kill' strategies could be used for control of malaria vectors as the addition of an oviposition attractant to ponds containing larvicide-treated water did not increase the proportion of gravid females orienting towards this pond.

To explore the effect of pyriproxyfen exposure on adults, individual *An. gambiae s.s.* and *Cx. quinquefasciatus* females were exposed to pyriproxyfen at seven time points around blood feeding. Fecundity and ability to transfer pyriproxyfen to an oviposition substrate

were studied in the laboratory. The impact of pyriproxyfen was dependent on the time of exposure. Females were nearly completely sterilized when exposure occurred around the blood meal while pyriproxyfen was only transferred by females that were exposed while gravid and close to egg-laying time.

Consequently, a baiting station for gravid females was developed and semi-field experiments implemented to explore the transfer of pyriproxyfen by gravid *An. gambiae s.s.* from the baiting station to aquatic habitats. Horizontal transfer was observed but the extent of emergence inhibition was dependent on the distance of the habitat from the baiting station. Only the closest habitats received sufficient pyriproxyfen to control significant numbers of offspring.

In conclusion, this study demonstrated great potential of the two control agents for the control of vector immature stages and adults caused by sterilizing effects of pyriproxyfen. Results suggest that they are suitable for inclusion into integrated vector management programmes for malaria control. Auto-dissemination of pyriproxyfen however, appears not to be a feasible strategy for malaria vector control.

Acknowledgement

The completion of this PhD thesis is a result of collective contributions of many individuals and institutions for whom I am grateful and I wish to express gratitude.

I am profoundly grateful to Dr. Ulrike Fillinger, my primary supervisor and mentor who introduced me to the field of malaria research and mosquito control. I will forever be grateful for the patience, scientific guidance and commitment that you accorded me in every single step of my PhD studentship. I enjoyed the good discussions that you encouraged us to freely have with you even as we interrupted you from your other duties. Thank you for sharing an office with all your students as this enabled us to constantly consult you and share the results and challenges of our work.

I sincerely thank Prof. Steve Lindsay, my second supervisor for the tremendous support, advice and inspiration he gave me throughout my study. Your comments and suggestions helped shape this work. Thank you for the live football match we watched together with your family and Manuela between Sunderland and West Bromwich Albion at the Stadium of Light in Sunderland.

I thank Dr. Jenny Lindh for the insights, advices and the numerous inspiring discussions we have had throughout this study.

I am grateful to Dr. Bryson Ndenga of KEMRI for including me in his team during the field work in the western Kenya Highlands. I appreciate the regular discussions we had as we carried out the field work. I am grateful for your guidance to ensure the field work was done expertly. Special thanks to all the field personnel we worked with.

Special thanks go to my fellow PhD students Michael Okal, Sisay Dugassa, Manuela Herrera and Lynda Eneh for the frequent discussions on mosquitoes and malaria control that we had every single day in and out of office. The critical discussions and ideas we shared through our studentship have indeed modelled me to appreciate the power of sharing.

I thank the OviART team members Margaret Mendi, Paul Ouma, Elizabeth Masinde, Gregory Masinde, Rose Ongole, Joel Odero and Benard Oyembe for their worthy

technical assistance and hard work as we carried out experiments. I could not have done it without you.

To Prof. Baldwyn Torto and Xavier Cheseto I will always thank you for the new skills you taught me in chromatographic analysis.

I thank David Alila and Elisha Obudho of the icipe-Mbita insectary for ensuring constant supply of mosquitoes for experiments.

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List of acronyms

ai	Active ingredient
AMF	Aquatain Mosquito Formulation
EIR	Entomological Inoculation rate
GEE	Generalized estimating equations
icipe	International Centre of Insect Physiology and Ecology
IGRs	Insect growth regulators
IRS	Indoor residual spraying
ITNs	Insecticide-treated nets
IVM	Integrated Vector Management
KEMRI	Kenya Medical Research Institute
LLINs	Long lasting insecticidal nets
LSM	Larval source management
MMFs	Monomolecular films
PPF	Pyriproxyfen
RBM	Roll Back Malaria
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

1 Introduction

1.1 The burden of malaria in sub-Saharan Africa

Despite the advances in preventive and public health measures to combat malaria, the disease remains one of the most important vector-borne disease (WHO 2014b). Latest WHO global estimates approximate 3.2 billion people to be at risk of malaria with about 198 million cases and 584,000 malaria-related deaths occurring in 2013, 80% of cases and 90% of these deaths occurring in Sub-Saharan Africa (WHO 2014c). The most vulnerable groups to malaria are infants, children less than five years of age and pregnant women (Crawley 2004; Schantz-Dunn and Nour 2009). In addition to the effect on public health, malaria continues to be a big hindrance to the socio-economic development of communities in resource-deprived African countries (Gallup and Sachs 2001). Malaria has a complex intricate relationship with poverty in most endemic communities. Individuals with low socio-economic status associated with low income, poor housing, difficulty to access effective medication, low educational status and poor knowledge of malaria and its control are generally at a greater risk of malaria infection (Jones and Williams 2004; Nkuo-Akenji *et al.* 2006; Somi *et al.* 2007; Ayele *et al.* 2013). The disease is a big impediment to the cognitive development of school-going children due to frequent absenteeism from class and reduces the productive life of adults of working age (Abdalla *et al.* 2007). The economic burden that the disease exerts at both the family and national level is enormous (Teklehaimanot and Mejia 2008). It is estimated that treatment and control of malaria in endemic countries in Africa accounts for approximately US$ 12-15 billion loss in gross domestic product (GDP) subsequently slowing down growth in these countries by more than 0.5-9% every year (Gallup and Sachs 2001; Sachs 2001).

1.2 Biological and environmental determinants of malaria transmission in Africa

Malaria transmission is a complex process that involves the interaction of the *Anopheles* mosquito, human host, parasite and environmental characteristics (Coosemans *et al.* 1992). *Plasmodium*, the parasite that causes malaria, is a parasitic protozoa transmitted to humans through the bite of an infectious female *Anopheles* mosquito. Four species of *Plasmodium* are known to infect humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and

P. malariae. Of these four, *P. falciparum* poses the greatest public challenge due to its greater virulence, is the most prevalent in sub-Saharan Africa and responsible for most deaths from malaria (Hayward *et al.* 1999; Guerra *et al.* 2008; WHO 2014c). However, *P. vivax* has a widest geographical distribution due to its ability to develop in the *Anopheles* mosquito at lower temperatures and survive at higher altitudes (Guerra *et al.* 2008; Guerra *et al.* 2010). *Plasmodium* has a complicated life-cycle that involves infecting successively a human host and the female *Anopheles* vector (Figure 1.1).
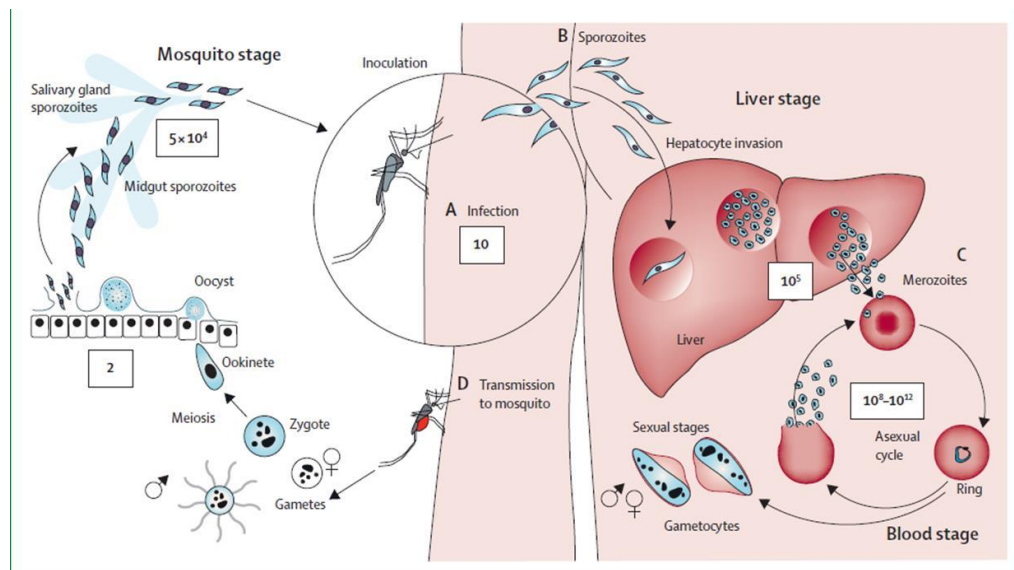
Figure 1.1: Basic life-cycle of the malaria parasite (White *et al.* 2014)

(A) An infected female *Anopheles* mosquito first inoculates sporozoites into a susceptible human host during a blood meal. (B) The sporozoites infect liver cells and differentiate into merozoites. (C) Merozoites burst from leave liver cells and infect red blood cells. Infected red blood cells burst releasing merozoites that infect other red blood cells. Some merozoites leave asexual reproduction and differentiate into haploid sexual stages called male and female gametocytes. (D) Female *Anopheles* mosquito picks up gametocytes from infected human during a blood meal. In the mosquito midgut the female gamete is fertilized by the male gamete to produce a diploid zygote. The zygote develops into an ookinete which transverses the mosquito midgut epithelium to form an oocyst. The oocyst undergoes maturation and eventually divides by meiosis to form multiple haploid sporozoites. The immature sporozoites penetrate the oocyst wall into the haemolymph in which they are transported to the salivary glands where they complete their differentiation. The mature sporozoites can then infect a susceptible human host when the mosquito next takes a blood meal. The development period of the parasite in the

Anopheles mosquito lasts 10-12 days depending on the prevailing temperature (White *et al.* 2014).

The malaria epidemiology is influenced by several factors including (1) vector bionomics such as vector abundance, biting habits, longevity, biting frequency and abundance and proximity of larval habitats to humans, parasite species (Garrett-Jones and Shidrawi 1969; Coosemans *et al.* 1992); (2) climatic conditions such as humidity, temperature and rainfall and topography (Lindsay *et al.* 1998; Githeko *et al.* 2006; Kazembe 2007; Arab *et al.* 2014); and (3) human population density and behaviour (such as agricultural practices and human mobility and availability of alternative blood meal sources) (Bruce-Chwatt 1966; Keiser *et al.* 2005a; Lefevre *et al.* 2009; Iwashita *et al.* 2014). At least three factors help explain the exceptionally high endemicity of malaria in sub-Saharan Africa: (1) the prevalence of the most competent and efficient vectors from the *Anopheles gambiae* and *An. funestus* species complexes exhibiting high anthropophagic behaviour (Gillies and Coetzee 1987; Sinka *et al.* 2010; Sinka *et al.* 2012); (2) presence of the most virulent form of the parasite, *Plasmodium falciparum* (Snow *et al.* 2005); and (3) favourable climatic conditions like warm temperature and high humidity that provide optimum conditions for reproduction and faster development of both vector and parasite, vector survival which are necessary for infection(Lindsay *et al.* 1998; Zhou *et al.* 2004; Paaijmans *et al.* 2009; Beck-Johnson *et al.* 2013). In areas where these conducive conditions are found the entomological inoculation rate (EIR) can exceed 1000 infectious bites per person per year (Beier *et al.* 1999; Okello *et al.* 2006).

The close association of these *Anopheles* species to man especially their propensity to obtain blood meals from human hosts, adaptation to enter, rest and feed inside human dwellings and the closeness of their larval habitats to dwellings contribute to their efficiency as malaria vectors (Coluzzi 1999; Constantini *et al.* 1999). The occurrence of malaria transmission throughout the year in sub-Saharan Africa is attributed partly to the seasonality in the abundance of *Anopheles* species in the *An. gambiae s.l.* and *An. funestus*; whilst the density of *An. gambiae s.l.* especially *An. gambiae s.s.* and *An. coluzzii* increases during and after the rainy seasons, *An. funestus* persists throughout the year due to the permanent nature of its larval habitats (Gillies and Coetzee 1987; Lindsay *et al.* 1998). Moreover even *An. gambiae s.s.*, *An. coluzzii* and *An. arabiensis* exhibit differences in their prevalence over ecological zones (Coluzzi *et al.* 1979). The

occurrence of two or more of these dominant species in sympatry over much of sub-Saharan Africa is another reason for the high transmission rates across the region (White 1974; Gillies and Coetzee 1987; Okello *et al.* 2006; Sinka *et al.* 2012).

1.3 Current successes and challenges in malaria vector control in Africa

Malaria mortality rates in sub-Saharan Africa have been reduced by 54% between 2000 and 2013 (WHO 2014c). This success has been attributed to improved tools for malaria control, increased international and donor funding as well as increased commitment by political leadership (WHO 2013c; WHO 2014c). The tools include improved access to rapid diagnostic and prompt treatment of clinical cases (WHO 2005b; WHO 2009; WHO 2014c) supported by the scaling up of vector control interventions mainly insecticide-treated nets (ITNs, including long-lasting insecticidal nets or LLINs) and indoor residual spraying (IRS) (Lengeler 2004; WHO 2006c; WHO 2007; Pluess *et al.* 2010; WHO 2014c). While conventional ITNs require regular retreatment by dipping in solution of a synthetic pyrethroid at least once per year to maintain their protective efficacy, LLINs have wash-resistant formulation of insecticide coated or incorporated into the netting fibres during production and thus retain their efficacy over a 3-6 years period even after repeated washing (Hill *et al.* 2006).

Vector control has been identified as a key component by Roll Back Malaria (RBM) in their global malaria control strategy and ultimate interruption of transmission of the disease (WHO 1999b; WHO 2003). When used appropriately ITNs and IRS have the combined effect of reducing the success and frequency of malaria vectors obtaining blood meals from human hosts as well reducing vector populations through their insecticidal properties (Lindsay *et al.* 1989 ; Lindsay *et al.* 1991; Magesa *et al.* 1991; Pates and Curtis 2005; Killeen *et al.* 2007). The impact on shortening the life stage of the adult female *Anopheles* has the greatest impact on reducing its vectorial capacity and subsequently malaria transmission (Macdonald 1956). Studies indicate that when used singly or in combination these tools can dramatically reduce the burden of malaria by killing adult female mosquitoes when they seek for blood meals from protected human hosts or while resting on insecticide-treated material after taking a blood meal or while resting on

insecticide-treated surface (Alonso *et al.* 1991; Binka *et al.* 1996; Lengeler 2004; Eisele *et al.* 2010; Pluess *et al.* 2010; Okumu and Moore 2011). Other than providing personal protection, wide scale use of ITNs and IRS can confer community benefits in reducing disease transmission even in individuals who do not use these interventions due to mass killing effects of *Anopheles* mosquitoes (Hawley *et al.* 2003; Klinkenberg *et al.* 2010; Zhou *et al.* 2010). Although ITNs and IRS effectively eliminate malaria transmission in areas of low malaria transmission their efficacy to reduce malaria parasite prevalence to the <1% threshold is limited in areas where the disease is holoendemic (Lengeler 2004; Griffin *et al.* 2010).

Because both ITNs and IRS are insecticide-based their effectiveness and sustainability is largely dependent on the continuous susceptibility of malaria vectors to the available insecticides. Thus insecticide resistance in malaria vectors is a major concern for public health programmes and national malaria control programmes in Africa where the efforts to eliminate the disease heavily relies on use of insecticides to control the *Anopheles* populations (WHO 2002; Ranson *et al.* 2009; Ranson *et al.* 2011; WHO 2013a). The increased use of pesticides in agriculture for crop protection has been identified as a major contributor for the rapid spread of insecticide resistance in mosquito populations (Chouaibou *et al.* 2008; Nwane *et al.* 2009). At present, only insecticides belonging to four chemical groups namely organophosphate, organochlorines, carbamates and pyrethroids are available for use in IRS while only pyrethroids are recommended for impregnating bed nets, mainly because of their low toxicity to humans and rapid knockdown mortality on mosquitoes (WHO 2006b). Two insecticide resistance mechanisms namely target-site mutations in structural genes of the central nervous system of the insect such as sodium channels and GABA receptors that decreases sensitivity of the target proteins as well as increased metabolic detoxification the insecticide have been identified to be responsible for the observed resistance (Ranson *et al.* 2011; Liu 2015). A further threat to the use of insecticide is the observed development of cross-resistance and multiple-resistance mechanisms that limits the use of alternative insecticides (Ranson *et al.* 2009; Nwane *et al.* 2013; Liu 2015). To manage insecticide resistance the WHO recommends four strategies: rotations with insecticides having different modes of action, combining interventions that use insecticides with different modes of action, mosaic spraying of different insecticides in different geographical areas and use mixtures of insecticides with different modes of action (WHO 2012a). In addition

there are calls for the development of new vector control tools and public health insecticides for use in malaria vector control (WHO 2012c).

Moreover despite scaling up of ITNs and IRS to full coverage defined by the World Health Organization (WHO) as the provision of one ITN for every two persons at risk of malaria (WHO 2012d; WHO 2014c) and more or less fully susceptible vectors in many areas malaria transmission persists although at a lower level than before (Killeen 2014). This has been described as residual malaria transmission (Durnez and Coosemans 2014; Killeen 2014) and has been among other factors associated with outdoor behaviours of vectors where these intradomiciliary tools have little or no impact (Russell *et al.* 2011; Bayoh *et al.* 2014; Durnez and Coosemans 2014). This residual transmission can be sustained by primary vectors like *An. arabiensis* and secondary vectors such as *An. rivulorum* that show higher probability of biting and resting outdoors and are therefore less amenable with these indoor tools (Kitau *et al.* 2012; Okumu *et al.* 2013; Killeen 2014). The increasing reports of historically endophilic and endophagic malaria vectors such as *An. gambiae s.s.* and *An. funestus* adapting to rest and bite outdoors or early before people get into bed in an attempt to escape indoor based interventions presents yet another challenge as contact between vectors and the insecticides is reduced (Reddy *et al.* 2011; Russell *et al.* 2011; Kabbale *et al.* 2013; Sougoufara *et al.* 2014). These are exacerbated by the readiness of anthropophagic malaria vectors such as *An. gambiae s.s.* and *An. funestsus* to obtain blood meals from non-human hosts when easily accessible and their preferred choice not available (Lefevre *et al.* 2009; Mayagaya *et al.* 2015).

These challenges point to the fact that current frontline vector control tools will not be sufficient to attain the ultimate target of disease elimination. Thus unsurprisingly there are now an increasing number of calls for development of additional vector control methods to aid in further suppressing malaria transmission (Ferguson *et al.* 2010; WHO 2012c; Hemingway 2014; Killeen 2014; WHO 2014a).

1.4   Integrated vector management

Integrated vector management (IVM) is described as *'the utilization of all appropriate technological and management techniques to bring about an effective degree of vector suppression in a cost-effective manner'* (Beier *et al.* 2008). IVM aims to strengthen the impact of vector control through the use of multiple interventions that complement each

other and reduce the overdependence on insecticides (Beier *et al.* 2008; WHO 2008b). Two key features of IVM are evidence based combination of vector control interventions and continuous capacity building at the local level to organize and implement malaria control activities (WHO 2004; WHO 2008b). Other important attributes of IVM strategies are inter-sectoral cooperation with the understanding that effective vector control is not the preserve of the health sector, combined use of intervention tools based on knowledge of factors determining the biology of local vectors and disease transmission, participation of the local community supported by legislation and regulation (WHO 2004; Beier *et al.* 2008; WHO 2008b). To conduct an effective and evidence-based vector control programme requires locally informed decisions because the epidemiology of malaria is heterogeneous (Van den Berg and Takken 2007; WHO 2008b). Moreover continuous monitoring, evaluation and surveillance are important components of integrated approaches for malaria vector control to detect small changes in biological and environmental determinants of the disease (Beier *et al.* 2008; WHO 2008b). Thus IVM recommends reconsideration of the intervention measures over time based on the prevailing environment, epidemiology and availability of resources (WHO 2004; Shaukat *et al.* 2010).

To successfully control malaria the current tools must be used effectively and the impact of the tools on malaria transmission measured precisely (Shaukat *et al.* 2010). Successful historical malaria control programmes in different parts of the world were implemented through an integrated approach (Killeen *et al.* 2002a; Utzinger *et al.* 2002). Indications are that use of only the frontline vector control tools namely ITNs and IRS will not be adequate to reduce malaria prevalence to the pre-elimination threshold level of >1% in many areas of Africa where the disease is holoendemic (Ferguson *et al.* 2010; Griffin *et al.* 2010). Strategies that target both the aquatic immature and adult stages of mosquitoes have demonstrated great promise for malaria control in sub-Saharan Africa (Utzinger *et al.* 2001; Utzinger *et al.* 2002; Chanda *et al.* 2008; Fillinger *et al.* 2009a). Although ITNs and IRS have been shown to be most effective tools for reducing entomological inoculation rate (EIR), anti-larval measures can amplify the effects of these adulticidal measures (Killeen *et al.* 2000).

1.5 Larval Source Management (LSM)

Source reduction and larviciding, the two main strategies in LSM historically played an important role in the control of malaria and other mosquito-borne diseases (Takken *et al.* 1990; Sufian 2005). Source reduction include measures aimed at temporarily or permanently removing mosquito larval habitats such as drainage and filling of water bodies, whilst larviciding involves the regular application of chemical or biological agents to water to kill mosquito larvae and pupae. These anti-larval measures were the main strategies in the intervention programmes that successfully suppressed malaria in the Tennessee River Valley, Palestine and Italy (Kitron and Spielman 1989). The successful elimination of the notorious African malaria vector *An. arabiensis* from vast areas of Brazil and Egypt was done primarily by application of the highly toxic Paris Green (copper (II) acetate triarsenite) into larval habitats (Soper 1966; Killeen *et al.* 2002a). Notably, source reduction-led measures that eliminated the lethal and debilitating effects of yellow fever and malaria made a significant contribution to the successful completion of the Panama Canal in 1914 (Patterson 1989).

In light of the increasing calls for adoption of integrated approaches to control malaria vectors coupled with concerns of insecticide resistance development by major malaria vectors to insecticides used indoors, there is renewed interest in LSM as a supplementary intervention for malaria vector control in sub-Saharan Africa (WHO 1982; WHO 2013b). The potential of LSM for mosquito control in sub-Saharan Africa has being documented (Utzinger *et al.* 2001; Majambere *et al.* 2007; Fillinger *et al.* 2008; Geissbühler *et al.* 2009; Tusting *et al.* 2013). The major advantage of LSM is that it targets aquatic mosquitoes at their most vulnerable stage when they cannot escape the interventions (Killeen *et al.* 2002b). In addition it has the potential of attacking mosquitoes with both outdoor and indoor resting/biting behaviour (Killeen *et al.* 2002b). LSM-based programmes were effective in reducing malaria transmission in a number of settings in sub-Saharan Africa especially when combined with adulticidal measures (Utzinger *et al.* 2002; Fillinger *et al.* 2009a; Geissbühler *et al.* 2009; Maheu-Giroux and Castro 2013). For instance source reduction employing vegetation clearance, modification of river boundaries and drainage of swamps were the main strategies coupled with IRS with DDT and quinine administration used to successfully suppress malaria in the Zambian copper belt for two decades between 1930-1950 (Utzinger *et al.* 2001; Utzinger *et al.* 2002). In Western Kenya the addition of larviciding with microbial larvicides to ITNs provided

additional benefit in reducing malaria incidence in children in an experimental trial (Fillinger *et al.* 2009a).

The success of larval control interventions is largely dependent on a thorough knowledge of the characteristics of the larval habitats of the target mosquito species (Walker and Lynch 2007). The lack of interest in larval control measures after the discovery of the powerful insecticidal properties of dichlorodiphenyltrichloroethane (DDT) in the 1940s that led to increased focus in adulticidal measures for malaria vector control is a major contributor to the limited knowledge in the larval ecology of malaria vectors (Najera 1999; Najera *et al.* 2011). This was based on an earlier Macdonald model developed for mosquito-borne pathogen transmission that predicted higher impact in reducing *Anopheles* vectorial capacity by targeting adult mosquitoes to reduce their life span over mere reduction in mosquito density (Macdonald 1956). Thus this model supported the adoption of indoor residual spraying with the residual insecticide dichlorodiphenyltrichloroethane (DDT) to kill indoor resting vector populations during the global malaria eradication programme (GMEP) between 1955-1969 (Najera *et al.* 2011). However recent models show that old Macdonald model did not include the mosquito larval stage and therefore unsuitable to evaluate measure that target larval stage of mosquitoes (Smith *et al.* 2012). In addition the limited timescales of larval ecology studies often conducted during the rainy or dry season only can be also partly be attributed to contribute to the limited knowledge on the larval ecology of the major Afrotropical malaria vectors (Gimnig *et al.* 2001; Bogh *et al.* 2003; Shililu *et al.* 2003a; Carlson *et al.* 2004).

Immature stages of *An. gambiae s.l.* are often associated with temporary, man-made water pools that are exposed to sunlight and are not organically polluted (Service 1971; Gimnig *et al.* 2001; Minakawa *et al.* 2004). Nevertheless *An. gambiae s.l.* shows great adaptability to a large range of water bodies, temporary to permanent (Fillinger *et al.* 2004; Majambere *et al.* 2008), clean to highly polluted (Sattler *et al.* 2005; Awolola *et al.* 2007), clear to turbid (Minakawa *et al.* 1999; Mala *et al.* 2011), with or without algae (Minakawa *et al.* 1999; Gimnig *et al.* 2001), with and without emergent plants on the edge or within the habitat (Minakawa *et al.* 2004; Mereta *et al.* 2013). Different studies from a range of eco-epidemiological settings and frequently from very time-limited observations have revealed controversial results concerning the factors associated with

the choice of female *An. gambiae s.l.* to lay eggs in specific water bodies (Robert *et al.* 1998; Fillinger *et al.* 2004; Mwangangi *et al.* 2007; Mireji *et al.* 2008). In general, it is difficult to predict with precision which habitats will be colonized by *An. gambiae s.l.* and which ones will produce most adults (Mwangangi *et al.* 2007; Fillinger *et al.* 2009b; Ndenga *et al.* 2011). For vector control targeting the immature stages with larvicides this meant in the past targeting all available habitats in the intervention area. However, recent mathematical modelling approaches predict that an over 70% reduction in transmission could be achieved by targeting only 50% of the habitats (Gu and Novak 2005; Smith *et al.* 2013). However, empirical evidence for this is lacking.

Anopheles funestus can share the same habitats as *An. gambiae s.l.* but are also found in much larger, deeper and permanent habitats that are highly vegetated (Gimnig *et al.* 2001; Minakawa *et al.* 2005; Mwangangi *et al.* 2007). Such areas are especially challenging for larviciding programmes since they are difficult to access on foot and the vegetation does not allow penetration of the insecticide to the water surface.

During the rainy seasons *Anopheles* develop frequently in the water collections created by human activities such as drains, burrow pits, rice fields avoiding fast flowing water channels (Dukeen 1986; Fillinger *et al.* 2004). In the dry season, the aquatic habitats colonized are often permanent water bodies such as the edges of rivers and streams with slow flowing water and the few permanent man-made pools such as drainage canals and concrete pits or open tanks (Dukeen 1986; Carlson *et al.* 2004; Jawara *et al.* 2008; Govoetchan *et al.* 2014).

The productivity of habitats has been described either as the presence or absence of larvae, or as the mean density or abundance of larvae or pupae or as the number of emerged adults per surface area, the latter being the best indicator for habitat productivity (Mutuku *et al.* 2006; Fillinger *et al.* 2009b; Ndenga *et al.* 2011). The study of the emergence of adult vectors from various habitat types is difficult and time consuming and has not been done frequently. Results available from very different ecological settings are inconsistent (Mutuku *et al.* 2006; Fillinger *et al.* 2009b; Kweka *et al.* 2011). In some areas small and unstable habitats were found to be more productive for *Anopheles* mosquitoes per given surface area (Ndenga *et al.* 2011) whilst others have shown more stable sites produce more *Anopheles* mosquitoes (Mutuku *et al.* 2006). More research is needed in order to develop approaches to target larval habitats for vector control targeted

in space (at selected sites only). An alternative approach of targeting larval habitats in time has been recently suggested to target larvicides in time when vector densities increase (Fillinger *et al.* 2009a) and has been favourably costed (Worrall and Fillinger 2011). Furthermore, rather than reducing the number of habitats to be treated frequently, another approach to reduce costs and effort of larviciding programmes could be the application of residual larvicides that require less frequent applications. Such larvicides have so far not been rigorously tested for the control of afro-tropical malaria vectors. Nevertheless, the use of persistent larvicides also has a risk of vector production from untreated habitats that are either newly-created or filled with water after larvicide application. Research is required to identify the optimum re-treatment intervals of persistent larvicides for effective control of malaria vectors.

1.5.1   Larvicides

Mosquito larvicides are grouped based on their modes of action: organophosphates, spinosyns, microbials, surface films and insect growth regulators.

1.5.1.1 Organophosphates

Organophosphate insecticides (i.e. temephos) have been extensively evaluated for mosquito larval control in America, Africa and other parts of the world (Bang *et al.* 1972; Lowe *et al.* 1980; Shililu *et al.* 2003b). Organophosphates kill mosquito larvae by modifying the normal functioning of the nerve cells by inhibiting the activity of cholinesterase enzymes at the neuromuscular junction thereby interfering with neuromuscular transmission. However, resistance to temephos has been reported in many places globally (Hemingway *et al.* 1988; Cheikh 1993). In addition, organophosphates have slight to moderate toxic effects to non-target aquatic organisms and are therefore less suited for modern IVM programmes (Pinkney *et al.* 1999).

1.5.1.2 Spinosyns

Spinosyns are compounds with insecticidal properties that are produced from the fermentation of soil bacteria of genus *Saccharopolyspora*. Spinosyns have a unique neurotoxic mode of action of disrupting the neuronal activity by exciting motor neurons that cause involuntary muscle contractions to eventually cause paralysis and death of the insect (Salgado 1998; Kirst 2010). Their unique mode of action coupled by the greater selectivity on target insect species and minimal impacts on environment and other non-

target species including mammals make them more appealing for insect control (Kirst 2010). These larvicidal agents have however found limited use for mosquito control. Spinosad, produced by the fermentation of the soil bacterium *Saccharopolyspora spinosa* to produce a mixture of spinosyns A and D has been shown to be highly toxic to mosquito larvae (Perez *et al.* 2007; Hertlein *et al.* 2010; Kirst 2010). Spinosad has been reported to have minimal negative effects on the environment (Hale and Portwood 1996; Cleveland *et al.* 2001).

1.5.1.3 Microbials

Mosquitocidal bacterial toxins produced during sporulation of *Bacillus sphaericus* (*Bs*) and *Bacillus thuringiensis* var. *israelensis* (*Bti*) are highly effective against different mosquito species in a variety of habitats and are environmentally safe due to their high specificity (WHO 1999a; Fillinger *et al.* 2003; Fillinger and Lindsay 2006; Poopathi and Abidha 2010). The larvicidal activity of microbials is due to the presence of protein toxins that disrupt the larval midgut once activated by enzymes in midgut. They are thus described as stomach poisons (Poopathi and Abidha 2010). Microbials are highly specific larvicides with minimal impact on non-target aquatic insects (Poopathi and Abidha 2010).

There are reports of mosquitoes developing resistance to *Bs* in the field especially if many applications are made that subject mosquito to strong selection pressure (Silva-Filha *et al.* 1995; Yuan *et al.* 2000), but none to *Bti* probably due to its multiple toxin complex (Poopathi and Abidha 2010). While the efficacy of *Bti* is reduced in highly polluted water bodies, *Bs* remains highly effective often recycling in cadavers of mosquito larvae it kills (Sutherland *et al.* 1989; Karch *et al.* 1990). Their short residual activity which necessitates application at 1-2 week intervals (Fillinger and Lindsay 2006; Majambere *et al.* 2007) is frequently considered a challenge since larviciding programmes have to be established that exclusively implement this intervention. Even though this has been shown not to be more costly than other malaria control interventions (Worrall and Fillinger 2011), this is frequently considered too expensive and involving to be added to ongoing vector control tools (WHO 2012b)

1.5.1.4 Surface films

Modern surface films have a purely physical mode of action (Corbet *et al.* 2000; Nayar and Ali 2003) making them especially interesting for insecticide resistance management. Despite their potential, they have not been extensively studied and used in malaria vector

control. The earliest surface films used for mosquito control were petroleum-based oils. These kill mosquito larvae by flooding of the larvae trachea but also have toxic effects (Hagstrum and Mulla 1968; Berlin and Micks 1973). Concerns on environmental safety and non-target aquatic organisms coupled with formation of non-uniform films on the water surface and disturbance of the film by wind and aquatic vegetation limited their use for mosquito control (Mozley and Butler 1978; Lopes *et al.* 2009).

Monomolecular surface films (MMFs) are surface-active agents that modify the physico-chemical properties of the water by reducing the water surface tension (Corbet *et al.* 2000). The reduced water surface tension drowns eggs, immature and adult stages of mosquitoes due to the increased wetting effect (Garrett and White 1977). Substantial reduction of water surface tension is essential for larvicidal and pupicidal effects by blocking the trachea through increased wetting of the internal hydrophobic of the trachea that interferes with respiration (Garrett and White 1977; Reiter 1978; Reiter and McMullen 1978). The most effective MMFs are those that spread spontaneously on water surface, are non-volatile and insoluble in water and can reduce water surface tension to below 29 dynes/cm (Garrett and White 1977; Reiter and McMullen 1978).

Lecithin monolayers were the first MMFs to be evaluated for mosquito control but they were effective for only two days in the field (Reiter 1979). Arosurf MSF and Agnique MMF are two exthoxylated isosteryl alcohol-based surfactants that have demonstrated great potential for the control of different mosquito species in different habitat types providing 2-12 weeks complete adult emergence inhibition at low doses (Levy *et al.* 1981; Karanja *et al.* 1994; Nayar and Ali 2003). The disadvantage of these MMFs are that they are easily broken by wind and vegetation opening up pockets where larvae can develop (Levy *et al.* 1982; Nayar and Ali 2003); therefore they have not been widely advocated even though they exhibit a high margin of safety on non-target aquatic organisms (Mulla *et al.* 1983; Hester *et al.* 1991; Nayar and Ali 2003).

Aquatain Mosquito Formulation (AMF) is a silicone-based film. It was initially developed as an anti-evaporant to prevent water loss from large water storage dams and tanks. The uniqueness of the surface film created by AMF is its self-spreading property over extended water surface areas and around vegetation (Bukhari *et al.* 2011). Only few studies have been implemented with AMF to date showing rapid mortality in mosquito

larvae and pupae at low doses in the laboratory (Bukhari and Knols 2009; Webb and Russell 2012). It could be a promising agent for the control of immature malaria vectors in large and highly vegetated habitats such as rice paddies that are difficult to access. A recent study has shown that AMF reduced anopheline adult emergence in rice fields by 93% and persisted for two weeks (Bukhari *et al.* 2011). In Australia AMF provided effective control of immature stages of *Aedes* and *Culex* for four weeks (Webb and Russell 2012). The silicon film is described to re-form after breakages by wind and rainfall which would be an advantage over the alcohol-based films available (Bukhari *et al.* 2011; Webb and Russell 2012).

The impact of AMF on aquatic non-target invertebrates including mosquito predators has not been studied in detail. The few studies to date show no negative impact on non-target aquatic organisms (Bukhari *et al.* 2011; Webb and Russell 2012), however more work is required.

1.5.1.5 Insect growth regulators (IGRs)

Insect growth regulators (IGRs) comprise a group of insecticides such as chitin synthesis inhibitors, ecdysone agonists/antagonists and juvenile hormone analogues which interfere with the growth and development of target insects. IGRs are quite selective in their modes of action and potentially act only on target species. The major impact of IGRs is the inhibition of development of insect larvae into adult (Graf 1993; Tunaz and Uygun 2004). Chitin synthesis inhibitors such as diflubenzuron and novaluron act by altering the synthesis, polymerisation and deposition of chitin on the eggs and larvae of insects (Deul *et al.* 1976; Farnesi *et al.* 2012; Merzendorfer 2013). The interference with chitin deposition causes death of insect larvae during moulting when the procuticle is subjected to the stresses of ecdysis and cuticular expansion (Dean *et al.* 1998). Ecdysone agonists are substances such as tebufenozide and methoxyfenozide that act like the endogenous moulting hormone and thus induce precocious incomplete moults during the insect larval stage which subsequently kills the larvae (Retnakaran *et al.* 2003; Boudjelida *et al.* 2005). Ecdysone antagonist such as azadiractin on the other hand inhibit the effects of ecdysone (Dinan *et al.* 1997).

Juvenile hormones are a group of acyclic sesquitepenoids that regulate the processes of metamorphosis, development and reproduction in insects (Staal 1975; Wyatt 1997; Hartfelder 2000; Riddiford 2012). Juvenile hormone are also involved in regulating the

processes of caste differentiation in social insects (Hartfelder 2000). During the insect immature stages juvenile hormone is present during the larval or early nymphal stages and plays the role of maintaining the juvenile stage thus termed the 'status quo hormone' but is in low titres or disappears during moults that occur at the late larval and pupa stages (Wigglesworth 1934; Hartfelder 2000). Juvenile hormone analogues such as pyriproxyfen and methoprene are substances that mimic the actions of the naturally occurring juvenile hormone in insects by preventing the development of larvae to adult when applied during the immature mosquito stages (Siddall 1976). Thus exogenous exposure of juvenile hormone and its analogues during the late larval and pupae stages results in the development of supernumerary larvae, larval-pupal intermediates and /or pupal-adult intermediates that subsequently die (Jones and Hammock 1985). In addition exogenous application of juvenile hormone to adult females causes sterility in exposed insects, inhibit egg hatching and laying of non-viable eggs by exposed females (Judson and de Lumen 1976; Wyatt 1997). Moreover the impact of these juvenile hormone analogues on the reproduction of insects has been shown (Kamal and Khater 2010; Bai *et al.* 2011).

Pyriproxyfen (PPF), a juvenile hormone analogue has been shown to be effective in the control of a wide range of insects of medical, veterinary and agricultural importance (Jacobs *et al.* 1996; Yapabandara and Curtis 2002; Tunaz and Uygun 2004). PPF exhibits high level of activity against immature stages of mosquitoes at low doses (Kamimura and Arakawa 1991; Okazawa *et al.* 1991; Yapabandara and Curtis 2002; Sihuincha *et al.* 2005). PPF has exhibited residual activity of between two weeks and six months in test with different mosquito species and in a range of habitat types (Suzuki *et al.* 1989; Okazawa *et al.* 1991; Chavasse *et al.* 1995a; Nayar *et al.* 2002; Yapabandara and Curtis 2002; Sihuincha *et al.* 2005). The superiority of PPF for mosquito control is further highlighted by its effectiveness at much lower doses and the extended control it provides compared with other IGRs (Kawada *et al.* 1993; Ali *et al.* 1995; Ali *et al.* 1999; Nayar *et al.* 2002). Another special attribute of PPF is its persistence in treated habitats during periods of dryness (Okazawa *et al.* 1991; Yapabandara and Curtis 2002). Moreover PPF has exhibited relative degree of safety against non-target aquatic insects and the environment (Mulla *et al.* 1986; Schaefer *et al.* 1988; Schaefer and Miura 1990).

In addition to the larvicidal impact, PPF has been reported to reduce the fecundity, fertility and longevity in exposed mosquitoes (Itoh *et al.* 1994; Sihuincha *et al.* 2005; Aiku *et al.* 2006). This is a novel strategy of mosquito control as PPF-exposed females

fail to lay eggs or lay unviable eggs (Ohba *et al.* 2013; Kawada *et al.* 2014). These have been demonstrated on adult mosquitoes that emerge from immature stages exposed to sub-lethal doses as well as adults exposed to PPF (Loh and Yap 1989; Sihuincha *et al.* 2005).

A novel strategy of auto-dissemination of PPF by adult mosquitoes from resting to breeding sites has been demonstrated for *Aedes* mosquitoes (Devine *et al.* 2009; Caputo *et al.* 2012; Abad-Franch *et al.* 2015). Auto-dissemination is a novel insect control technique that utilizes the insect behaviour to transfer lethal doses of an insecticide from a contamination site to other insect populations. This has been successfully demonstrated in social and aggregating insects where the transfer of insecticide can happen either directly through insect-to-insect contact or indirectly following contact with a substrate that has been contaminated with other insects (Soeprono and Rust 2004; Buczkowski *et al.* 2008; Choe and Rust 2008). For mosquito control, this approach has been found to be suited for the control of selected *Aedes* which do not fly far from their breeding sites and where the breeding sites are generally small bodies of water (Schoof 1967; Burkot *et al.* 2007). The auto-dissemination of an insecticide by the mosquito female in search of an oviposition site could be beneficial for the control of mosquitoes that are not targeted by ITNs and IRS particularly those that exhibit outdoor resting and/or biting characteristics and for the control of immature stages in habitats that are difficult to locate and access. Nevertheless, PPF has not been rigorously tested in the field under operational field conditions for the control of malaria vectors in sub-Saharan Africa. To date, the only study to explore the potential of the auto-dissemination technique for control of malaria vectors in sub-Saharan Africa, provided proof of principle that adult *An. arabiensis* can transfer sufficient PPF from contaminated resting pots oviposition substrate leading to more than 80% reduction in adult emergence from laid eggs (Lwetoijera *et al.* 2014). Additional studies are needed to explore the potential of this technique for control of malaria vectors in sub-Saharan Africa.

1.6   Description of study areas

The research was implemented in western Kenya. Laboratory and semi-field experiments took place at the 1977 initiated Thomas Odhiambo Campus (TOC) of the International Centre of Insect Physiology and Ecology- (icipe). The campus is located within Mbita

Point Township on the shores of Lake Victoria in western Kenya, close to the equator (geographic coordinates 0^0 26' 06.19" S, 34^0 12' 53.13"E) at an altitude of 1240 m above sea level. Mbita area experiences a warm and humid climate suitable for supporting a rich insect biodiversity. This makes it a 'hot-spot' for research on crop pests as well as on vectors of human and animal diseases (www.icipe.org/mbita/). Icipe-TOC covers an area of 24.5 hectares of land which holds state-of-the-art laboratories and offices, a modern automatic weather station, 16 semi-field experimental systems (netting screened greenhouses), and open field plots for setting up standardized open field experiments. Icipe-TOC also has a large mosquito rearing facility holding three mosquito species originating from Mbita: *An. gambiae s.s.*, *An. arabiensis* and *Cx. quinquefasciatus*. All experimental work was implemented at icipe-TOC with insectary-reared mosquitoes either under ambient laboratory, semi-field or standardized field conditions.

Field work to evaluate the effectiveness of Sumilarv®0.5G for controlling wild populations of malaria vectors was conducted in the western Kenya highlands in Vihiga County (geographical coordinates, 0.0667° N, 34.6667°E) between 1448 m and 1666 m above sea level. This area is one of the most highly populated areas in Kenya with a population density of 1033 persons per km^2 in 2004 (NEMA 2011). The study area included six defined valleys that were approximately 1 km apart. The major economic activity at these sites is subsistence farming of crops such as maize, napier grass, cassavas and bananas. Malaria risk in the western Kenya highlands is traditionally regarded as limited by low temperature (Lindsay and Martens 1998). However, increasing malaria transmission in most of the highlands of East Africa was reported since the 1990s as a result of a rapid rise in population density and subsequent changes in land use in the form of deforestation and swamp cultivation (Lindsay and Martens 1998). Scaling up of malaria control measures led to a decrease in malaria since mid 2000s but still environmental changes threaten to continuously expose populations in these highland regions to malaria vectors. Many regions are epidemic prone particularly if the current interventions are not sustained (Zhou *et al.* 2004; Chaves *et al.* 2012). Malaria transmission in the western Kenya highlands is marked by temporal and spatial heterogeneity influenced by climate variability and topography (Githeko *et al.* 2006; Afrane *et al.* 2014). The topography of the area is characterized by steep and gently sloping hills and valleys. The majority of aquatic habitats that serve as mosquito larval habitats are located at the valley bottom and are thus easy to locate. This makes the

highlands an interesting environment for targeting mosquito larvae in their defined, focal and easy to access habitats. Larviciding with microbial larvicides has demonstrated great potential in reducing adult malaria vector densities by over 75% and provided a two-fold additional benefit in reducing malaria incidence when added to insecticide-treated nets (Fillinger *et al.* 2009a).

The climate of western Kenya generally consists of a bi-modal pattern of rainfall, with the long rainy season from March to June, which triggers the peak in malaria transmission and epidemics due to increased abundance of malaria vectors during this period and the short rainy season from October through to November (Fillinger *et al.* 2009a). The remaining months constitute the dry season when little rainfall is experienced. *Plasmodium falciparum* is the primary malaria parasite species (Munyekenye *et al.* 2005; Ernst *et al.* 2006), while the predominant vector species are *An. gambiae s.s.*, *An. arabiensis* and *An. funestus* (Ndenga *et al.* 2006; Omukunda *et al.* 2013).The frontline malaria vector control measures in this area include the use of long lasting insecticidal nets (LLINs) and spraying the inner surface of houses with residual insecticides (IRS) (MoH 2009). Recent studies suggest development of resistance to pyrethroid insecticides by the major malaria vectors in this study area and other regions in western Kenya to be caused by two resistance mechanisms: target site insensitivity and increased metabolism of insecticides (Ranson *et al.* 2000; Chen *et al.* 2008a). Reports from other studies indicate increased outdoor biting behaviours by major *Anopheles* vectors following increased use of long-lasting insecticidal nets by human population (Ototo *et al.* 2015).

1.7   Rationale

The current frontline vector control interventions for malaria control will not be sufficient to eliminate malaria in most parts of sub-Saharan Africa due to increased resistance of vectors to the insecticides used as well as the persistent residual transmission sustained by *Anopheles* vectors that evade these intradomiciliary interventions (Griffin *et al.* 2010; Ranson *et al.* 2011; Govella *et al.* 2013; Killeen 2013; Durnez and Coosemans 2014; Killeen 2014; Killeen and Chitnis 2014). Thus there are increasing calls to explore the potential of additional interventions for use in integrated approaches to safeguard the hard won gains and further suppress malaria transmission (WHO 2008b; WHO 2012c; Killeen 2014; WHO 2014a). These interventions should preferably be implemented outside of

houses to target both endophilic and exophilic vectors and should use insecticides with a completely different mode of action than those used indoors for adult mosquito control to manage insecticide resistance.

Larval source management (LSM), although one of the oldest tools in the fight against malaria, was at the beginning of the 21st century a largely forgotten and often dismissed intervention for malaria control in Africa (Fillinger and Lindsay 2011). Despite the lack of its application in Africa, LSM has been the main focus of mosquito control programmes for decades in the USA and Europe (Carlson 2006; Floore 2006; Abramides *et al.* 2011). Regardless of the scale and success of these operations in developed countries, this activity had been ignored by those interested in malaria control, until recently. Recent studies investigated the efficacy and technical feasibility of mosquito larval source management for malaria control in different eco-epidemiological settings in Africa (Shililu *et al.* 2003b; Fillinger and Lindsay 2006; Majambere *et al.* 2007; Geissbühler *et al.* 2009), the added benefit of integrating larval source management with personal protection measures ((Fillinger *et al.* 2009a; Maheu-Giroux and Castro 2013), the potential for more targeted approaches in space and time of this intervention (Killeen *et al.* 2000; Gu and Novak 2005; Gu *et al.* 2008; Fillinger *et al.* 2009a; Yakob and Yan 2009), the development of participatory approaches to implement community-based programmes (Fillinger *et al.* 2008; Maheu-Giroux and Castro 2013), and the costs of this intervention for different eco-epidemiological settings (Worrall and Fillinger 2011). Consequently, the World Health Organization published a new guideline for this intervention 40 years after the last edition (WHO 2013b) and a Cochrane Review was produced highlighting the potential of this intervention to contribute to malaria control especially in an integrated vector management approach (Tusting *et al.* 2013).

Although costs of larviciding programmes compare well with costs of LLINs and IRS programmes per person protected, it needs to be considered that resource-poor African countries might not be in a position to add this additional expense. Reviewing the costing by Worrall and Fillinger (2011), it is clear that the larvicide (in their evaluation *Bacillus* products), and the labour costs for regular application (weekly) drive the costs. It is therefore necessary to investigate larvicides with novel mode of actions that are easy to apply, safe for the environment and applicator and require less frequent applications. In this context it is especially important to investigate the optimal re-application interval

since the use of persistent larvicides for vector control might present a challenge when new aquatic habitats are created frequently in-between treatment cycles potentially producing large numbers of adult malaria vectors before the next application and therefore jeopardizing the overall impact on malaria control. Another challenge of larviciding programmes is the ground accessibility of habitats (Majambere *et al.* 2010). Therefore, novel strategies to apply larvicides especially in large and vegetated aquatic habitats need to be explored.

1.8   Overall aim and objectives

The aim of this thesis was to investigate two novel insecticides for the control of *An. gambiae s.l.* the major malaria vector in sub-Saharan Africa: the insect growth regulator pyriproxyfen (PPF) and a self-spreading silicone-based surface film (AMF).

The research was driven by the following hypotheses:

Hypothesis 1: *Anopheles gambiae s.s. and An. arabiensis* are highly susceptible to low dosages of PPF and AMF leading to over 80% emergence inhibition for at least one month after application and including sub-lethal effects on adults that survive exposure during larval development.

Hypothesis 2: Operational application of PPF to aquatic habitats in the field at three-weekly intervals inhibits over 80% of adult emergence from treated habitats.

Hypothesis 3: Exposure of adult *An. gambiae s.s.* to PPF around blood feeding time leads to sterilization of the females.

Hypothesis 4: Adult *An. gambiae s.s.* can auto-dissemination PPF.

Hypothesis 5: Attract and kill strategies can be developed for controlling gravid malaria vector by either (1) combining the residual larvicides PPF and AMF with a chemical

oviposition attractant at application; or (2) luring the gravid females to an attractive
baiting station to contaminate them with PPF for horizontal transfer to aquatic habitats.

2 Aquatain® Mosquito Formulation (AMF) for the control of immature *Anopheles gambiae sensu stricto* and *Anopheles arabiensis*: Dose-responses, persistence and sub-lethal effects

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2.1   Abstract

Background: Persistent monomolecular surface films could benefit larval source management for malaria control by reducing programme costs and managing insecticide resistance. This study evaluated the efficacy of the silicone-based surface film, Aquatain® Mosquito Formulation (AMF), for the control of the Afrotropical malaria vectors, *Anopheles gambiae sensu stricto* and *Anopheles arabiensis* in laboratory dose-response assays and standardized field tests.

Method: Tests were carried out following guidelines made by the World Health Organization Pesticide Evaluation Scheme (WHOPES). Sub-lethal effects of AMF were evaluated by measuring egg-laying and hatching of eggs laid by female *An. gambiae s.s.* that emerged from habitats treated with a dose that resulted in 50% larval mortality in laboratory tests.

Results: Both vector species were highly susceptible to AMF. The estimated lethal doses to cause complete larval mortality in dose-response tests in the laboratory were 1.23 (95% confidence interval (CI) 0.99-1.59) ml/m^2 for *An. gambiae s.s.* and 1.35 (95% CI 1.09-1.75) ml/m^2 for *An. arabiensis*. Standardized field tests showed that a single dose of AMF at 1ml/m^2 inhibited emergence by 85% (95% CI 82-88%) for six weeks. Females exposed as larvae to a sub-lethal dose of AMF were 2.2 times less likely (Odds ratio (OR) 0.45, 95% CI 0.26-0.78) to lay eggs compared to those from untreated ponds. However, exposure to sub-lethal doses neither affected the number of eggs laid by females nor the proportion hatching.

Conclusion: AMF provided high levels of larval control for a minimum of six weeks, with sub-lethal doses reducing the ability of female mosquitoes to lay eggs. The application of AMF provides a promising novel strategy for larval control interventions against malaria vectors in Africa. Further field studies in different eco-epidemiological settings are justified to determine the persistence of AMF film for mosquito vector control and its potential for inclusion in integrated vector management programmes.

2.2 Background

Historically, larval source management made a significant contribution to many successful malaria control programmes (Soper 1966; Kitron and Spielman 1989; Killeen *et al.* 2002a; Keiser *et al.* 2005b; Walker and Lynch 2007). The application of petroleum-based oils to water bodies to prevent emergence of adults is one of the oldest anti-larval measures used for mosquito control (Micks *et al.* 1967; Hagstrum and Mulla 1968). These petroleum-based oils kill the aquatic stages of mosquitoes by two mechanisms: specific toxicity and suffocation (Freeborn and Atsatt 1918; Richards 1941) and provide effective control for two weeks or more (Darwazeh *et al.* 1972; Mulla and Darwazeh 1981). However, a major limitation of petroleum-based oils was the formation of a thick and non-uniform film that often required the addition of oil-soluble surface active agents to ensure uniform spreading of the film (Murray 1940; Toms 1950). Additionally, there are concerns about the damaging environmental consequences of these oils on non-target aquatic organisms when applications are made at high doses (Mozley and Butler 1978; Lopes *et al.* 2009). Monomolecular surface films (MMFs) that consist of non-ionic surfactants were developed as potential alternatives to petroleum-based oils for mosquito control (Garrett and White 1977; Nayar and Ali 2003). A unique feature of MMFs is that they spread spontaneously and rapidly over a water surface to form a uniform ultrathin film about one molecule in thickness – a monolayer (Garrett and White 1977; Nayar and Ali 2003). Importantly, the effective doses used for mosquito control can be reduced 70 times when petroleum-based oils are replaced by MMFs (Garrett and White 1977), which saves on shipment, storage and application costs. Unlike petroleum-based oils and other control agents, MMFs are not toxic to immature mosquitoes (Reiter 1978; Reiter and McMullen 1978). Their mode of action is physical, rather than chemical, and they work by lowering the water surface tension that affects all stages of the mosquito life-cycle; it is ovicidal, larvicidal, pupicidal and adulticidal (Garrett and White 1977; Reiter and McMullen 1978). The reduced surface tension wets and drowns eggs, suffocates larvae and pupae and kills emerging and ovipositing females by drowning (Reiter and McMullen 1978; Levy *et al.* 1982). This is an advantage over conventional insecticides that are only effective against larva (Poopathi and Abidha 2010) or pupae (Mian and Mulla 1982). Importantly, the physical mode of action reduces the chance of mosquitoes developing resistance.

Lecithin monolayers were the first MMFs to be evaluated for mosquito control but were only effective for two days when used to control *Anopheles gambiae sensu lato* in Western Kenya (Reiter 1979). Arosurf ®MSF and Agnique®MMF are two commercially available MMFs made from renewable plant oils that are effective at controlling mosquitoes for up to five weeks in a variety of habitat types (Mulla *et al.* 1983; Karanja *et al.* 1994; Nayar and Ali 2003; Batra *et al.* 2006). However, MMFs are yet to gain wider acceptance in mosquito control programmes because of concerns about the disturbance of the film by environmental influences such as wind, rainfall and vegetation cover resulting in a patchy distribution of the chemical and mosquito emergence (Levy *et al.* 1981; Levy *et al.* 1982; Nayar and Ali 2003).

Aquatain® Mosquito Formulation (AMF) is a silicone-based film with a unique self-spreading ability. AMF was initially developed as an anti-evaporant to prevent water loss from large water reservoirs. The advantage of the AMF film is its resilience to breakages by wind and rainfall as well as its ability to penetrate vegetation cover and floating debris on the water surface. These properties combined with its safety to humans make it a promising agent for mosquito control especially in large and highly vegetated habitats that have often proven difficult to treat with insecticides (Bukhari *et al.* 2011). Surprisingly, to date only two studies have been published evaluating the potential of AMF for the control of *An. gambiae s.l.*, the major malaria vector in sub-Saharan Africa; one laboratory (Bukhari and Knols 2009) and one field (Bukhari *et al.* 2011) study.

We aimed to supplement the available knowledge by testing the efficacy of AMF for the control of *An. gambiae sensu stricto* and *An. arabiensis* in Phase I and Phase II trials following the standardized procedures by the World Health Organization Pesticide Evaluation Scheme (WHOPES) (WHO 2005a). The specific aims of the study were to: (1) determine and compare the susceptibility of *An. gambiae s.s.* and *An. arabiensis;* (2) establish the initial and residual activity of AMF under standardized field conditions; and (3) test delayed effects of exposure to sub-lethal doses of AMF during larval development on a female's ability to lay eggs, the number of eggs laid and the number of eggs hatched.

2.3   Methods

2.3.1   Study area

The study was carried out at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (icipe-TOC) located on the shore of Lake Victoria in Homabay county, western Kenya (geographic coordinates 0^0 26' 06.19" S, 34^0 12' 53.13"E; altitude 1,137 m above sea level). The area is characterized by two rainy seasons, the long rains between March and June and the short rains between October and December. The average annual rainfall for 2010 to 2013 was 1, 645 mm (icipe-TOC meteorological station). The laboratory study was conducted in June, 2011 while the standardized field tests were conducted in June-August 2012.

2.3.2   Mosquitoes

Insectary-reared third instar larvae of *An. gambiae s.s.* and *An. arabiensis* (Mbita strains) were used for all experiments in this study. The mosquito immature stages were maintained in a netting-screened greenhouse-like building (semi-field system; 7.1m wide, 11.4 m long and 2.8 m high at the wall and 4.0 m high at the highest point of the roof) (Dugassa *et al.* 2012) with an average daily temperature of 25-28ºC, relative humidity of 68-75% and natural lighting. Mosquito maintenance is described more fully elsewhere (Das *et al.* 2007). Briefly, mosquito larvae were reared in round plastic tubs (diameter 60 cm) filled with 5 l water (5 cm deep) from Lake Victoria filtered through a charcoal-sand filter. The mosquito larvae were fed with fish food (Tetramin©Baby) twice daily. Mosquito larvae for experiments were randomly collected from different tubs to ensure that larvae introduced into each experimental cup or pond were of equal size (Araujo *et al.* 2012).

2.3.3   Insecticide

AMF was provided by the manufacturer Aquatain Products Pty Ltd., Australia. AMF contains 78% polydimethylsiloxane (silicone), the active ingredient. The manufacturer's recommended application rate for mosquito control is 1 ml/m^2.

2.3.4 Dose-response tests

Tests were carried out on tables located in a semi-field system under ambient climatic conditions but protected from rain (Dugassa *et al.* 2012). In range-finding tests, mortality rates were evaluated at doses between 0.01-1 ml/m^2 compared to untreated controls. Thereafter, dose-response tests were carried out with dosages that yielded between 10% and 95% larval mortality in the range finding tests to determine the lethal doses, LD_{50}, LD_{90} and LD_{99}. Thus, the following dosages were evaluated: 0.05 ml/m^2, 0.1 ml/m^2, 0.2 ml/m^2, 0.4 ml/m^2 and 0.5 ml/m^2. These were compared to larval mortality in untreated controls.

To carry out the tests, batches of 25 third-instar larvae were introduced into plastic tubs (diameter 0.42 m) filled with 5 l (depth 5 cm) of unchlorinated tap water originating from Lake Victoria. Thereafter, the appropriate volume of AMF was applied into the treatment tubs to obtain the above doses. Application of AMF was done using a micropipette. *Anopheles gambiae s.s.* and *An. arabiensis* were evaluated in parallel. The tests were conducted over three rounds on separate dates. Each test round lasted for 48 hours. Data on number of dead larvae was collected every 24 hours. Test larvae were fed on Tetramin©Baby fish food every 24 hours. In each round there were four replicates per test dosage and control for each mosquito species. Thus in total for each mosquito species there were 12 replicates per test dosage and control.

2.3.5 Standardized field tests

Tests were carried out in an open sunlit area within icipe-TOC campus that had been cleared of vegetation. Artificial ponds were created by sinking 40 plastic tubs, (diameter 0.42 m, depth 10 cm) into the ground. Ponds were arranged 1.5 m apart in eight rows with each row having five ponds. Each plastic tub was filled with 8 l of unchlorinated water and 2 l of soil to provide suitable biotic and abiotic parameters for mosquito larvae. Artificial ponds were used because tests were implemented during the dry season when natural breeding habitats of *An. gambiae s.l.* are often limited in number (Jawara *et al.* 2008; Mala *et al.* 2011; Govoetchan *et al.* 2014). These tests were also conducted with insectary-reared *An. gambiae s.s.* and *An. arabiensis* larvae due to the low density of vectors in the study area during the dry season (Fillinger *et al.* 2004). Both species were tested in parallel. Batches of 50 third-instar larvae were introduced into each pond before

AMF was applied into treatment ponds; 20 ponds contained *An. gambiae s.s.* and 20 ponds contained *An. arabiensis.* The ponds were assigned into treatments and controls by lottery. Twenty ponds (10 per species) were treated with AMF at the manufacturer's recommended dose of 1 ml/m^2. Since the surface area of water in each pond was 0.14 m^2, a volume of 0.14 ml (140 µl) was applied at the edge of the pond using a micropipette. The remaining 20 ponds (10 per species) were left untreated and served as controls. After AMF application an emergence trap modified from Fillinger *et al*., (2009b) was placed on top of each pond to prevent adult mosquitoes escaping and to avoid natural colonization of ponds by wild mosquitoes. A cone-shaped frame made of metallic rods was covered by mosquito netting with a sleeve to allow aspiration of any emerged adults (Figure 2.1).

The residual effect of a single dose of AMF was evaluated for six weeks by introducing new batches of 50 insectary-reared third-instar larvae into each pond each week. New batches of mosquito larvae were introduced into a pond using a plastic disposable transfer pipette (Fisherbrand, capacity 3 ml). This was done by first inserting the mouth of the pipette into the water before releasing the mosquito larvae gently into the water. After one week all larvae had developed into adults or died. After introducing larvae into each pond the number of live larvae and pupae and emerged adults was recorded daily. This was done by first assessing the emergence trap on each pond for presence of any emerged adult. If any adult was found in the trap it was aspirated into a holding plastic cup with the opening covered with mosquito netting. Emerged adults from separate ponds were held in separate holding plastic cups. At the end of a round, after six weeks, water from the ponds was discarded and set-up afresh for the next treatment round. The tests were conducted in three rounds. Rainfall was recorded at the icipe-TOC meteorological station weekly.

Figure 2.1: Standardized field test set up. Netting-covered emergence trap on top of artificial pond.

2.3.6 Delayed effects in adults emerging from sub-lethal dosages

Forty artificial ponds (diameter 0.42 m) were set-up as described above in a semi-field system. Here the ponds were arranged in four parallel rows with 10 ponds in each row. Batches of 50 insectary-reared third instar *An. gambiae s.s.* larvae were introduced into each pond. Thereafter, 20 of the ponds were randomly selected and treated with AMF at 0.12 ml/m^2, the dose that killed 50% of larvae in laboratory dose-response tests. To obtain this dose, 16.8 µl of AMF was applied at the edge of each treatment pond using a micropipette. The remaining 20 ponds were left untreated to serve as controls. Adult emergence from ponds was monitored as described above. The number of days to pupation was recorded. In addition the behaviour and movement of the larvae in water was observed. Tests were carried out in three rounds on separate dates with each round running for one week, sufficient for all larvae to successfully develop into adults or die. Every week, ponds were discarded to set-up the next treatment round with fresh batches of larvae.

Male and female mosquitoes that emerged from ponds were brought to the laboratory and transferred into 30 x 30 x 30 cm cages provided with 6% glucose solution *ad libitum*. Adults collected from control and treatment ponds were maintained in separate cages. Females in the cages were provided with a blood meal on a human arm on two consecutive days when they were 3-5 days old. On the third day after the last blood meal,

gravid females were individually introduced into 15 x 15 x 15 cm cages that contained a glass cup (diameter 7 cm) filled with 100 ml unchlorinated tap water to serve as oviposition substrate. Mosquitoes were left overnight to lay eggs and the number of eggs laid by individual females the following morning was recorded. Eggs were left in the oviposition cups for three days to hatch. The number of eggs that hatched into larvae was recorded. Here the egg-laying capacity and hatching of eggs laid by 50 individual females collected from control ponds and 50 females from treatment ponds was evaluated in each round. Thus in total 150 individual females from control and 150 females from treated ponds were used in this test.

2.3.7 Statistical analysis

IBM SPSS Statistics 20 software was used for data analyses. Dose-response data were analysed using log-dosage probit regression analysis. All replicates of the dose-response tests were pooled by doses for each mosquito species to estimate the lethal dose that killed 50% of the population (LD_{50}) and the LD_{90} and LD_{99}. Test dosages were included in the model as covariates and mosquito species as factors. Relative median potency estimates were used to compare the susceptibility of mosquito species. Generalized estimating equations (GEE) fitted to a negative binomial distribution with a log-link function and an exchangeable correlation matrix were used to estimate the impact of treatment of ponds on adult emergence. The pond identity number was included as the repeated measure variable since data on larval mortality was repeatedly collected from the same pond. Treatment, mosquito species, application round, water turbidity (categorized as clear or turbid) and presence or absence of rain during the test week were included in the model as fixed factors. Interactions between treatment and turbidity, and treatment and rain were also included in the model. A GEE model was also used to estimate the delayed effect of exposure of *An. gambiae s.s.* to sub-lethal dosages in the larval habitat on egg-laying and hatching of eggs. The parameter estimates of the GEE models were used to predict the weekly mean adult emergence, mean number of eggs laid per female and mean number of eggs that hatched into larvae and their associated 95% confidence intervals by removing the intercept from the models. Weekly percent reductions in adult emergence from treated ponds was calculated with Abbott's formula (Abbott 1987). The time to pupation of larvae introduced into ponds in tests to evaluate sub-lethal effects of AMF was calculated using the formula: $(Ax1) + (Bx2) + (Cx3)\cdots+(Hx8)/(\text{Total number}$

of pupae collected) where A, B, C····H are the number of pupae collected on day 1, 2, 3 to 8.

2.3.8 Ethical considerations

Ethical approval for arm-feeding mosquitoes was obtained from the Kenya Medical Research Institute's Ethical Review Committee. An experimental permit to import and test AMF was granted by the Pest Control Products Board, Nairobi, Kenya.

2.4 Results

2.4.1 Dose-response tests

Larval mortality was similar in the three experimental rounds for each mosquito species; therefore rounds were pooled for each mosquito species for calculation of mean larval mortality and effective lethal doses. The relative median potency estimates showed that both mosquito species were equally susceptible to AMF. Larval mortality occurred at all doses tested (Figure 2.2).
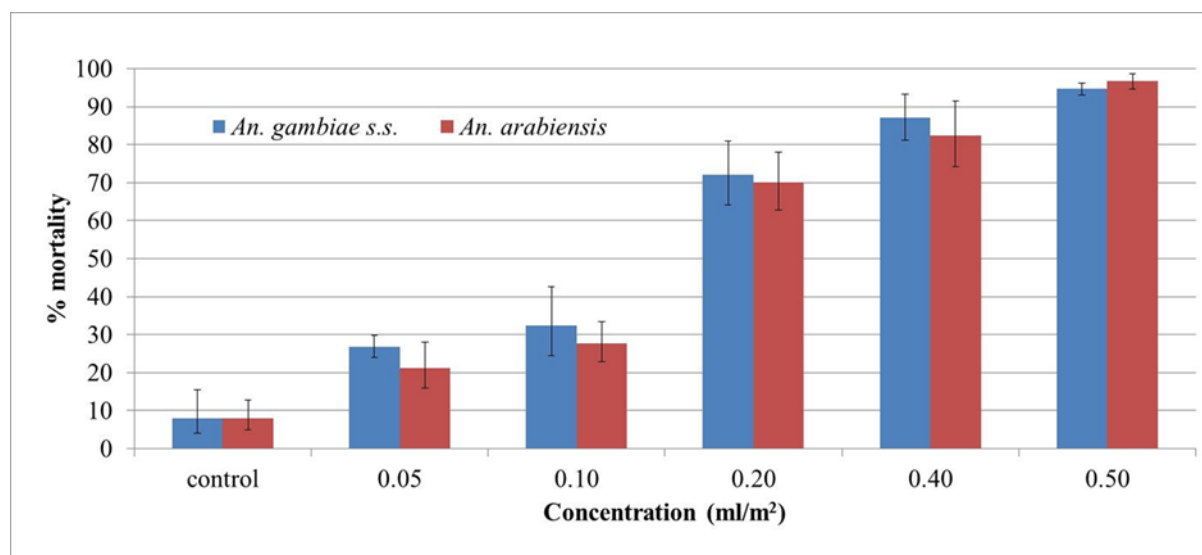
Figure 2.2: Mean mortality of larvae exposed to increasing doses of AMF in dose–response tests. Error bars represent 95% confidence intervals.

Probit analysis predicted that approximately 0.5 ml/m^2 was required to kill 90% of all exposed larvae whilst slightly over 1 ml/m^2 of AMF was needed to kill all larvae after 48

hours of exposure (Table 2.1). It was observed that at the two lower doses of AMF, 0.05 and 0.1 ml/m^2, some parts of the water surface remained untreated. Observation of the larvae in tubs treated at dosages above 0.1 ml/m^2 showed a reduced activity compared to larvae in control tubs and very slow response rates when disturbed e.g. when passing a hand over water surface or tapping the larval container. Larvae exposed to higher doses of AMF were often observed to coil into a circle with their mouthparts placed on the abdomen in a tail nibbling effect.

Table 2.1: Effective doses of AMF against third instar *An. gambiae s.s.* and *An. arabiensis*

	An. gambiae s.s. ml/m^2	*An. arabiensis* ml/m^2
LC$_{50}$ (95% CI)	0.12 (0.11-0.13)	0.13 (0.11-0.15)
LC$_{90}$ (95% CI)	0.43 (0.37-0.51)	0.47 (0.41-0.56)
LC$_{99}$ (95% CI)	1.23 (0.99-1.59)	1.35 (1.09-1.76)

2.4.2   Standardized field tests

The effect of AMF on larval mortality under field conditions was not significantly different between *An. gambiae s.s.* and *An. arabiensis* (Table 2.2) thus data for the two species were pooled to show weekly larval mortality in Figure 2.3 and to calculate weekly percent mortality (Table 2.3).

Table 2.2: GEE analysis of factors affecting adult emergence from ponds

Explanatory variables	Odds ratio (95% CI)	p-value
Treatment		
treatment ponds	0.15 (0.12-0.18)	<0.0001
control ponds	1	
Mosquito species		
An. gambiae s.s.	0.94 (0.85-1.04)	0.235
An. arabiensis	1	
Round		
round 3	1.05 (0.93-1.19)	0.408
round 2	1.09 (0.95-1.24)	0.223
round 1	1	
Weeks		
week 6	2.61 (1.70-4.02)	<0.0001
week 5	2.37 (1.60-3.51)	<0.0001
week 4	2.71 (1.78-4.10)	<0.0001
week 3	1.35 (1.12-1.64)	0.002
week 2	1.01 (0.93-1.10)	0.778
week 1	1	
Water turbidity		
Turbid	0.65 (0.51-0.82)	<0.0001
Clear	1	
Rainfall		
Rain	0.80 (0.68-0.95)	0.013
no rain	1	
Interaction between treatment and turbidity		
treatment*turbid	2.72 (1.99-3.72)	<0.0001
treatment*clear	1	
Interaction between treatment and rainfall		
treatment*rain	1.45 (0.95-2.11)	0.053
treatment*no rain	1	

*symbol for interaction between factors

AMF applied at 1 ml/m^2 provided complete larval mortality for two weeks. Emergence from treatment ponds occurred from week 3, but this remained below 10% over the six week monitoring period (Figure 2.3). The emergence of adults coincided with the

observation of small breakages of the surface film in some of the ponds from the third week onwards. On average, 84.7% (95% 75.7-93.3%) of larvae introduced weekly into control (untreated) ponds successfully developed into adults. Results were very consistent from round to round (Figure 2.3).
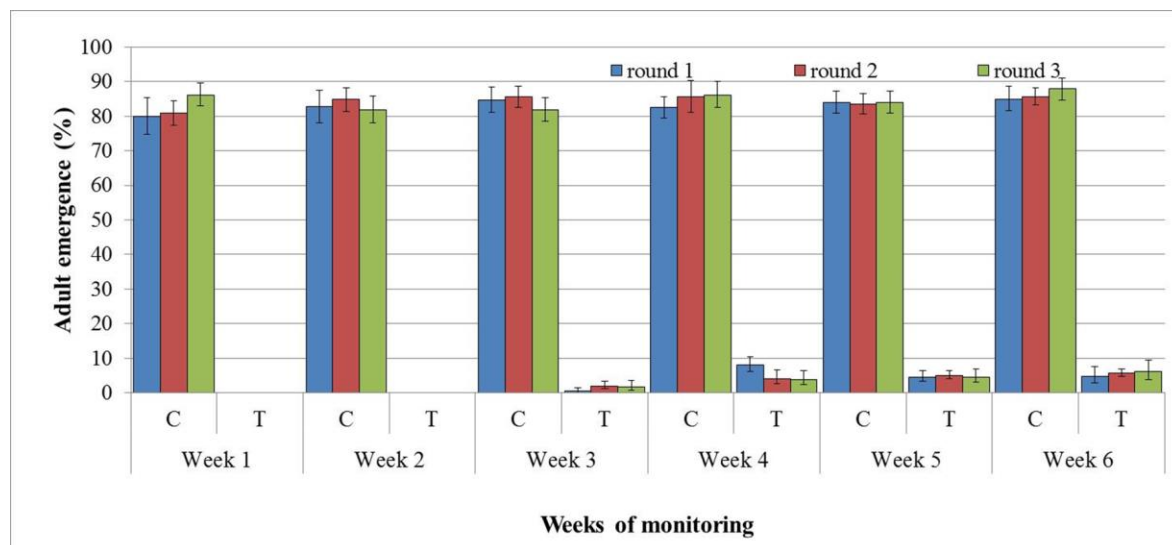
Figure 2.3: Weekly emergence of *An. gambiae s.l.* from control (C) and treatment (T) in standardized-field tests. Error bars represent 95% confidence intervals.

Adjusting for other factors, it was 6.7 times less likely for an adult to emerge from treated ponds compared to control ponds (Table 2.2). However, the probability of emergence increased over time and was 1.4-2.6 times higher from ponds that had received treatment 3-6 weeks earlier compared to freshly treated ponds (Table 2.2). Both turbidity and rainfall affected adult emergence from ponds irrespective of the treatment. It was 1.5 times less likely for adults to emerge from turbid ponds than from clear ponds and 1.25 times less likely to emerge if it had rained during the exposure week (Table 2.2). In addition to the main effect, turbidity and rainfall interacted with the treatment in such a way that both factors increased the probability of emergence from AMF treated ponds, or in other words, slightly decreased the impact of the intervention (Table 2.2). The overall impact of the interaction can be estimated by multiplying the odds ratios (Katz 2006). This means for instance that while it was 6.7 times less likely for adults to emerge from treated ponds that were clear in the first week of round 1, it was only 3.8 times less likely for adults to emerge from treated ponds that were turbid in the same time period. Similarly, while it was 4.5 times less likely for adults to emerge from treatment ponds

when it failed to rain during week 3 of round 2, the likelihood of emergence was only 3.8 times less from similar treatment ponds at same time period when it rained.

Table 2.3: Weekly percent mortality of *An. gambiae s.l.* larvae in treatment ponds

	Week 1	Week2	Week 3	Week 4	Week 5	Week 6
round 1	100	100	97 (96-99)	90 (87-92)	94 (92-96)	94 (91-96)
round 2	100	100	97 (96-98)	95 (92-97)	93 (92-95)	93 (92-94)
round 3	100	100	95 (94-99)	95 (93-97)	94 (92-96)	93 (90-95)

2.4.3   Delayed effects in adults emerging from sub-lethal dosages

Results from individual rounds were similar (p=0.16) and therefore pooled for analysis. The mean percent adult emergence was 92.9% (95% CI 92.4-93.3%) from untreated ponds and 55.8% (95% CI 44.9-66.5%) from treated ponds. Significant differences were observed in the mean pupation time of larvae introduced in control and treatment ponds. Of those larvae that survived, the mean pupation time was estimated as 3.4 days (95% CI 3.0-3.7) in control ponds and 4.9 days (95% CI 4.4-5.3) in ponds treated with sub-lethal dose of AMF. Furthermore, live larvae in treated ponds often showed signs of weakness as they exhibited slow movement when disturbed on the water surface in contrast to those unexposed.

Females that emerged from ponds treated with sub-lethal doses of AMF were 2.2 times less likely (OR 0.45; 95% CI 0.26-0.78) to lay eggs compared with females from untreated ponds. However, if females laid eggs the mean number of eggs laid per female did not differ significantly between treatment groups (p=0.31). The mean number of eggs laid per female was 49.3 (95% CI 41.3-58.8) when adults emerged from control ponds and 45.4 (95% CI 37.4-55.1) when females emerged from larvae that developed in ponds treated with a sub-lethal dose of AMF. Similarly, there were no significant differences in the hatching of eggs laid by females emerged from treated and control ponds (p=0.18). The mean number of hatched eggs was 41.0 (95% CI 38.0-44.2) when eggs were laid by females emerging from control ponds and 36.8 (95% CI 33.8-40.1) for eggs laid by females emerging from ponds treated with a sub-lethal dose of AMF.

2.5   Discussion

The dose-response tests and consequent standardized field tests confirmed that the manufacturer's recommended dosage of 1 ml/m^2 is effective for the control of the two malaria vectors, *An. gambiae s.s.* and *An. arabiensis*. Furthermore, the dose-response tests highlight the high susceptibility of these two species with half the recommended dosage (0.5 ml/m^2) already leading to 90% mortality and approximately a quarter of it still leading to greater than 50% mortality. *Anopheles gambiae s.s.* and *An. arabiensis* were equally susceptible to AMF which is not surprising given the physical mode of action of this larvicide and the similar larval behaviour of both vector species (Clements 2000) exposing them to the surface film while feeding.

The standardized field tests showed over 80% emergence inhibition from AMF-treated ponds over the entire six week observation period, confirming the stability of the silicone-based surface film over time. Our results confirm the extended residual activity of AMF and other MMFs reported in the field (Nayar and Ali 2003; Batra *et al.* 2006; Webb and Russell 2012). Studies have shown that Arosurf® MSF and Agnique® MMF are effective for control of different genera of mosquito for 7-21 days (Mulla *et al.* 1983; Nayar and Ali 2003). The efficacy of AMF was found to last 4-6 weeks for the control of *Culex* and *Aedes* larvae in small-scale field trials in Australia (Webb and Russell 2012). It is important, however, to consider that our test habitats were small, confined and undisturbed and phase III trials should now be conducted to evaluate AMF in different habitat types and sizes to establish the residual activity under different environmental conditions to give final recommendations for application intervals for different habitat types. The only field study to evaluate AMF for control of Afrotropical malaria vectors found the film to be effective in reducing emergence of anopheline and culicine mosquitoes when applied at 1 ml/m^2 in rice paddies in Western Kenya (Bukhari *et al.* 2011). However, a double dose (2 ml/m^2) was necessary to effectively suppress larval densities of both mosquito genera (Bukhari *et al.* 2011). Differences in susceptibility of life stages of mosquito immatures to surface films have been reported elsewhere (Nayar and Ali 2003; Bukhari and Knols 2009).

Turbid water and rainfall reduced the efficacy of AMF for mosquito control. The water in our artificial ponds could have been turbid due to algae, bacteria and other suspended

particles in the water column (Cuker 1987). Possibly turbidity increased the rate of degradation of the AMF film therefore reducing film efficacy from the effect of increased water temperatures (Levy *et al.* 1981; Paaijmans *et al.* 2008; Gouagna *et al.* 2012; Mereta *et al.* 2013). It might also be that the reduced efficacy of the film in turbid water is caused by natural films formed by suspended particles that limit the spread of AMF film (Garrett and White 1977). Rainfall in general increased larval mortality irrespective of the treatment likely due to flush out effects (Paaijmans *et al.* 2007). However, larvae from treated ponds that experienced rain during the week of exposure were more likely to survive than larvae from treated ponds without rain, probably because rain breaks up the surface film and provides pockets of film free environments for larval development (Levy *et al.* 1981). It has been reported in other studies that rainfall is a major factor that limits the efficacy of surface films for mosquito control (Levy *et al.* 1981; Levy *et al.* 1982), though in our study rainfall reduced the activity of AMF only slightly. However, this tool would be especially promising when applied to aquatic habitats in the dry season due to the minimal climatic and environmental influences at this time providing long-lasting control with a single application.

Exposure of larval stages to sub-lethal doses of AMF increased larval development time and reduced the proportion of gravid females egg-laying. Similar effects have been reported for organophosphates, spinosyns, insect growth regulators and microbials (Loh and Yap 1989; Robert and Olson 1989; Wang and Jaal 2005; Antonio *et al.* 2009; Kamal and Khater 2010; Sanil and Shetty 2012). These effects would be an additional benefit to larviciding programmes as they reduce the frequency of larvicide application thereby reducing intervention costs (Worrall and Fillinger 2011). Longer larval development time predisposes mosquito larvae to several risks that reduce their survival including predation, disturbances by human activities and instability of breeding habitats (Padmanabha *et al.* 2010; Padmanabha *et al.* 2011; Oliver and Brooke 2013). It has been previously shown that nutrient deprivation is a common cause of prolonged mosquito larval development (Lehmann *et al.* 2006; Telang *et al.* 2007; Yoshioka *et al.* 2012; Oliver and Brooke 2013; Takken *et al.* 2013). Thus, it is most likely in the current study the prolonged larval development was caused by poor nutrition of larvae in treatment ponds. This is because as observed in our dose-response tests and previous studies (Reiter and McMullen 1978; Corbet *et al.* 2000), mosquito larvae exposed to MMFs spend a great deal of time attempting to wash off the liquid that blocks their respiratory structures and thus have

little time to feed. Adults that emerge from poorly fed larvae are often small in size with low teneral reserves (Koenraadt *et al.* 2010; Oliver and Brooke 2013; Takken *et al.* 2013), with the effect of reduced egg-laying capacity (Steinwascher 1982; Briegel 1990), a phenomenon observed in the current study. Additional effects of reduced survival and insemination in females have been observed in adults deprived of nutrients during the larval stage (Ameneshewa and Service 1996; Oliver and Brooke 2013), which can potentially reduce the vectorial capacity.

2.6   Conclusion

The high susceptibility of *An. gambiae s.s.* and *An. arabiensis*, the long residual activity, sub-lethal effects on larval development and reproduction combined with the physical mode of action makes AMF a novel, and potentially important tool for larval control interventions against malaria vectors in Africa. Further field studies in different eco-epidemiological settings are justified to determine the efficacy and persistence of AMF film for mosquito vector control and its potential for inclusion in integrated vector management programmes. Furthermore, although AMF and other MMFs have been shown to have minimal effect on most non-target aquatic insects since they spend much less time on the water surface (Mulla *et al.* 1983; Nayar and Ali 2003; Bukhari *et al.* 2011), concerns on the safety of those that rely on the water surface for respiration and movement needs to be investigated. AMF might be a useful control agent to be considered for rotation or in combination with other larvicides to reduce insecticide-resistance development.

3 Dose-response tests and semi-field evaluation of lethal and sub-lethal effects of slow release pyriproxyfen granules (Sumilarv®0.5G) for the control of the malaria vectors *Anopheles gambiae sensu lato*

Oscar Mbare, Steven W Lindsay, Ulrike Fillinger

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3.1   Abstract

Background: Recently research has shown that larviciding can be an effective tool for integrated malaria vector control. Nevertheless, the uptake of this intervention has been hampered by the need to re-apply larvicides frequently. There is a need to explore persistent, environmentally friendly larvicides for malaria vector control to reduce intervention efforts and costs by reducing the frequency of application. In this study, the efficacy of a 0.5% pyriproxyfen (PPF) granule (Surmilarv®0.5G, Sumitomo Chemicals) was assessed for the control of *Anopheles gambiae sensu stricto* and *Anopheles arabiensis*, the major malaria vectors in sub-Saharan Africa.

Methods: Dose-response and standardized field tests were implemented following standard procedures of the World Health Organization's Pesticide Evaluation Scheme to determine: (i) the susceptibility of vectors to this formulation; (ii) the residual activity and appropriate retreatment schedule for field application; and, (iii) sub-lethal impacts on the number and viability of eggs laid by adults after exposure to Sumilarv®0.5G during larval development.

Results: *Anopheles gambiae s.s.* and *An. arabiensis* were highly susceptible to Sumilarv®0.5G. Estimated emergence inhibition (EI) values were very low and similar for both species. The minimum dosage that completely inhibited adult emergence was between 0.01-0.03 parts per million (ppm) active ingredient (ai). Compared to the untreated control, an application of 0.018 ppm ai prevented 85% (95% confidence interval (CI) 82%-88%) of adult emergence over six weeks under standardized field conditions. A fivefold increase in dosage of 0.09 ppm ai prevented 97% (95% CI 94%-98%) emergence. Significant sub-lethal effects were observed in the standardized field tests. Female *An. gambiae s.s.* that were exposed to 0.018 ppm ai as larvae laid 47% less eggs, and females exposed to 0.09 ppm ai laid 74% less eggs than females that were unexposed to the treatment. Furthermore, 77% of eggs laid by females exposed to 0.018 ppm ai failed to hatch, whilst 98% of eggs laid by females exposed to 0.09 ppm ai did not hatch.

Conclusion: *Anopheles gambiae s.s.* and *An. arabiensis* are highly susceptible to Sumilarv®0.5G at very low dosages. The persistence of this granule formulation in

treated habitats under standardized field conditions and its sub-lethal impact, reducing the number of viable eggs from adults emerging from treated ponds, enhances its potential as malaria vector control tool. These unique properties warrant further field testing to determine its suitability for inclusion in malaria vector control programmes.

3.2   Background

Malaria control interventions with long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) have resulted in substantial reductions of malaria cases in sub-Saharan Africa (Steketee and Campbell 2010; Okumu and Moore 2011). Since both LLINs and IRS target the fraction of the vector population that enter houses (Robert and Carnevale 1991; Pinder *et al.* 2011) their efficacy is threatened by vectors developing resistance to insecticides used indoors (WHO 2000; Chouaibou *et al.* 2008; Kawada *et al.* 2011a) and behavioural adaptations where vectors shift their biting patterns to bite in early evening and in the morning when people are out of the nets (Faye *et al.* 1997; Reddy *et al.* 2011). There has also been a shift in the vector species' composition in parts of East Africa with LLINs dramatically reducing the numbers of largely endophilic *Anopheles gambiae s.s.* but having little or no impact on *Anopheles arabiensis* that tends to bite and rest outdoors (Bayoh *et al.* 2010; Russell *et al.* 2010; Kitau *et al.* 2012) resulting in *An. arabiensis* becoming the dominant vector. Since IRS and LLINs cannot totally suppress malaria transmission there is a growing interest in the use of additional tools in an integrated vector management approach (Clive 2002; WHO 2004; Beier *et al.* 2008; Chanda *et al.* 2008; Fillinger *et al.* 2009a).

Larval source management has been re-evaluated for malaria control (Fillinger *et al.* 2003; Shililu *et al.* 2003b; Fillinger and Lindsay 2006; Majambere *et al.* 2007; Geissbühler *et al.* 2009; Bukhari *et al.* 2011), with results indicating the added benefit larval control could have when used together with interventions that target adult mosquitoes (Chanda *et al.* 2008; Fillinger *et al.* 2009a; Shaukat *et al.* 2010). One of the advantages of larval source management is that it targets the aquatic stages of the vectors thus controlling both indoor and outdoor biting and resting and insecticide resistant mosquitoes (Fillinger and Lindsay 2011). Commercially available chemical larvicides and microbials are highly effective in the control of the major malaria vectors of sub-Saharan

Africa (Majori *et al.* 1987; Karch *et al.* 1991; Karch *et al.* 1992; Ragoonanansingh *et al.* 1992; Ravoahangimalala *et al.* 1994; Skovmand and Bauduin 1996; Seyoum and Abate 1997; Fillinger *et al.* 2003; Shililu *et al.* 2003b). However, relatively few studies evaluated them under operational conditions (Barbazan *et al.* 1998; Fillinger and Lindsay 2006; Shililu *et al.* 2007; Fillinger *et al.* 2008; Fillinger *et al.* 2009a; Majambere *et al.* 2010) and a major limitation is their short activity under most environmental conditions, frequently requiring weekly re-application (Skovmand and Sanogo 1999; Fillinger *et al.* 2003; Majambere *et al.* 2007; Fillinger *et al.* 2008). Larvicide and labour are the major costs in large-scale larval control programmes and these could be substantially reduced if re-application intervals could be reduced without jeopardizing the impact of the intervention (Worrall and Fillinger 2011). In addition, the toxic effects of chemical-based larvicides to non-target aquatic insects limits their use for regular larviciding programmes (Fales *et al.* 1968; Fortin *et al.* 1987).

Sumilarv®0.5G (Sumitomo Chemicals) is a granule insecticide developed for mosquito control. The active ingredient is pyriproxyfen (PPF) (4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether), a juvenile hormone analogue that acts as an insect growth regulator. PPF generally inhibits adult emergence of target insects (Kamimura and Arakawa 1991; Yapabandara and Curtis 2002; Vythilingam *et al.* 2005). However it also has delayed effects on female reproduction of adult mosquitoes exposed to sub-lethal doses at the larval (Loh and Yap 1989; Kamal and Khater 2010) or adult stage (Itoh *et al.* 1994; Sihuincha *et al.* 2005). Sumilarv®0.5 has exceptional residual activity of up to six months for the control of *Aedes, Culex* and *Anopheles* mosquitoes in their natural breeding habitats (Chavasse *et al.* 1995a; Yapabandara and Curtis 2002; Sihuincha *et al.* 2005; Vythilingam *et al.* 2005). Furthermore, PPF has been evaluated as a safe insecticide for application in drinking water (WHO 2008a) with minimal impacts on non-target aquatic insects and the environment (Mulla *et al.* 1986; Schaefer *et al.* 1988; Schaefer and Miura 1990; Schaefer *et al.* 1991; WHO 2008a). Nevertheless, Sumilarv®0.5G has never been evaluated for the control of immature stages of *An. gambiae s.l.*, the major malaria vector in sub-Saharan Africa.

The objectives of the present study were to evaluate the efficacy of this granular formulation of PPF for the control of *An. arabiensis* and *An. gambiae s.s.* by determining: (i) the minimum effective dose in dose-response tests; (ii) the optimum application dose

to be applied under field conditions; (iii) the residual period of the optimum dose; and, (iv) the effects of sub-lethal doses on egg production and larval hatching. All tests were based on the World Health Organization Pesticide Evaluation Scheme (WHOPES) guidelines for laboratory and field testing of mosquito larvicides (WHO 2005a).

3.3　Methods

3.3.1　Study area

The study was conducted at the International Centre of Insect Physiology and Ecology-Thomas Odhiambo Campus (*icipe*-TOC) in Mbita (0° 26′ 06.19” S; 34° 12′ 53.13” E) close to Lake Victoria, Western Kenya (altitude 1,137 m). Here, the major malaria vectors are *An. arabiensis* with a small number of *An. gambiae s.s.* and *An. funestus* (Kawada *et al.* 2011b). The area is characterized by a tropical climate with an average annual minimum temperature of 16^oC and an average maximum temperature of 28^oC (*icipe*-TOC meteorological station data for 2010 to 2012). The area experiences two major rainy seasons, the long rains between March and June and the short rains between October and December. The average annual rainfall for 2010 to 2012 was 1,150mm (*icipe*-TOC meteorological station). The laboratory tests were conducted in July-August 2011 while the standardized field tests were conducted between October 2011 and January 2012.

3.3.2　Mosquitoes

Both laboratory and standardized field tests used insectary-reared third instar larvae of *An. arabiensis* and *An. gambiae s.s.* (Mbita strains). Larvae were reared in round plastic tubs (diameter 60 cm) filled with water (5 l, 5 cm high) from Lake Victoria filtered through a charcoal-sand filter. Mosquito larvae were fed with fish food (Tetramin©Baby) twice daily. Third instar mosquito larvae were selected from different tubs so that the larvae were of a similar range in size in each tub tested (Araujo *et al.* 2012). Mosquito larvae were reared at ambient climate and light conditions in a netting-screened greenhouse with an average daily temperature of 27°C, an average 76% relative humidity and a natural 12 hours of dark and 12 hours of light cycle.

3.3.3 Insecticide

Sumilarv®0.5G was provided by the manufacturer Sumitomo Chemicals Company, Japan, for all tests. It is a granular formulation containing 0.5% active ingredient (weight: weight).

3.3.4 Dose-response tests

Tests were done in the shade, under ambient climate and light conditions in a netting-screened greenhouse. Prior to the dose-response tests, range-finding tests were implemented by exposing test larvae to a wide range of test concentrations and a control. This served to find the activity range of the insecticide for each test species. Concentrations between 10 parts per million (ppm) active ingredient (ai) and 0.0000001 ppm ai were tested. After determining the emergence inhibition (EI) of the larvae in the wider range, nine concentrations were chosen, yielding between 10% and 95% EI in the range-finding tests in order to determine the EI_{50}, EI_{90} and EI_{99} in dose response bioassays. The following concentrations were tested: 0.005 ppm ai, 0.001 ppm ai, 0.0005 ppm ai, 0.0001 ppm ai, 0.00007 ppm ai, 0.00004 ppm ai and 0.00001 ppm ai, 0.000005 ppm ai, 0.000001 ppm ai.

A stock solution was prepared by grinding the granular formulation into a very fine powder following the procedure of Sihuincha *et al.* (2005). Using a pestle and mortar, 5 g of Sumilarv®0.5G (25 mg ai) was ground and added to 500 ml of non-chlorinated tap water. This gave a stock solution of 10,000 ppm Sumilarv®0.5G (50 ppm ai). The mouth of the vial was covered with aluminium foil and the solution left to agitate for one hour on a shaker (Gerhardt Analytical Systems). Since Sumilarv®0.5G is a slow release formulation the mixture was left overnight to allow the active ingredient to be released into solution. In the morning the mixture was again agitated on a shaker for 30 minutes to prepare a homogenous mixture since some of the inert ingredients of the formulation (potentially still containing some active ingredient) had settled overnight. Serial dilutions were made immediately after shaking in non-chlorinated tap water to produce the test concentrations.

Anopheles arabiensis and *An. gambiae s.s.* were evaluated in parallel. Each test concentration and a control were replicated four times per round per mosquito species. Two hundred millilitres of each test solution was set up in 300 ml plastic cups. Three

rounds of tests were implemented. Separate batches of 25 insectary-reared third instar larvae of both test species were introduced into each test concentration and the control (non-chlorinated tap water). Thus in total 300 larvae of each species were tested per test concentration and control (total of 3000 larvae). Larvae were fed with Tetramin© Baby fish food every 24 hours and cups covered with netting to prevent any emerging adults from escaping. The number of live and dead larvae, pupae and adults was recorded every 24 hours for 10 days. Live pupae from each cup were transferred into a separate cup with approximately 20 ml of water from the respective cup of collection. These cups were covered with netting and pupae monitored for emergence. Separate pipettes were used to collect pupae from treated and control cups to avoid cross-contamination.

3.3.5 Standardized field tests

Standardized field tests (WHO 2005a) were carried out in an open field with grass approximately 3 cm in height between October 2011 and March 2012. Thirty artificial ponds were set up in an open field by sinking enamel-coated bowls (diameter 42 cm, depth 10 cm) into the ground (Figure 3.1). Ponds were arranged 2 m apart in six rows. Each bowl was filled with 8 l of non-chlorinated tap water. Into each pond 2 l of soil collected from the surrounding field was added and mixed well to resemble a natural habitat.

Figure 3.1: Set-up of standardized field test. (A) Enamel-coated bowl sunk into the ground and filled with water and soil to simulate a natural pond. (B) Netting-covered emergence trap on top of a pond to prevent escape of emerged adults.

Batches of 50 insectary-reared third instar larvae were introduced into each pond. Sumilarv®0.5G treatment was applied after introduction of larvae. Treatment of the ponds was allocated randomly using a lottery system. In each treatment round, 10 of the ponds served as untreated controls; in five of them *An. arabiensis* were introduced and in the other five *An. gambiae s.s.* Two application rates of Sumilarv®0.5G were tested per mosquito species. The application rate was based on the surface area of the water, which was 0.14 m^2 per pond. Sumilarv®0.5G was spread evenly over the entire water surface by hand. Five ponds were treated with 1 mg ai per m^2 (equalling 0.018 ppm ai considering the volume of 8 l of water) while five other ponds were treated with 5 mg ai per m^2 (or 0.09 ppm ai) per mosquito species. A netting-covered emergence trap was placed on top of each pond to prevent wild mosquitoes from laying eggs in the sites and to prevent the escape of any emerging adult mosquitoes (Figure 3.1 B). The residual activity of Sumilarv®0.5G was evaluated by introducing new batches of 50 insectary-reared third instar larvae into each pond at weekly intervals. After one week all the larvae had either emerged as adults or died. The efficacy of Sumilarv®0.5G was evaluated for six weeks. This experiment was implemented three times (referred to as rounds in the analyses).

To assess larval mortality, the number of larvae present in each habitat was counted daily. First, the emergence trap over each pond was assessed for the presence of any newly emerged adults and any adults collected with an aspirator and placed into a disposable cup covered with netting. Any pupae in the ponds were transferred into plastic cups holding 50 ml of the water from the respective pond. Pupae collections were done in the morning and evening so that any emergence or emergence inhibition could be recorded daily in the laboratory.

To monitor environmental parameters that may influence the efficacy of the insecticide, daily data on turbidity and pH of water in each pond was collected. Ponds were visually categorized into clear (ground visible) or turbid ponds. The water pH was measured using a pH meter (Phywe International, Germany).

3.3.6 Sub-lethal effects

Tests to assess the impact of sub-lethal doses of Sumilarv®0.5G were carried out under ambient conditions in a netting-screened greenhouse. The number of eggs laid and the number of eggs hatched (number of offspring produced) per adult mosquito that emerged from treated ponds were compared to that of the adults that emerged from the untreated ponds in standardized field tests. All pupae used in these tests were collected from the ponds in week six of each test round. Pupae collected from ponds treated at the two Sumilarv®0.5G dosages and untreated ponds were introduced into separate cages (30 x 30 x 30 cm) covered with mosquito netting. Emerged adults (both male and female) were maintained in the same cages with 6% glucose solution *ad libitum*. When the adults were two to four days old they were blood fed twice on a human arm on two successive days. To carry out tests a single gravid mosquito was introduced into a cage (15 x 15 x 15 cm) with an oviposition cup (diameter = 7 cm) containing 100 ml of non-chlorinated tap water. The number of eggs laid by each mosquito overnight and the number of eggs hatched over one week were counted. Sub-lethal effects of the treatment dosage of 1mg ai per m^2 were tested with 20 individual females per round of semi-field test for *An. arabiensis* and *An. gambiae s.s.*, respectively (total 3 x 20 = 60 females per species). There were 20 replicates of mosquitoes collected from untreated (control) ponds. Due to the persistent high immature mortality of the 5 mg ai per m^2 treatment only 10 females per species and round could be tested (total 3 x 10 = 30 females per species).

3.3.7 Statistical analyses

Data analyses were done with SPSS statistical software version 19. All data from the replicates of the dose-response tests were pooled by doses for each mosquito species for the estimation of the EI$_{50}$, EI$_{90}$ and EI$_{99}$ values using the log dosage-probit regression analysis with the test dosages as covariates and species as factors in the model. Relative median potency estimates were used to compare the susceptibility of the two species. Generalized estimating equations (GEE) were used to estimate the overall emergence inhibition of the two Sumilarv®0.5G dosages for the six weeks treatment period in standardized field tests. The number of successful emerged adults was the dependent variable and was fitted to a negative binomial distribution with a log-link function and an exchangeable correlation matrix. The treatments, test rounds, mosquito species, water turbidity (clear, turbid), water pH (grouped in two categories: pH < 8, pH ≥8) and the

occurrence of rain during the test week (no rain, rain) were added to the model as fixed factors. Since the same pond was evaluated repeatedly for larval mortality over the six-week period, the unique pond ID was included as the repeated measures variable. Interaction terms were included in the model between treatments and turbidity, treatments and pH, and treatments and rain. GEE models were also used to estimate the impact of sub-lethal concentrations on the number of eggs laid and the number of eggs that hatched from emerged *An. gambiae s.s.* adults. The parameter estimates of the GEE models were used to calculate the weekly mean adult emergence, mean number of eggs laid per female and mean number of laid eggs that hatched into larvae and the associated 95% confidence intervals (CIs) by removing the intercept from the models. For the calculation of percent reduction the weekly emergence inhibition in the treated ponds was corrected using Abbott's formula based on emergence in the untreated ponds as denominator (Abbott 1987). Percent reduction was therefore calculated as follows:

$$\% \text{ treatment EI} = \frac{[\% \text{ untreated EI} - \% \text{ treated EI}] \times 100\%}{\% \text{ untreated EI}}$$

3.4   Results

3.4.1   Dose-response tests

The dose-response tests showed that Sumilarv®0.5G affected adult mosquito emergence in *An. arabiensis* and *An. gambiae s.s.* at very low and over a very wide range of concentrations (0.000001-0.005 ppm ai). Data from the three rounds of dose-response tests showed similar trends in emergence inhibition for each species, and were therefore pooled per dose (Figure 3.2) to estimate emergence inhibition (EI) rates; EI $_{50}$, EI$_{90}$ and EI$_{99}$ (Table 3.1).
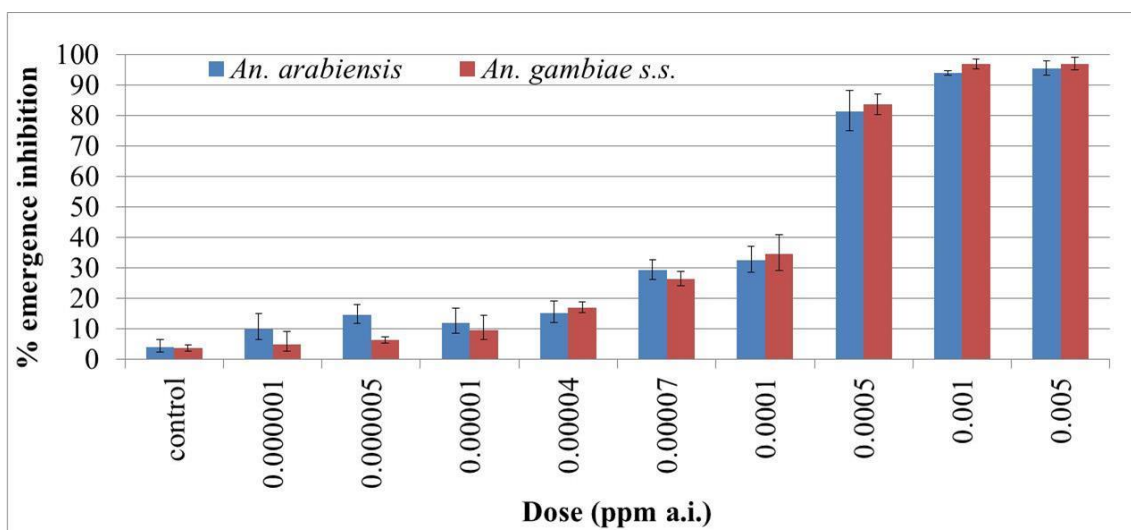
Figure 3.2: Average percent emergence inhibition (error bars: 95% confidence intervals) of *An. arabiensis* and *An. gambiae s.s.* in response to increasing concentrations (ppm ai) of Sumilarv®0.5G.

The minimum dosage that completely inhibited adult emergence was estimated to be between 0.01-0.03 ppm ai (Table 3.1). *Anopheles arabiensis* and *An. gambiae s.s.* were equally susceptible to Sumilarv®0.5G.

Table 3.1: Estimated doses (ppm ai) of Sumilarv®0.5G for 50%, 90% and 99% emergence inhibition (EI) in *Anopheles gambiae s.s.* and *Anopheles arabiensis*

	An. arabiensis ppm ai	*An. gambiae s.s.* ppm ai
IE50 (95%CI)	0.00012 (0.00009-0.00016)	0.00013 (0.00010-0.00017)
IE90 (95%CI)	0.00248 (0.00154-0.00450)	0.00139 (0.00092-0.00232)
IE99 (95%CI)	0.02860 (0.01379-0.07296)	0.00973 (0.00526-0.02159)

3.4.2 Standardized field tests

There was no difference in adult emergence from treated ponds between *An. arabiensis* and *An. gambiae s.s.* (p=0.3) and data for both species were pooled for analysis. The weekly adult emergence per round from the treated and untreated ponds is shown in Figure 3.3 and emergence inhibition calculated in Table 3.2. Complete emergence

inhibition was observed for two weeks in rounds one and three of the high treatment dose of 5 mg ai per m^2 (0.09 ppm ai). However at the lower dosage of 1 mg ai per m^2 (0.018 ppm ai) which corresponded with the minimum effective dosage established in the dose-response tests complete emergence inhibition was only observed in week one in round one and three. Ponds treated at 5 mg ai per m^2 provided better residual impact than the lower treatment dosage of 1 mg ai per m^2 (Figure 3.3 and Table 3.2).
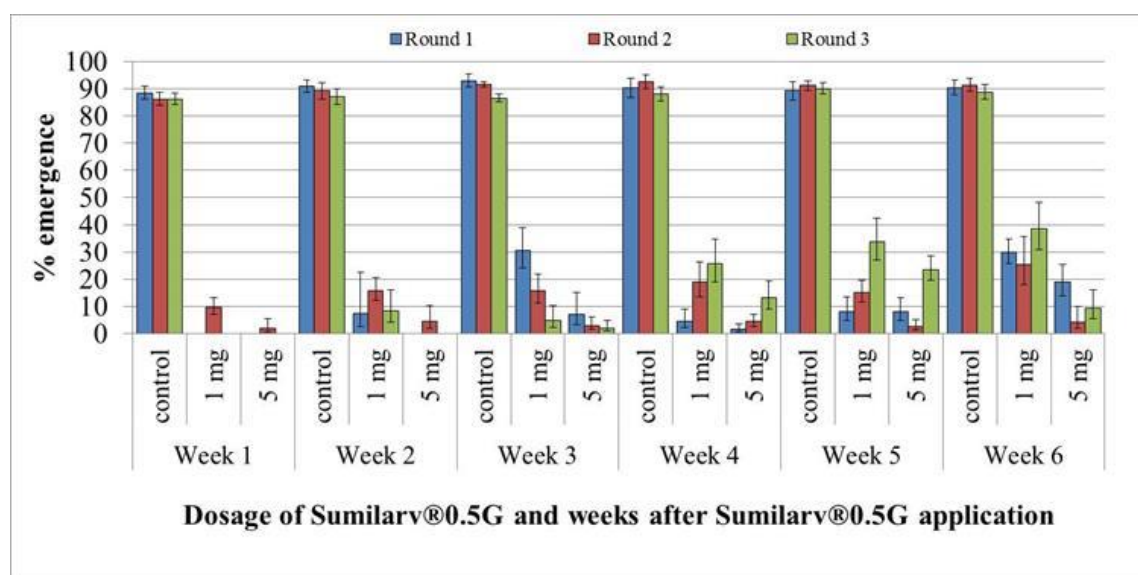
Figure 3.3: Mean adult emergence (error bars: 95% confidence intervals) of *Anopheles gambiae s.l.* in standardized field tests after application of 1 mg or 5 mg ai per m^2 Sumilarv®0.5G in artificial ponds.

Table 3.2: Weekly percent emergence inhibition (95% CI) of *An. gambiae s.l.* from treated ponds

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
1 mg ai per m^2						
Round 1	100	98 (94-99)	65 (55-72)	95 (90-98)	93 (85-97)	66 (59-71)
Round 2	88 (83-92)	86 (76-90)	83 (76-88)	78 (69-85)	79 (73-84)	72 (62-80)
Round 3	100	92 (80-97)	94 (86-98)	71 (62-78)	62 (54-69)	57 (47-64)
5 mg ai per m^2						
Round 1	100	100	94 (80-98)	98 (96-99)	91 (82-95)	84 (73-90)
Round 2	99 (95-100)	95 (81-99)	97 (96-98)	96 (90-99)	97 (94-99)	95 (90-98)
Round 3	100	100	98 (95-99)	85 (79-89)	74 (69-78)	90 (83-94)

Adjusting for other factors the GEE model estimated that Sumilarv®0.5G inhibited 85% of adult emergence over a period of six weeks at an application dose of 1 mg ai per m^2 and 97% at a dose of 5 mg ai per m^2 compared to emergence from untreated ponds (Table 3.3).

Table 3.3: Multivariable analyses (GEE) of factors affecting the emergence of adult malaria vectors over a six week period from ponds treated with Sumilarv®0.5G

Explanatory variable	Odds ratio (OR)	95% CI	p value
Treatment			
5 mg ai per m^2	0.03	0.02-0.04	<0.0001
1 mg ai per m^2	0.15	0.12-0.18	<0.0001
Control	1		
Round			
round 3	1.19	1.00-1.41	0.050
round 2	1.03	0.78-1.34	0.859
round 1	1		
Vector species			
An. arabiensis	0.95	0.86-1.05	0.278
An. gambiae s.s.	1		
Water turbidity			
turbid	1.01	0.95-1.07	0.765
Clear	1		
Water pH			
≥8	0.99	0.91-1.08	0.820
<8	1		
Rain during test week			
Rain	1.05	0.92-1.20	0.449
no rain	1		
Interaction between treatment and turbidity			
5 mg ai per m^2*turbid	1.93	1.12-3.26	0.017
5 mg ai per m^2*clear	1		
1 mg ai per m^2*turbid	1.4	1.08-1.79	0.011
1 mg ai per m^2*clear	1		
Interaction between treatment and pH			
5 mg ai per m^2*pH≥8	1.9	1.13-2.85	0.002
5 mg ai per m^2*pH<8	1		
1 mg ai per m^2*pH≥8	1.25	1.06-1.47	0.008
1 mg ai per m^2*pH<8	1		
Interaction between treatment and rain			
5 mg ai per m^2*rain	1.23	0.89-1.69	0.211

5 mg ai per m^2*no rain	1		
1 mg ai per m^2*rain	0.87	0.70-1.07	0.87
1 mg ai per m^2*no rain	1		

The overall impact of 5 mg ai per m^2 on inhibiting emergence was significantly higher than the impact of 1 mg ai per ^{2} ($p<0.001$). Despite consistent rainfall during the first round of the standardized field tests and occasional rainfall during the following two rounds (Figure 3.4), rain did neither affect the emergence of adults from control and treatment ponds nor the impact of the treatments (Table 3.3). There were also no main effects of water turbidity or pH on adult emergence but interactions were identified between the treatments and water turbidity, and the treatments and water pH. Turbid water and high pH reduced the impact of the treatments leading to slightly higher adult emergence from treatment ponds under these conditions (Table 3.3). The impact of the interactions can be calculated by multiplication of the odds ratios (Katz 2006). This means for example emergence inhibition was 85% at 1 mg ai per m^2 when ponds were clear and had a pH <8, emergence inhibition was reduced to 79% when the same treatment pond was turbid with a pH <8 and to 74% when the same treatment pond was turbid and had a pH ≥8. Similarly for the 5 mg ai per m^2 ponds in round one, overall emergence inhibition is 97% when treatment ponds are clear with pH <8, emergence inhibition is reduced to 95% when the treatment ponds are turbid with pH <8 and further reduced to 90% when the treatment ponds are turbid and with pH ≥8.
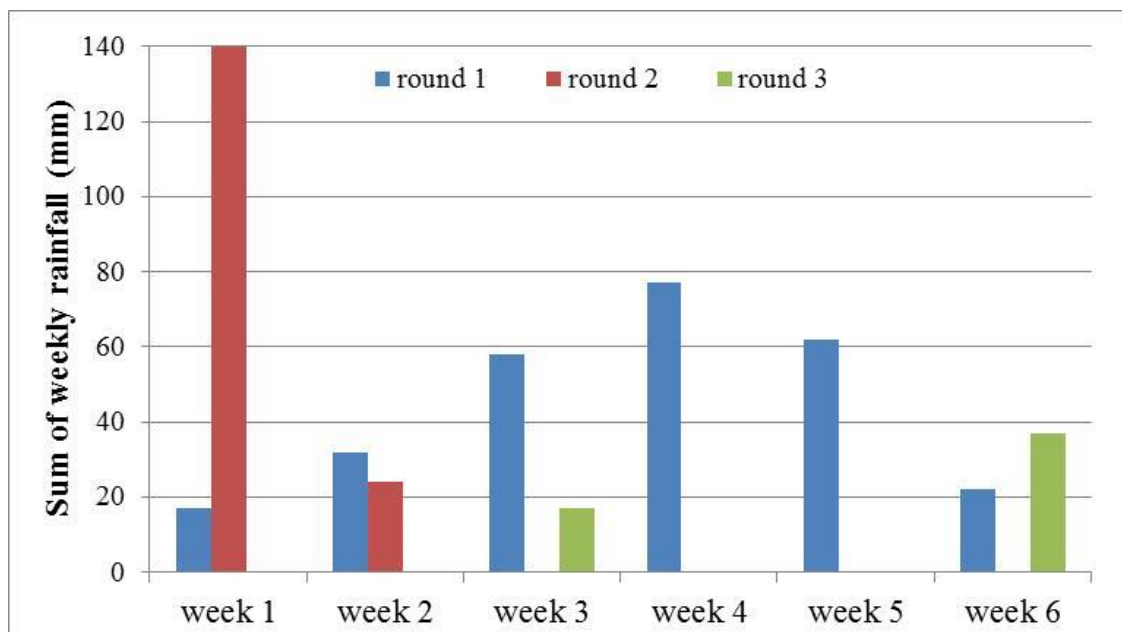
Figure 3.4: Weekly rainfall during the three rounds of standardized field tests.

3.4.3 Sub-lethal effects

The impact of sub-lethal effects could not be evaluated for *An. arabiensis* that emerged from pupae since neither females from untreated ponds nor females from treated ponds laid eggs, possibly due to unsuitable mating conditions provided for this species (Marchand 1985). Exposure of *An. gambiae s.s.* to both Sumilarv®0.5G dosages during the larval stage resulted in: (i) a reduced probability of the adult female laying eggs; (ii) reduced mean number of eggs laid per female; and, (iii) reduced mean number of eggs that hatched into larvae (Table 3.4). Treatment rounds were not significantly different (p=0.687), and data for all rounds for *An. gambiae s.s.* were pooled for analysis. Mosquitoes that emerged from treated ponds were 65-68% less likely to lay eggs compared to mosquitoes that emerged from untreated ponds. The mean number of eggs laid per female *An. gambiae s.s.* was reduced by 47% from females emerging from ponds treated at 1 mg ai per m^2 and by 74% from females emerged from ponds treated at 5 mg ai per m^2 compared to that in the untreated controls (Table 3.4). The impact of the higher dosage was twice the impact measured from the lower dosage (odds ratio (OR) 2.1, 95% CI 1.2-3.7, p=0.02). Furthermore, it was 90% less likely for an egg to hatch that was laid by a female exposed to the higher Sumilarv®0.5G dosage compared to eggs laid by females that emerged from low dosage ponds (OR=0.10, 95% CI 0.04-0.23, p<0.0001). The probability of an egg hatching was reduced by 77% for eggs laid by a female exposed to the lower treatment dosage and 98% for eggs laid by a female exposed to the higher dosage as compared to eggs in females that emerged from the untreated control ponds.

Table 3.4: Sub-lethal effects of Sumilarv® 0.5G on egg laying and hatching of *An. gambiae s.s.*

	control	1 mg ai per m^2	5 mg ai per m^2
Number of females exposed	60	60	30
Number of females that laid eggs	43	27	14
Mean eggs laid/female (95% CI)	43.8 (35.6-53.8)	23.1 (16.5-32.3)	11.2 (6.9-18.2)
Mean eggs hatched/female(95% CI)	37.4 (30.5-45.8)	8.7 (6.0-12.4)	0.8 (0.4-1.8)

3.5   Discussion

Anopheles arabiensis and *An. gambiae s.s.* were equally and highly susceptible to Sumilarv®0.5G under laboratory and standardized field conditions. Sumilarv®0.5G inhibited over 80% of the total adult emergence over a period of six weeks at both application dosages. However, weekly emergence rates increased steadily over the six-week test period at the lower dosage that corresponded with the EI_{99} in the laboratory and weekly emergence inhibition was frequently lower than the 80% that is recommended by WHOPES for successful immature control (WHO 2005a). Laboratory tests were conducted under standardised conditions without major abiotic and biotic influences and therefore EI values represent only minimum dosages. Application rates frequently have to be increased up to several times the minimum dose to obtain sufficient immature control under field conditions (Becker and Rettich 1994; WHO 2005a). The higher dosage of 5 mg ai per m^2 or 0.09ppm ai inhibited well over 80% of adult emergence in all but one test week. This dosage was 4.5 times the average EI_{99} in the laboratory. Further field tests to establish the optimum dose for operational control in a variety of different habitats are necessary but based on the results presented here it is likely that the optimum dosage lies between the two tested here and therefore coincides with the maximum dosage recommended by the manufacturer (0.05 ppm ai) for operational control of other mosquito species.

The estimated emergence inhibition rates from the dose-response tests were four times higher than those previously reported by Kawada *et al.*, (1993) for *An. gambiae,* but within the range of rates estimated for *Culex* and *Aedes* species (Hatakoshi *et al.* 1987; Ali *et al.* 1999; El-Shazly and Refaie 2002; Andrighetti *et al.* 2008; Al-Sarar *et al.* 2011). These differences may arise from the different PPF formulations used in separate studies (Kawada *et al.* 1988), but also from the material of the test containers (Vythilingam *et al.* 2005). Kawada *et al.* (1993) used a 5% emulsifiable concentrate formulation while in the present study a granular formulation was used and had to be crushed in a mortar for the laboratory tests, which might have not led to an equal amount of active ingredients being released into the stock solution. Also, in the present study plastic cups were used for bioassays while Kawada *et al.* (1993) used aluminium cups. There is a concern that the active ingredient PPF adheres to plastic (Caputo *et al.* 2012) leading to a longer residual effect from such treated containers due to a continuous slow release from the plastic

(Schaefer *et al.* 1991). In the short term however, plastic might reduce the amount of active ingredient in the water, which could be responsible for the higher estimates of EI concentrations found in this study. The extremely low concentrations of active ingredient needed for the control of mosquitoes with Sumilarv®0.5G is worth noting. The estimated effective dose of PPF is approximately 10 times lower than those reported for microbial larvicides (Fillinger *et al.* 2003; Majambere *et al.* 2007). This is not surprising since PPF is a juvenile hormone analogue, and insect hormones, like all hormones, operate at extremely low concentrations as chemical messengers(Ali *et al.* 1995; Al-Sarar *et al.* 2011). Thus, far smaller quantities of Sumilarv®0.5G would be required for larviciding programmes compared to microbial larvicides, thereby helping to lower costs associated with transporting and storing larvicides (Worrall and Fillinger 2011).

The residual impact of Sumilarv®0.5G on *An. gambiae s.l.* emergence observed here corresponds well with reports from previous studies on other mosquito species (Nayar *et al.* 2002; Vythilingam *et al.* 2005; Andrighetti *et al.* 2008) but application dosages required to achieve the same effect seem slightly higher for *An. gambiae s.l.* Sumilarv®0.5G at 0.02 ppm ai and 0.05 ppm ai provided almost complete emergence inhibition of *Ae. aegypti*, *Ae. albopictus* and *Ae. taeniorhynchus*, *Culex nigripalpus* and *An. quadrimaculatus* for six weeks under standardized field conditions (Nayar *et al.* 2002). This slow-release formulation has even been shown to exhibit prolonged residual activity for control of *Aedes* larvae even when the treatments were diluted by using replacement of treated water with untreated water in the treated containers (Itoh 1993; Vythilingam *et al.* 2005). Similarly, here we observed that rainfall did not negatively affect the impact of the treatments. Exceptional performance of Sumilarv®0.5G was reported for the control of *An. culicifacies* in confined gem pits in Sri Lanka (Yapabandara and Curtis 2002) where a single application of PPF at 0.01 ppm ai was sufficient to inhibit adult emergence for approximately six months. Similarly, Sihuincha *et al.* (2005) reported complete emergence inhibition of *Ae. aegypti* for five months from water tanks in Peru at an application rate of Sumilarv®0.5G of 0.05 ppm ai. Overall it can be concluded from previous work that the efficacy and residual activity of different PPF-containing products depends on the formulation, dose, habitat types treated, prevailing weather conditions and target mosquito species (Schaefer *et al.* 1988; Nayar *et al.* 2002; Andrighetti *et al.* 2008).

The current study showed that the efficacy of Sumilarv®0.5G is reduced in turbid water and water with a pH ≥8. Water is turbid because it carries a suspension of fine particles of both organic and inorganic matter in the water column. Some of the turbidity observed here might have been due to algae and bacteria growth in the established habitats, which in turn might have increased the water pH. It is possible that the active ingredient, PPF, is adsorbed onto particles in the water column and was less accessible to larvae. Turbidity and pH of aquatic habitats are important parameters that are associated with the abundance, development and survival of *Anopheles* larvae (Ye-Ebiyo *et al.* 2003). *Anopheles* larvae are known to exploit aquatic habitats with varying degrees of water turbidity and pH (Gimnig *et al.* 2001; Ye-Ebiyo *et al.* 2003). Suspended particles including algae in the water column in turbid ponds provide mosquitoes with food that enhances their development and survival thus increase emergence from turbid ponds (Gimnig *et al.* 2002; Kaufman *et al.* 2006). Mulligan and Schaefer (1990) found PPF to adsorb onto organic matter which might have been responsible for larvae to be exposed to reduced doses. This needs to be considered and monitored in field operations where it might be necessary to increase the application dose or reduce retreatment intervals to ensure a consistent emergence inhibition above 80% as recommended by WHOPES (WHO 2005a).

An added benefit to the direct effect of Sumilarv®0.5G on immature stages were the sub-lethal effects that affected the offspring of adult females that successfully emerged from treated ponds. At 5 mg ai per m^2 the reproduction of females was reduced by well over 90%. Similar effects of insect growth regulators have been shown for *Aedes* and *Culex* (Loh and Yap 1989; Mohsen and Zayia 1995; Kamal and Khater 2010). The laying of non-viable eggs by female *An. gambiae s.s.* emerging from treated ponds might further extend the efficacy and residual effect of PPF, and may help further reduce intervention costs by extending the retreatment intervals. It would be particularly helpful in the context of an auto-dissemination strategy (Gaugler *et al.* 2012) of Sumilarv®0.5G where potentially only sub-lethal doses are transferred to a habitat by female gravid mosquitoes. The delayed sub-lethal effects of insect growth regulators were also shown to affect the sex ratio and to reduce blood feeding rates in exposed mosquitoes (Loh and Yap 1989; Vasuki 1992). Similar effects were shown for adults exposed to PPF (Itoh *et al.* 1994; Sihuincha *et al.* 2005; Ohashi *et al.* 2012). Ohashi *et al.* (2012) demonstrated that *An. gambiae s.s.* was completely sterilized, with no female laying eggs after exposure to PPF-

treated nets. Insect growth regulators have been shown to suppress ovarian development and egg development in mosquitoes (Judson and de Lumen 1976; Fournet *et al.* 1993). Judson and de Lumen (1976) showed that exposure of *Ae. aegypti* females to juvenile hormone analogues suppressed egg development by inhibiting development of ovarian follicles. Fournet *et al.* (1993) similarly showed that the ovarian development of *Ae. aegypti* females that emerged from larvae exposed to insect growth regulators was affected.

As with every insecticide it is important to be cautious about using PPF formulations as a stand-alone intervention since tolerance to PPF has been found in dipterans (Crowder *et al.* 2008; Karatolos *et al.* 2012). It is also of concern to know whether the progeny of gravid females that are exposed to sub-lethal level doses of PPF and survive have greater tolerance to PPF than other mosquitoes. If this is the case, resistance may spread.

PPF exhibits favourable characteristics for utilization as a larvicide for mosquito control. The recommended application rate in drinking water limit of 300 ppb (0.3 ppm) (WHO 2008a) is several folds higher than the recommended dose of 0.01-0.05 ppm for mosquito control and also has minimal environmental impacts at recommended rates for mosquitoes (Mulla *et al.* 1986; Schaefer *et al.* 1988).

3.6   Conclusion

Anopheles arabiensis and *An. gambiae s.s.* are highly susceptible to Sumilarv®0.5G at very low dosages. The persistence of Sumilarv®0.5G in treated habitats under standardized field conditions and its sub-lethal impact, reducing the number of viable eggs from adults emerging from treated ponds, enhances its potential as a malaria vector control tool in integrated vector management strategies. These unique properties of Sumilarv®0.5G warrant further field testing in a range of natural *An. gambiae s.l.* larval habitats and under operational conditions to recommend if and how this insect growth regulator could be included in vector control programmes for malaria control in sub-Saharan Africa.

Based on the results of this study the maximum dosage recommended by the manufacturer for other mosquito species of 0.05 ppm ai is recommended as the minimum

dosage for further field testing for *An. gambiae s.l.* control. Although the residual effect observed for the test concentrations lasted for a six-week period, initially a shorter retreatment interval should be evaluated under natural conditions where habitat types and water quality are highly heterogeneous and might affect the residual activity. Furthermore, the estimation of retreatment intervals should also consider the probability of new habitats emerging during treatment cycles that could then harbour mosquito larvae that might successfully emerge before the target area receives another round of Sumilarv®0.5G application. Initial application cycles should be determined for the predominant habitat type in the target area, the season of application and the development time of immature vectors. In areas where temporary habitats dominate or areas with high rainfall an initial application cycle of two to three weeks should be tested whilst in areas of more semi-permanent to permanent habitats or during dry seasons a three to four-weekly application cycle might be appropriate for an initial field operation informed by a monitoring and evaluation programme.

4 Operational field evaluation of the efficacy of slow release pyriproxyfen granules (Sumilarv®0.5G) for the control of immature stages of malaria vectors in the western Kenya highlands

Oscar Mbare, Bryson Ndenga, Baldwyn Torto, Steven W. Lindsay, Ulrike Fillinger

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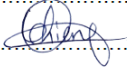
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4.1   Abstract

Background: A major constraint of larviciding programmes for malaria control in sub-Saharan Africa is the high cost of frequent application. The objective of this study was to evaluate the efficacy of a persistent insect growth regulator (Sumilarv®0.5G; active ingredient pyriproxyfen) for the control of immature stages of malaria vectors under operational field conditions in the western Kenya highlands.

Methods: Six study sites were randomly assigned to non-intervention and intervention arms and Sumilarv®0.5G applied 1 g per m^2 water surface area for one year to all aquatic habitats in the intervention arm at three-weekly intervals. All habitats were surveyed weekly for the presence of mosquito immature stages. Ten sentinel habitats randomly selected in each study site were surveyed weekly for mosquito immature abundance and co-habiting organisms and environmental characteristics. The impact of Sumilarv®0.5G on adult productivity was directly assessed through collection of late instar *Anopheles* larvae and pupae from habitats and water collections seeded with insectary-reared larvae. The persistence of Sumilarv®0.5G in treated aquatic habitats that temporarily fell dry was investigated. Adult malaria vector productivity of untreated aquatic habitats created between successive Sumilarv®0.5G application rounds was assessed.

Results: Sumilarv®0.5G was highly effective in the control of mosquito larvae and pupae when applied in these highland sites in three-weekly intervals. The chance of finding an aquatic habitat colonized by *Anopheles* larvae was reduced 5-7 fold in intervention sites compared with non-intervention sites in both low and high transmission seasons. Similarly, the abundance of late instar *Anopheles* was reduced 10 fold in intervention sites during the high transmission season. Less than 20% of pupae collected from intervention sites developed into adult vectors during both the low and high transmission seasons. Over 80% emergence inhibition of *Anopheles* larvae collected from habitats that temporarily fell dry for 56 days was recorded. Collection of Sumilarv®0.5G-treated water samples from aquatic habitats for exposure of insectary-reared larvae in the laboratory was less effective in estimating treatment effect than the collection of late instar larvae and pupae. The three-week re-application of Sumilarv®0.5G was short

enough to prevent the successful completion of larval development into adults in new aquatic habitats created in-between Sumilarv®0.5G application round.

Conclusion: Sumilarv®.5G demonstrates effective control of mosquito immatures for inclusion into integrated malaria control strategies. Use of water samples collected from treated aquatic habitats underestimates the impact of Sumilarv®0.5G and is not recommended as a monitoring tool.

4.2   Background

The recent declines in malaria transmission in sub-Saharan Africa is largely attributed to the improved access to rapid diagnosis and prompt treatment of clinical cases supported by the scaling up of vector control interventions namely long-lasting insecticidal nets (LLINs) and indoor residual spraying (WHO 2014c). The residual malaria transmission maintained by exophagic and exophilic vectors less exposed to these indoor interventions continues to raise concern over the overdependence on these indoor vector control tools (Killeen 2014)

Today, malaria control programmes are encouraged to adopt integrated vector management measures that emphasize utilization of multiple tools in appropriate settings with an aim of increasing efficiency and cost-effectiveness (WHO 2004). Larval source management (LSM) that aims to minimize adult mosquito propagation by managing aquatic habitats to minimize adult vector production can be effective for malaria transmission under certain eco-epidemiological settings (Utzinger *et al.* 2001; Keiser *et al.* 2005b; Fillinger *et al.* 2009a; Tusting *et al.* 2013; WHO 2013b). Larviciding for malaria vector control in sub-Saharan Africa is recommended as a supplementary method to frontline vector control measures in areas where aquatic habitats are few, fixed and findable (WHO 2013b). Such conditions exist in the western Kenya highlands where aquatic habitats are well defined and accessible as most habitats are concentrated on the valley bottom (Minakawa *et al.* 2005; Omukunda *et al.* 2012). Thus malaria epidemiology in the western Kenya highlands is stratified and focal with greater prevalence of the disease in populations residing at the valley bottoms while populations living uphill are less likely to be infected (Githeko *et al.* 2006; Atieli *et al.* 2011; Afrane

et al. 2014). A recent trial in these highlands found that weekly larviciding with microbials provided additional protection against *Plasmodium* infections than that achieved by using insecticide-treated nets alone (Fillinger *et al.* 2009a). However although the cost of larviciding with microbials is similar to that for indoor residual spraying (Worrall and Fillinger 2011), reducing the frequency of application would increase the cost-effectiveness of larviciding programmes (Yapabandara and Curtis 2002; Fillinger and Lindsay 2011). Field studies in different eco-epidemiological settings have shown pyriproxyfen (PPF) , an insect growth regulator, to be highly effective in the control of mosquitoes providing up to 6 months residual activity (Kamimura and Arakawa 1991; Chavasse *et al.* 1995a; Yapabandara and Curtis 2002; Sihuincha *et al.* 2005). Surprisingly, PPF has not been rigorously evaluated in the field for the control of Afrotropical malaria vectors.

Our recent trials carried out under controlled field condition showed that Sumilarv®0.5G, a granular formulation of PPF, could control the major Afrotropical malaria vectors for up to six weeks (Mbare *et al.* 2013). However, a potential challenge in the use of persistent larvicides is the potential of adult vector production from untreated habitats newly created before the next larvicide application round, especially during periods of rain. In addition monitoring the impact of PPF is challenging since the insecticide does not produce acute toxic effect on mosquito larvae but has delayed effects in preventing adult emergence from exposed pupae (Invest and Lucas 2008). Thus the impact of PPF has been variably evaluated by assessing adult emergence of field collected mosquito larvae and pupae (Suzuki *et al.* 1989; Kamimura and Arakawa 1991), larvae exposed to water samples collected from the field (Yapabandara and Curtis 2002) or insectary-reared larvae exposed directly in treated aquatic habitats in the field (Mulla *et al.* 1974; Yapabandara *et al.* 2001).

This study aimed to evaluate whether a three-weekly application of Sumilarv®0.5G (granular PPF formulation) to aquatic habitats in western Kenya highlands was effective in inhibiting emergence of adult malaria vectors. The specific objectives of the study were to determine: (1) adult mosquito emergence inhibition of larvae in treated aquatic habitats; (2) best monitoring tool to assess impact of PPF; (3) effect of PPF on non-target aquatic organisms; (4) persistence of PPF in treated habitats during dry periods; and (5)

risk of adult vector production from untreated habitats created between successive Sumilarv®0.5G application rounds.

4.3   Methods

4.3.1   Study area

This study was conducted in the western Kenya highlands along the Luanda-Siaya, Luanda-Majengo and Luanda-Busia roads in Vihiga County. Six study sites were selected for this study: Ebulako, Mudabala, Muluhoro, Ivona, Mugogo and Inavi (Figure 4.1, Table 4.1).
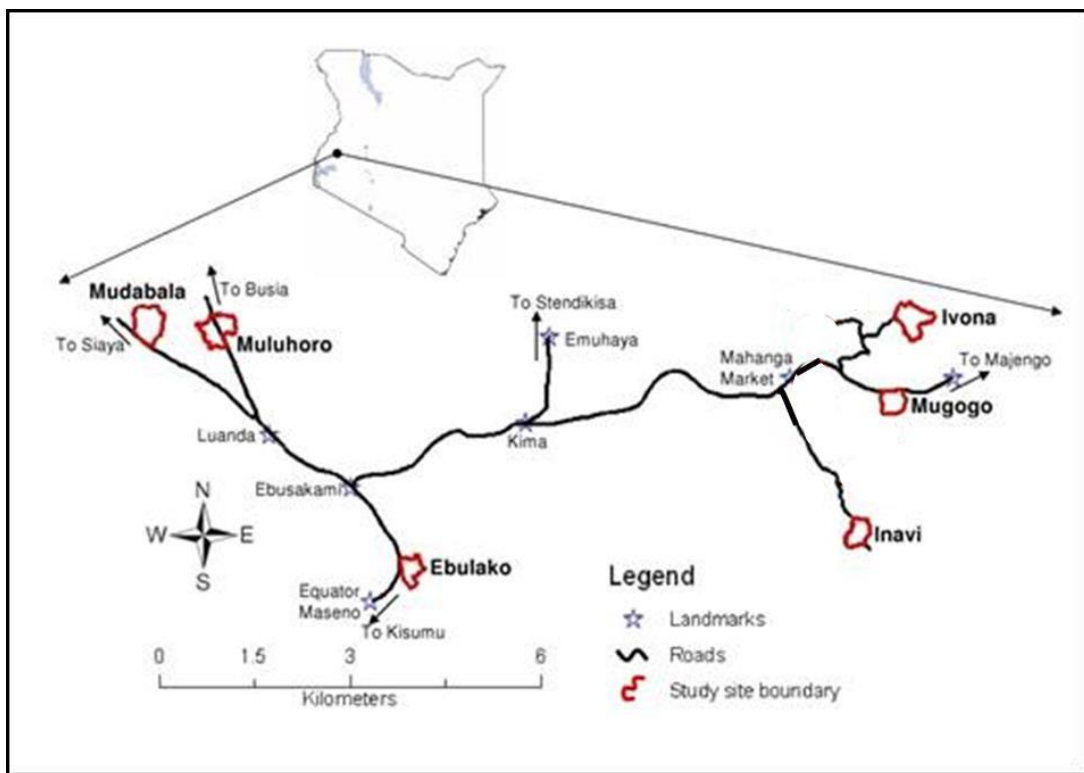
Figure 4.1: Location of the six study sites: Ebulako, Mudabala, Muluhoro, Ivona. Mugogo and Inavi. Inset Kenya.

Each study site was 12-22 hectares (ha) in area and they were at least 1 km apart. The study sites were all valleys characterized by undulating topography with steep and gently sloping hills. The valleys were relatively defined, surrounded on all sides by increased elevation to minimize risk of vectors invading from outside areas. Most aquatic habitats

were on the valley bottom. Site characteristics including coordinates and altitude are given in Table 4.1.

Table 4.1: Characteristics of the study sites

Site	Treatment arm	Longitude	Latitude	Altitude (meters above sea level)	Area (ha)	No. of aquatic habitats (habitats per ha)
Ebulako	non-intervention	36.67880	0.5790	1527-1567	12 ha	142 (12/ha)
Muluhoro	non-intervention	36.67563	0.4260	1448-1490	19 ha	205 (11/ha)
Inavi	non-intervention	36.68597	0.1177	1615-1666	12 ha	141 (12/ha)
Mudabala	Intervention	36.67477	0.4449	1484-1520	22 ha	193 (9/ha)
Ivona	Intervention	36.68677	0.4371	1533-1567	20 ha	196 (10/ha)
Mugogo	Intervention	36.68652	0.3029	1560-1601	13 ha	93 (7/ha)

The area is characterized by tropical climate with a mean annual daily temperature of 20.8° C and average annual rainfall of approximately 2000 mm and two rainy seasons; the long rains between March and June and the short rainy season between October and November (Munyekenye *et al.* 2005; Ndenga *et al.* 2006; Afrane *et al.* 2008). The area experiences seasonal malaria transmission with a peak in transmission during and immediately after the long rains when habitats fill with water as other habitats are created by humans for use in agriculture. The peak in malaria transmission occurs between March and June while the remainder of the year is considered a low transmission season (Fillinger *et al.* 2009a). The area is densely populated with subsistence farming of crops such as maize, napier grass, cassavas and bananas being the major economic activity. The dominant vectors of malaria in the study area are *Anopheles gambiae sensu stricto*, *An. arabiensis* and *An. funestus* (Ndenga *et al.* 2006; Zhou *et al.* 2011; Omukunda *et al.* 2013).

4.3.2   Mapping and surveying of larval habitats

Weekly visits were made between June 2011 and December 2013 to each study site to survey aquatic habitats within the site boundaries which were given unique identification numbers (Figure 4.2) and their locations recorded using a Global Positioning System (GPS) unit (Garmin Ltd. 2003, Olathe, Kansas, USA).

Figure 4.2: Example of an identification number of aquatic habitat (112)

A local field assistant familiar with each study site was involved in the identification of aquatic habitats within the study sites. During the weekly visits any new habitat not previously mapped was included in the list of habitats and assigned identification number following the last number given during the previous visit.

Figure 4.3 shows typical aquatic habitats encountered in the study sites which are described as follows: (1) natural swamps are water-saturated sites covered with vegetation and not used for agriculture, (2) cultivated swamps are water-saturated sites on which crops were grown, (3) river fringes are protected slow flowing or still water on the edges of a river, (4) puddle are transient collections of water that mostly occurred after the rains, (5) drains are interconnected channels of water which are often constructed for agricultural purposes and (7) burrow pits are large holes where the soil has been removed for brick making or creation of fish ponds.

Habitats found without water during the weekly visits were recorded as dry. The length of habitats with water was estimated and categorized as <10 m, 10-100 m or >100 m. The depth of habitat was measured using a metre rule and categorized as below or above 0.5 m. The type of vegetation found in a habitat was recorded as floating, submerged or emergent while the proportion of aquatic habitat surface area covered by vegetation, biofilm and filamentous algae was visually estimated and recorded. The flow of water in an aquatic habitat was recorded as stagnant, slow or fast. A rain gauge (Comptus Beta) was used to measure daily rainfall in each site.

Figure 4.3: Habitats found in the study sites. A-natural swamp, B-cultivated swamp, C-river fringe, D-puddle, E-drain, F-burrow pit

4.3.2.1 Mosquito positivity rate of all aquatic habitats per site

This activity was carried out weekly in all aquatic habitats in the study sites. A standard 350 ml capacity mosquito dipper (Clarke Corporation, Illinois, USA) (Figure 4.4 A) was used to sample habitats with water for the presence or absence of mosquito larvae and pupae (Service 1971). Habitats that were less than 1 m in their longest length had the entire water surface sampled. Habitats whose lengths exceeded 1 m had at least 10 dips taken from where it is most likely to find mosquito larvae (e.g along edges of habitats with tufts of grass and low vegetation) (Fillinger *et al.* 2004). At least one dip was taken for each metre length of water surface. A habitat was considered positive for a given mosquito species it had at least one of the species sampled in any of the dips taken. Larvae sampled in a dipper were first classified as *Anopheles* and culicines based on crude morphological criteria (Rozendaal 1997). *Anopheles* and culicines were further

classified as early (first and second) and late (third and fourth) instars based on size. Pupae were not identified to genus level due to the difficulty to distinguish them under field conditions (Fillinger *et al.* 2004; Fillinger *et al.* 2008). All organisms were returned to the water.

4.3.2.2 Mosquito immature abundance in sentinel aquatic habitats

This activity was carried out in 10 sentinel aquatic habitats randomly selected using a computer generated random number list in each of the six study sites. The 10 sentinel habitats in each study site were selected at the start of the study from 23 aquatic habitats in Ebulako, 144 aquatic habitats in Mudabala, 80 aquatic habitats in Muluhoro, 141 aquatic habitats in Ivona, 103 aquatic habitats in Mugogo and 115 aquatic habitats in Inavi. A sentinel habitat was substituted with another aquatic habitat nearby when it fell dry. The substitution was done 10 times in Ebulako, 23 times in Mudabala, 8 times in Muluhoro, 14 times in Ivona and 7 times in Mugogo. Sentinel habitats in Inavi were not substituted as they remained wet throughout the study period. During the weekly visits the sentinel habitats were surveyed to measure for larval abundance per surface area. Sweep nets were used for sampling due to their greater efficiency in collecting mosquito larvae and pupae as compared to dipping (Robert *et al.* 2002). The sweep net was made of cotton material mounted on a circular metallic frame (length=0.4 m, width=0.2 m, height =0.3 m) and attached to a handle made of a metal rod (length=1.5 m) (Figure 4.4 B). Aquatic habitats that were less than 20 m long (irrespective of their width) had their entire water surface area sampled with a sweep net while habitats that were > 20 m long were sampled for a maximum length of 20 m of the water surface area. Approximately 1 m^2 of water surface area was swept with one sweep. Large habitats had only the edges sampled within 1 meter from the edge as mosquito larvae and pupae often aggregate in such areas (Fillinger *et al.* 2004; Fillinger and Lindsay 2006). The net was gently drawn through the water until it became filled with water. The water and all its contents was then emptied into a large white basin to ensure visibility of large organisms during counting. The number of mosquito larvae and pupae and other aquatic organisms collected such as insects of the orders odonata, coleoptera and hemipteran were recorded. Late instar *Anopheles* larvae and pupae sampled in a habitat were transferred into separate 200 ml glass jars half filled with water from the respective habitat. The jars were labelled with habitat identification number and the number of immature mosquitoes in the jar. The top of the jar was loosely tightened with the lid and transported in a cool box to the

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insectaries at Kenya Medical Research Institute (KEMRI) at Kisian, Kisumu County (-0°4'40"N   34°40'38"E). After counting the other organisms were returned to the habitats. To avoid contamination jars containing larvae and pupae from non-intervention and intervention study sites were transported in separate boxes.

Figure 4.4: Tools to sample mosquito larvae and pupae. (A) Mosquito dipper and (B) sweep net

4.3.3   Insecticide

Sumilarv®0.5G was provided by the manufacturer, Sumitomo Chemical, Japan. It is a granular formulation containing 0.5% active ingredient (ai) (weight: weight). The active ingredient in Sumilarv®0.5G is pyriproxyfen (PPF).

4.3.4   Random allocation of study sites into non-intervention and intervention study sites

Before the intervention period started in December 2011 the study sites were randomly assigned to non-intervention and intervention arms of the study by lottery. The sites assigned to the non-intervention arm were Ebulako, Muluhoro and Inavi while Mudabala, Ivona and Mugogo were assigned to the intervention arm of the study (Table 4.1).

4.3.5   Application of insecticide

Sumilarv®0.5G was applied by hand broadcasting to all aquatic habitats within the boundaries of the three sites assigned to the intervention arm of the study from December

2011 until December 2012 (intervention year) (Figure 4.5). Based on the findings from standardized field tests (Mbare *et al.* 2013) Sumilarv®0.5G was applied to the habitats at 1 g per m^2 assuming a 10 cm depth of water every three weeks. Thus application of the insecticide was based on the surface area of water in a habitat. Sumilarv®0.5G was applied under fully operational conditions. This means water depths and the size of the habitats were not measured prior to application. The personnel applying Sumilarv®0.5G received training prior to field application to ensure that the correct quantity was applied. For training purposes a 5 m x 2 m plastic sheet was divided into ten parts, each with a surface area of 1 m^2. One gram of Sumilarv®0.5G was weighed and every person given the opportunity to equally spread it over a 1 m^2 area. The spread material was then collected for weighing. This was repeated until all personnel were able to estimate accurately 1 g of Sumilarv®0.5G granules for application on 1m^2 surface area.

Figure 4.5: Application of Sumilarv®0.5G into aquatic habitats in intervention arm by hand broadcasting

4.3.6   Investigating the vector productivity of aquatic habitats in the intervention period

During the intervention period aquatic habitats were visited on day 6, 12 and 19 after Sumilarv®0.5G application into aquatic habitats in the intervention sites. This

corresponds to 1, 2 and 3 weeks respectively after application of the insecticide to aquatic habitats in the intervention sites.

Since the impact of PPF cannot be directly assessed by dipping for larval presence or use of sweep nets to assess for larval and pupal abundance due to its lack of acute toxic effects (Invest and Lucas 2008), three methods were tested to assess adult emergence of exposed larvae.

4.3.7 Late immature collections from sentinel aquatic habitats to assess for adult emergence

Late instar *Anopheles* larvae and mosquito pupae were sampled weekly from sentinel aquatic habitats and monitored in an insectary to see if they developed into adults or not. Larvae were fed daily on fish food (Tetramin©Baby) using a dipstick. In the insectary the lid of the jars were replaced by mosquito netting to cover the top of jars so as to prevent escape of any emerged adult (Figure 4.6). To avoid contamination larvae and pupae from non-intervention and intervention sites were maintained on separate benches. On a daily basis the number of emerged adults on each jar was counted. If any emerged adult was found it was aspirated into a 1.5 ml eppendorf tube (greiner bio-one) and left to die.

Figure 4.6: Laboratory evaluation of emergence of larvae and pupae collected from aquatic habitats in the field

Adult mosquitoes that emerged were morphologically identified to genus level using the keys developed by Gillies and Coetzee (Gillies and Coetzee 1987). *Anopheles gambiae sensu lato* were further identified to species level as *An. gambiae s.s.* and *An. arabiensis* using a ribosomal (Deoxyribonucleic nucleic acid) DNA Polymerase Chain Reaction

(PCR) method (Scott *et al.* 1993). The legs of the mosquitoes were used as templates in the PCR. Positive controls for *An. gambiae s.s.* and *An. arabiensis* obtained from the mosquito colony in the insectary were used in each PCR run. The PCR master mix was prepared by adding universal forward primer (10pmol/µl) (Eurofins MWG Operon), *An. gambiae s.s.* reverse primer 10pmol/µl) (Eurofins MWG Operon), *An. arabiensis* reverse primer (10pmol/µl) (Eurofins MWG Operon) to nuclease-free water (Sigma Aldrich) in a 1.5 ml eppendorf tube. Table 4.2 shows the volumes of each substance added to the eppendorf tube for master mix preparation. The volumes of each substance to be added in the master mix were calculated by multiplying by the number of samples to be identified.

Table 4.2: Substances used to prepare PCR master mix where n refers to the number of samples to be identified

Substance	nx1 (µl)
Nuclease-free water	23.2
Universal forward primer	0.6
Reverse primer GA (*Anopheles gambiae s.s.*)	0.6
Reverse primer AR (*Anopheles arabiensis*)	0.6

The PCR master mix was aliquoted into 200 µl PCR tubes with beads (PuReTaq^{TM} Ready-To-Go^{TM} PCR Beads in a plate, x 96 reactions, GE Healthcare, UK). The legs of the mosquitoes were put in PCR tubes as template while in the negative control tube nuclease-free water was added. A single leg was taken from each mosquito and inserted into individual PCR tubes that were labelled with the mosquito identification number. The PCR reaction was performed in a thermo cycler (Techne). The thermo cycler programme is presented in Table 4.3.

Table 4.3: PCR programme on thermo cycler

Programme	Adjusted value
Heated lid	105 ºC
Pre-heat lid	Off
Initial denaturation	94 ºC for 5 minutes
No. of cycles	30
Denaturation	94 ºC for 30 seconds
Annealing	50 ºC for 30 seconds
Extension	72 ºC for 30 seconds
Final extension	72 ºC for 30 seconds
Hold	10 ºC

Gel electrophoresis was used to separate the PCR products according to size. The electrophoresis was run on 1.5% gel to which the chemical ethidium bromide (EtBr) (Sigma Aldrich) was added to aid in visualizing DNA molecule under ultraviolet (UV) light. The electrophoresis was run for 45 minutes in Tris Acetate-EDTA (TAE) buffer solution (Sigma Aldrich). Later the gel was observed and visualized under UV light in a gel documentation system (Syngene In Genius Bio Imaging). The different DNA fragments were identified based on their sizes; the DNA fragment of *An. arabiensis* is 315 base pairs while that of *An. gambiae s.s.* is 390 base pairs.

4.3.7.1 Water collections from randomly selected aquatic habitats

Weekly collection of water samples were made from 10 randomly selected aquatic habitats (excluding sentinel habitats) per study site for exposing insectary-reared third instar larvae to the water in the laboratory and adult emergence observed. In each weekly visit a computer generated random number list was used to randomly select new batches of aquatic habitats for collection of water samples. The objective here was to: (1) compare this method to the immature collection method; and (2) monitor the efficacy of the intervention in randomly selected habitats ensuring that a large variety of habitats were surveyed over time and that personnel charged with the responsibility of Sumilarv®0.5G application could not predict where water samples might be taken. The sentinel habitats were known to personnel and might have been treated more rigorously than other habitats. Approximately 150 ml of water was collected from each habitat into 200 ml glass jars and transported to the laboratories at KEMRI. Thus each week 60 water samples were collected from aquatic habitats. In the laboratory 10 insectary-reared third

instar *An. gambiae s.s.* larvae (Kisumu strain) were introduced and monitored daily for emergence. Larvae were fed on fish food and monitored for emergence/emergence inhibition as described above.

4.3.7.2 Floating cup method

Comparing results from the immature collection method and water collection method four months into the intervention year it became apparent that the two methods differed widely in their efficacy estimate of the intervention. Therefore, a small study was designed to compare three methods at the same time in the same habitats. Ten aquatic habitats (excluding sentinel habitats) were randomly selected from a non-intervention site (Ebulako) and an intervention site (Mudabala). Vector productivity of habitats were compared by: (1) collection of late instar *Anopheles* larvae and pupae in their habitat water to monitor emergence in the laboratory; (2) introduction of insectary-reared late instar *An. gambiae s.s.* larvae into water samples collected from the aquatic habitats in the field; and (3) the use of floating cups for direct exposure of insectary-reared third instar *An. gambiae s.s.* to treated water in the aquatic habitats. The floating cups were modified after Mulla *et al.* (1974). Briefly, the floating cup was made of a stainless steel cup (500 ml) whose open top and bottom were covered with fine mosquito netting to prevent predators and other mosquito larvae from entering the cup whilst at the same time allowing water exchange from the habitat into the cup. The cup was inserted in a Styrofoam ring to float. To avoid the floating cup from being washed away it was tethered to vegetation at the edge of the habitat (Figure 4.7). One floating cup was placed in each of the 10 water bodies in each site. Ten insectary-reared third instar *An. gambiae s.s.* larvae were introduced into each of them. Larvae were introduced into the cups in the aquatic habitats 6, 12 and 19 days after Sumilarv®0.5G application. The cups were observed daily for any emerged adult. It took approximately 5-6 days for late instar larvae introduced into the cups to die or successfully emerge as adults. Emerged adults were aspirated and transferred into a paper cup with a lid where they were left to die.

These tests were carried out on three separate Sumilarv®0.5G application rounds. During each Sumilarv®0.5G application round a new batch of aquatic habitats to carry out the tests were selected.

Figure 4.7: Floating cup for exposure of insectary-reared larvae directly in aquatic habitats

4.3.7.3 Persistence of Sumilarv®0.5G in habitats over dry periods

This activity was done only in sites assigned to the intervention arm of the study. The aim here was to assess the persistence of Sumilarv®0.5G in treated habitats that refilled with water after a dry period. Aquatic habitats located in sites assigned to the intervention arm were monitored during the weekly visits to determine when they fell dry. When any of the habitats fell dry, the date on which it was first found dry was recorded. The habitats were then monitored weekly to ascertain when they refilled with water. When the habitats refilled with water the following were done: (1) sampling with sweep net to collect mosquito larvae and pupae to observe for adult emergence in the laboratory; and (2) collection of water samples for introduction of insectary reared *An. gambiae s.s.* larvae to assess for adult emergence as described above.

4.3.7.4 Risk of vector production from untreated habitats

This activity was done only in sites assigned to the intervention arm of the study. Here, the aim was to assess whether the three-weekly application of Sumilarv®0.5G was short enough to prevent adult vector production from untreated aquatic habitats newly created in-between successive application cycles. During the weekly surveys, sites in the intervention arm of the study were monitored for any new aquatic habitat that were

created or filled up with water between any two successive Sumilarv®0.5G application cycles. If a new aquatic habitat was found it was mapped and given a unique identification number. Sumilarv®0.5G was not applied to the new habitat until the next application cycle when all aquatic habitats in the intervention sites were treated. During the period when the new aquatic habitats remained untreated sampling was done with a sweep net as described above. Late instar *Anopheles* larvae and pupae were collected and observed for emergence in the laboratory as already described.

4.3.7.5 Liquid chromatography-mass spectrometry (LC-MS) analysis of treated water samples

In order to determine whether PPF could be detected in treated habitats, water samples were taken and a method developed to analyse these samples by liquid chromatography-mass spectrometry using electron spray ionization (LC/EIS-MS). Water samples were collected from randomly selected aquatic habitats (excluding sentinel aquatic habitats) on days 6, 12 and 19 after Sumilarv®0.5G application from aquatic habitats in a non-intervention site (Ebulako) and an intervention site (Mudabala). Ten aquatic habitats were selected from intervention site and five habitats from the non-intervention site. On each weekly visit 10 water samples from intervention site and five samples from non-intervention sites were collected. Thus during the three-week survey period a total of 45 water samples were collected. Water samples were collected as close as possible from the bottom of the aquatic habitats and emptied into 1 L capacity glass jars. The water samples were transported within 24 hours in a cool box to the laboratories at International Centre of Insect Physiology and Ecology (icipe)-Nairobi. Water samples collected from non-intervention and intervention study sites were transported in separate cool boxes to avoid contamination.

In the laboratory, 500 ml of water samples were extracted in chloroform (Sigma Aldrich) to separate the aqueous and organic layers. The organic layer where PPF was expected to dissolve was concentrated by evaporating it to dryness in a rotary evaporator (HEIDOLPH INSTRUMENTS, Germany). The residue was dissolved in1 ml methanol (Sigma Aldrich) and stored at 4°C. To assist in quantification of PPF a known concentration (0.00002 µg) of 4-benzyliphenyl (Sigma Aldrich) was added into each extracted water sample as an internal standard just before the LC/EIS-MS run. First the

standards of pure Sumilarv®0.5G and 4-benzylbiphenyl were initially run separately in the LC-MS system to confirm the retention times of PPF and the internal standard. The peaks of PPF and 4-benzylbiphenyl at the retention times were identified based on the molecular masses of their individual ions (molecular masses of pyriproxyfen-322 and 4-benzylbiphenyl-247).

The LC/ESI-MS used consisted of a quaternary LC pump (Model 1200) coupled to Agilent MSD 6120-Single quadruple MS with electrospray source (Palo Alto, CA). The mass spectrometry component of the system was used to verify the peak assigned to pyriproxyfen or 4-benzylbiphenyl as the active ingredients based on their identification on molecular masses of the ions. The system was controlled using ChemStation software (Hewlett-Packard). Reverse-phase liquid chromatography was performed using an Agilent Technologies 1200 infinite series LC, equipped with a Zorbax Eclipse Plus C_{18} column, 4.6 x 100 mm x 3.5 µm (Phenomenex, Torrance, CA). The following gradient using A (5% formic acid in LC-grade ultra pure H_2O) and B (LC-grade methanol) (Sigma, St. Louis, MO) was used; 0-5 min, 95-100% B; 5-10 min, 100% B; 100-5 min. The mobile phase liquid was acetonitrile (Sigma Aldrich). The flow rate was held constant at 0.7 mL min^{-1}. The sample injection volume was 100 µl, and data were acquired in a full-scan positive-ion mode using a 100 to 500 m/z scan range. The dwell time for each ion was 50 ms. Other parameters of the mass spectrometer were as follows: capillary voltage, 3.0 kV; cone voltage, 70 V; extract voltage, 5 V; RF voltage, 0.5 V; source temperature, 110ºC; nitrogen gas temperature for desolvation, 350ºC; and nitrogen gas flow for desolvation, 400 L/h.

4.3.8 Data Analysis

All data were analysed using generalized linear models with R statistical software version 2.14.2. Generalized estimating equations (GEE) fitted to a Poisson distribution, with a log-link function and an exchangeable correlation matrix were used to analyse data on abundance of immature mosquitoes in sentinel aquatic habitats. Since habitats were visited weekly, the unique habitat identification (ID) number was included in the GEE model as the repeated measure. GEE models were also used to analyse data on the proportion of aquatic habitats that contained mosquito larvae and pupae and proportion of larvae and pupae collected from habitats or introduced into water samples collected from

habitats that emerged into adults. Here the model was fitted to a binomial distribution, logit function and exchangeable correlation matrix. The habitat ID was included in the model as the repeated measure. The treatment arm (intervention, non-intervention sites) and malaria transmission season (high, low) and in some instances the week of monitoring after Sumilarv®0.5G application were included in the models as fixed factors. The non-intervention arm of the study was always used as reference. Interaction of terms between treatment arm and weeks were included in some models. All presented means and their 95% confidence intervals were calculated as the exponential of the parameter estimates for models with no intercept included. Generalized linear models fitted to binomial distribution were used to compare the proportion of larvae or pupae collected from non-intervention and intervention sites that successfully emerged as adults. Here the treatment arm (non-intervention, intervention sites) was included as fixed factor. The parameter estimates of the models were used to predict the mean proportions per intervention group and their 95% confidence intervals (CIs) by removing the intercept from the model. Multiple comparisons between intervention groups were also calculated based on the parameter estimate of the models.

4.3.9   Ethical approval

Ethical approval for the study was obtained from KEMRI/National Ethics Review Committee (SSC Protocol no. 1963). Authority to import Sumilarv®0.5G for the study research was obtained from the Pests Control Products Board (PCPB/I11/REG/VOL.1/11/22). Verbal informed consent was sought from farmers and the local administrators and residents to sample aquatic habitats for mosquitoes and to apply larvicides.

4.4   Results

4.4.1   Characteristics of study sites

A total of 970 aquatic habitats were mapped in all the study sites during the study period (Table 4.1). Habitat density per hectare was similar in most sites except Mugogo which had the fewest habitats (Table 4.1). Most aquatic habitats were man-made associated with agricultural activity. Most habitats were drains in agricultural fields (78%), followed by

burrow pits (12%), cultivated swamps (4%), puddles (3%), natural swamps (2%) and river fringes (1%). Puddles were the most temporary water bodies that only appeared following rainfall. Habitats in the western Kenya highlands were characterized by their permanence. At any sampling date on average 87% (95% CI 85-90%) of the habitats contained water. However there was a pronounced dry season between December 2011 and March 2012 when an average of 68% (95% CI 56-81%) of habitats had water per week; this increased to 88% (95% CI 86-90%) during the remaining time (Figure 4.8). The greatest reduction in wet habitats during the dry period (December 2011 - March 2012) was recorded in Muluhoro (site with greatest number of habitats) where a 29% decline in habitats with water occurred.
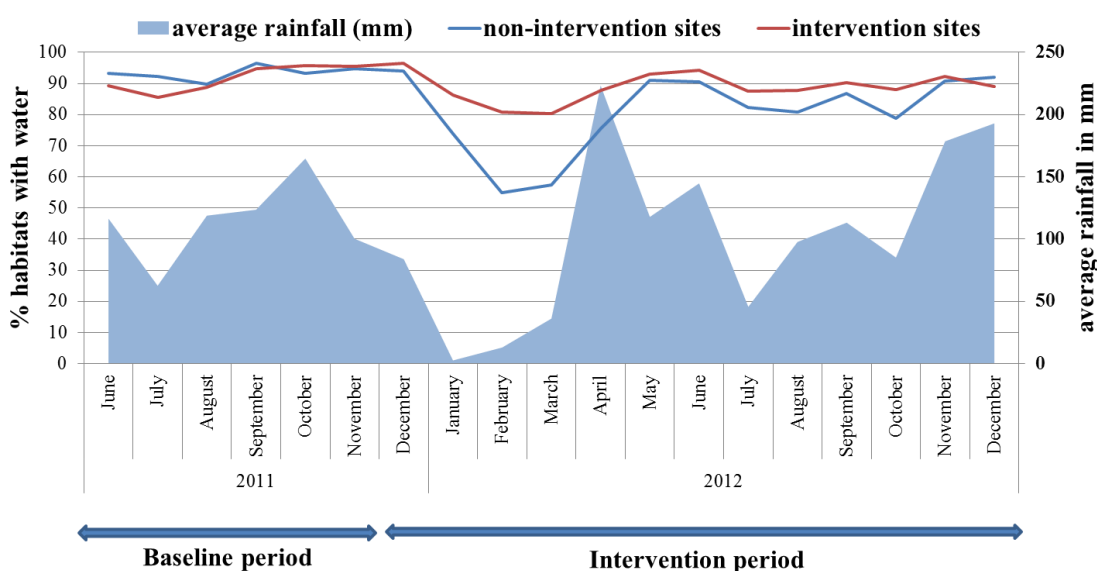
Figure 4.8: Proportion of habitats with water in relation to rainfall. The high malaria transmission season in the highlands is during the long rainy season from March to June. The remaining time of the year malaria transmission is low even though it rains (Fillinger *et al.* 2009a).

4.4.2 Baseline characteristics of the aquatic habitats

At baseline, the chances of finding early and late instar *Anopheles* in an aquatic habitat were similar in both treatment arms but significant differences were found in habitats colonized by culicines and mosquito pupae (Figure 4.9, Table 4.4).
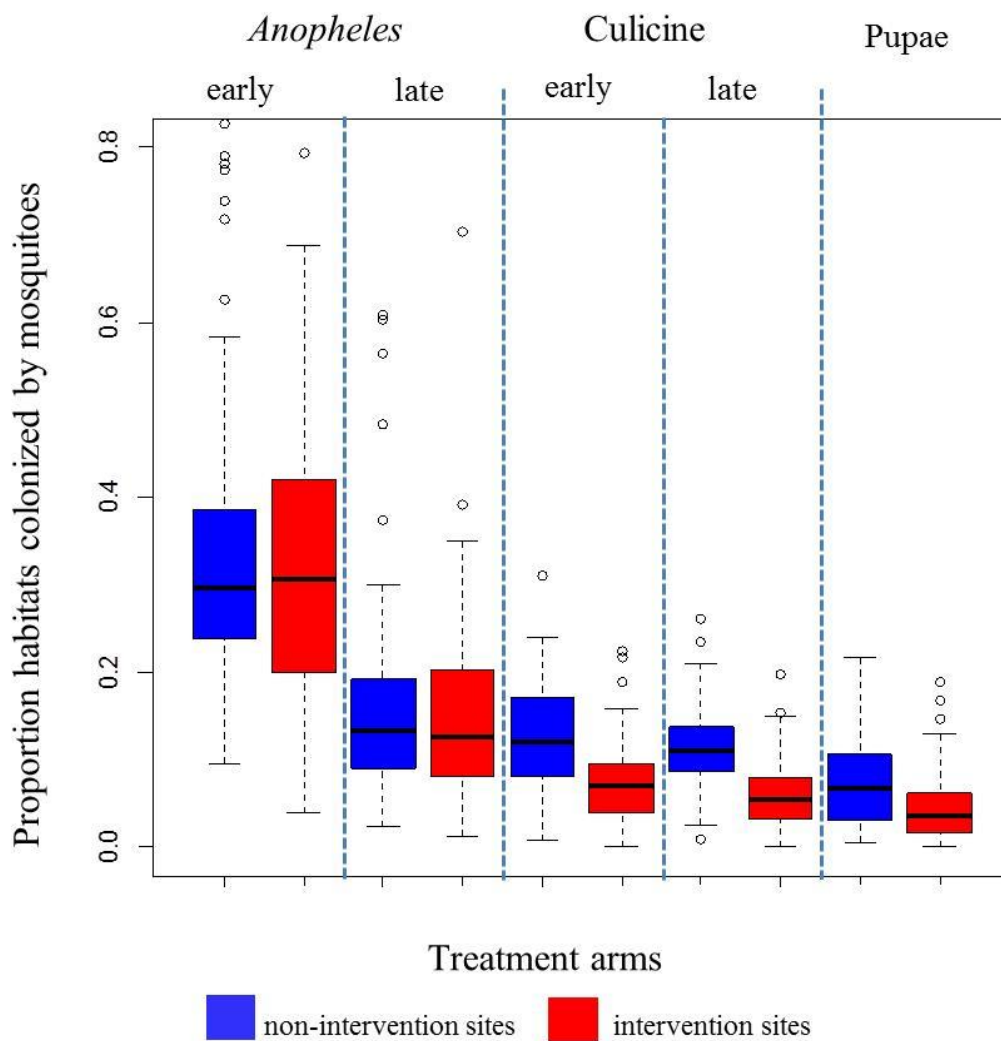
Figure 4.9: Box and whisker plots showing the median proportion and interquartile range of aquatic habitats being colonized by mosquito during the baseline surveys from July to November 2011.

The chance of finding culicine larvae was 1.6-1.8 times less in the intervention sites compared to non-intervention sites at baseline. It was also 1.6 times less likely to find pupae in aquatic habitats in the intervention sites than non-intervention sites suggesting that most of pupae were culicines (Table 4.4).

Table 4.4: Chances of finding mosquito immatures in aquatic habitats during baseline period

Mosquito genera	Treatment arm	Mean proportion habitats with immatures (95% CI)	Odds ratio (95% CI)	p-value
Anopheles early instar	non-intervention	0.48 (0.41-0.56)	1	
	intervention	0.43 (0.39-0.48)	0.91 (0.75-1.10)	0.333
Anopheles late instar	non-intervention	0.17 (0.13-0.22)	1	
	intervention	0.17 (0.15-0.20)	1.00 (0.75-1.32)	0.991
Culex early instar	non-intervention	0.14 (0.12-0.16)	1	
	intervention	0.09 (0.07-0.11)	0.63 (0.47-0.83)	0.001
Culex late instar	non-intervention	0.13 (0.12-0.15)	1	
	intervention	0.07 (0.05-0.08)	0.54 (0.45-0.65)	<0.001
Pupae	non-intervention	0.08 (0.06-0.10)	1	
	intervention	0.05 (0.03-0.06)	0.62 (0.45-0.87)	0.005

Immature abundance in the sentinel sites was similar for all groups at baseline (Table 4.5). Table 4.5 shows the analyses for the overall abundance of immatures estimated in all sentinel aquatic habitats in the study sites irrespective of whether they contained immatures or not. Furthermore, it shows the actual abundance of immatures per habitat that contained immatures. Interestingly, when only habitats with immature stages were considered, it was nearly three times more likely to find a pupa in the intervention sites than in the non-intervention sites at baseline. Possibly, the latter was again associated with a higher, though only borderline significant, abundance of late instar culicines (Table 4.5).

Table 4.5: Mean abundance of mosquito immature stages in sentinel aquatic habitats at baseline

Mosquito genera	Treatment arm	Mean abundance per m^2 (95% CI)	Rate ratio (95%CI)	p-value
Inclusive of all sentinel aquatic habitats (colonized and not)				
Anopheles early instar	non-intervention	1.50 (1.02-2.19)	1	
	intervention	1.87 (1.20-2.92)	1.25 (0.71-2.18)	0.434
Anopheles late instar	non-intervention	0.40 (0.24-0.65)	1	
	intervention	0.75 (0.44-1.29)	1.89 (0.96-3.72)	0.066
Culicine early instar	non-intervention	0.39 (0.23-0.66)	1	
	intervention	0.32 (0.16-0.65)	0.82 (0.36-1.89)	0.646
Culicine late instar	non-intervention	0.23 (0.14-0.38)	1	
	intervention	0.32 (0.19-0.55)	1.37 (0.71-2.64)	0.346
Pupae	non-intervention	0.07 (0.04-0.11)	1	
	intervention	0.14 (0.05-0.40)	2.21 (0.77-6.34)	0.140
Only sentinel aquatic habitats that contained mosquito immatures				
Anopheles early instar	non-intervention	4.37 (3.04-6.28)	1	
	intervention	4.96 (3.55-6.93)	1.14 (0.69-1.86)	0.613
Anopheles late instar	non-intervention	2.25 (1.40-3.60)	1	
	intervention	3.48 (2.50-4.85)	1.55 (0.90-2.65)	0.111
Culicine early instar	non-intervention	2.87 (1.90-4.35)	1	
	intervention	3.60 (1.96-6.62)	0.25 (0.60-2.59)	0.545
Culicine late instar	non-intervention	2.22 (1.52-3.24)	1	
	intervention	3.44 (2.46-4.81)	1.55 (0.95-2.51)	0.077
Pupae	non-intervention	0.89 (0.61-1.28)	1	
	intervention	2.32 (0.94-5.73)	2.62 (1.03-6.55)	0.042

Since there was no difference in habitat colonization and abundance of early and late instar *Anopheles*, the organisms of interest, the impact of Sumilarv®0.5G applications was assessed by comparing data from non-intervention sites with data from intervention study sites during the intervention year only. The reason for this is that the baseline collection period was short and did not cover the same seasons as the intervention period.

4.4.2.1 Investigating the impact of Sumilarv®0.5G applications during the intervention period

The impact of Sumilarv®0.5G applications in inhibiting adult vector production from aquatic habitats in the intervention study sites were compared during both the low and high malaria transmission seasons. The two transmission seasons are defined based on differences in adult *Anopheles* densities as previously described in the same study area (Fillinger *et al.* 2009a). The high transmission season occurs between March and June when adult *Anopheles* density substantially increases while the remainder of the year is considered the low transmission season due to the low density of *Anopheles*.

4.4.2.2 Mosquito positivity rate of aquatic habitats

In non-intervention sites, the probability of an aquatic habitat being colonized with early instar *Anopheles* larvae nearly doubles during the high transmission season compared to the low transmission season (also confirming the definition of these seasons). This effect can also be seen for late instars, although is less pronounced likely due to increased mortality (Figure 4.10, Table 4.6).
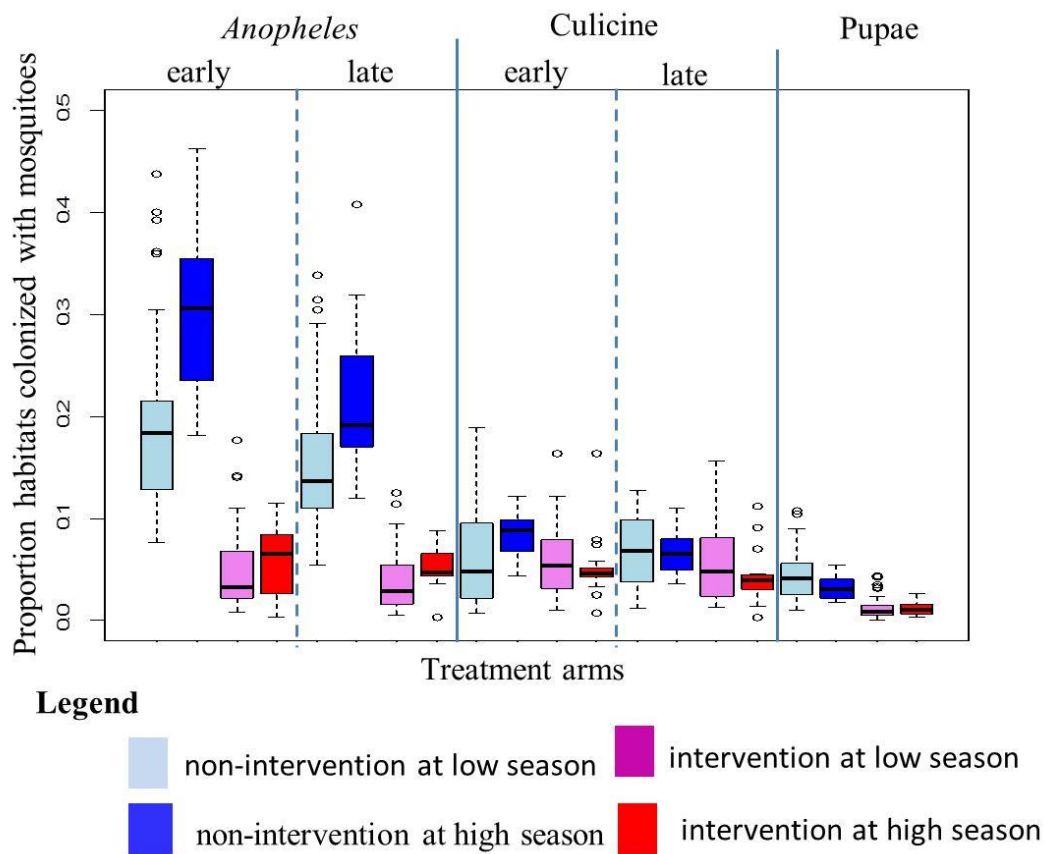
Figure 4.10: Box and whisker plots showing the median proportion and interquartile range of aquatic habitats being colonized by mosquito during the intervention surveys from December 2011 to December 2012

Compared to the non-intervention sites, the probability of finding a habitat positive for early instar *Anopheles* in intervention sites was reduced five-fold when low transmission seasons were compared and reduced seven-fold when high transmission seasons were compared. Similarly, habitats with late instar *Anopheles* larvae were five times less likely to be encountered in intervention sites than non-intervention sites irrespective of season (Table 4.6). Culicines occur less frequently than *Anopheles* larvae in the aquatic habitats of the western Kenya highlands which has also been shown previously (Ndenga *et al.* 2011). The impact of Sumilarv®0.5G application on culicines was consequently less conspicuous. There is however an indication that fewer habitats in intervention sites were colonised by culicines since the proportion of habitats with early instars decreases in the intervention period especially during the high malaria transmission season (Table 4.6).

Table 4.6: Chance of finding mosquito immatures in aquatic habitats during intervention period (excluding baseline data)

Mosquito genera	Treatment arm	Malaria season	Mean proportion of habitats colonized (95% CI)	Odds ratio (95% CI)	p-value
Anopheles early instar	non-intervention	Low	0.191 (0.185-0.197)	1	
	non-intervention	High	0.311 (0.300-0.322)	1.91 (1.79-2.03)	<0.001
	Intervention	low	0.048 (0.045-0.051)	0.21 (0.20-0.23)	<0.001
	Intervention	High	0.057 (0.052-0.063)	0.26 (0.23-0.28)	<0.001
Anopheles late instar	non-intervention	Low	0.174 (0.170-0.179)	1	
	non-intervention	High	0.226 (0.216-0.235)	1.38 (1.29-1.47)	<0.001
	Intervention	low	0.037 (0.034-0.040)	0.18 (0.16-0.20)	<0.001
	Intervention	High	0.051 (0.047-0.057)	0.26 (0.23-0.29)	<0.001
Culicine early instar	non-intervention	Low	0.062 (0.058-0.065)	1	
	non-intervention	High	0.084 (0.077-0.091)	1.39 (1.25-1.54)	<0.001
	Intervention	low	0.058 (0.055-0.062)	0.94 (0.86-1.03)	0.180
	Intervention	High	0.053 (0.048-0.058)	0.85 (0.76-0.96)	0.009
Culicine late instar	non-intervention	Low	0.083 (0.080-0.087)	1	
	non-intervention	High	0.067 (0.061-0.073)	0.79 (0.71-0.87)	<0.001
	Intervention	low	0.056 (0.052-0.060)	0.65 (0.60-0.70)	<0.001
	Intervention	High	0.043 (0.040-0.048)	0.50 (0.44-0.56)	<0.001
Pupae	non-intervention	Low	0.054 (0.052-0.057)	1	
	non-intervention	High	0.033 (0.029-0.037)	0.59 (0.51-0.68)	<0.001
	Intervention	low	0.012 (0.010-0.014)	0.21 (0.18-0.24)	<0.001
	Intervention	High	0.012 (0.010-0.015)	0.22 (0.18-0.27)	<0.001

Notably, habitats with pupae in non-intervention sites decreased during the high transmission season compared to the low transmission season which might be related to the reduced numbers of habitats with late instar culicines but might also be an indicator that heavy rainfall affects survival or more easily washes out pupae than larvae (Romoser and Lucas 1999; Paaijmans *et al.* 2007). Nevertheless, habitat positivity rates for pupae were five-fold reduced during the low transmission season and three-fold during the high transmission season in intervention sites compared to non-intervention sites (Table 4.6).
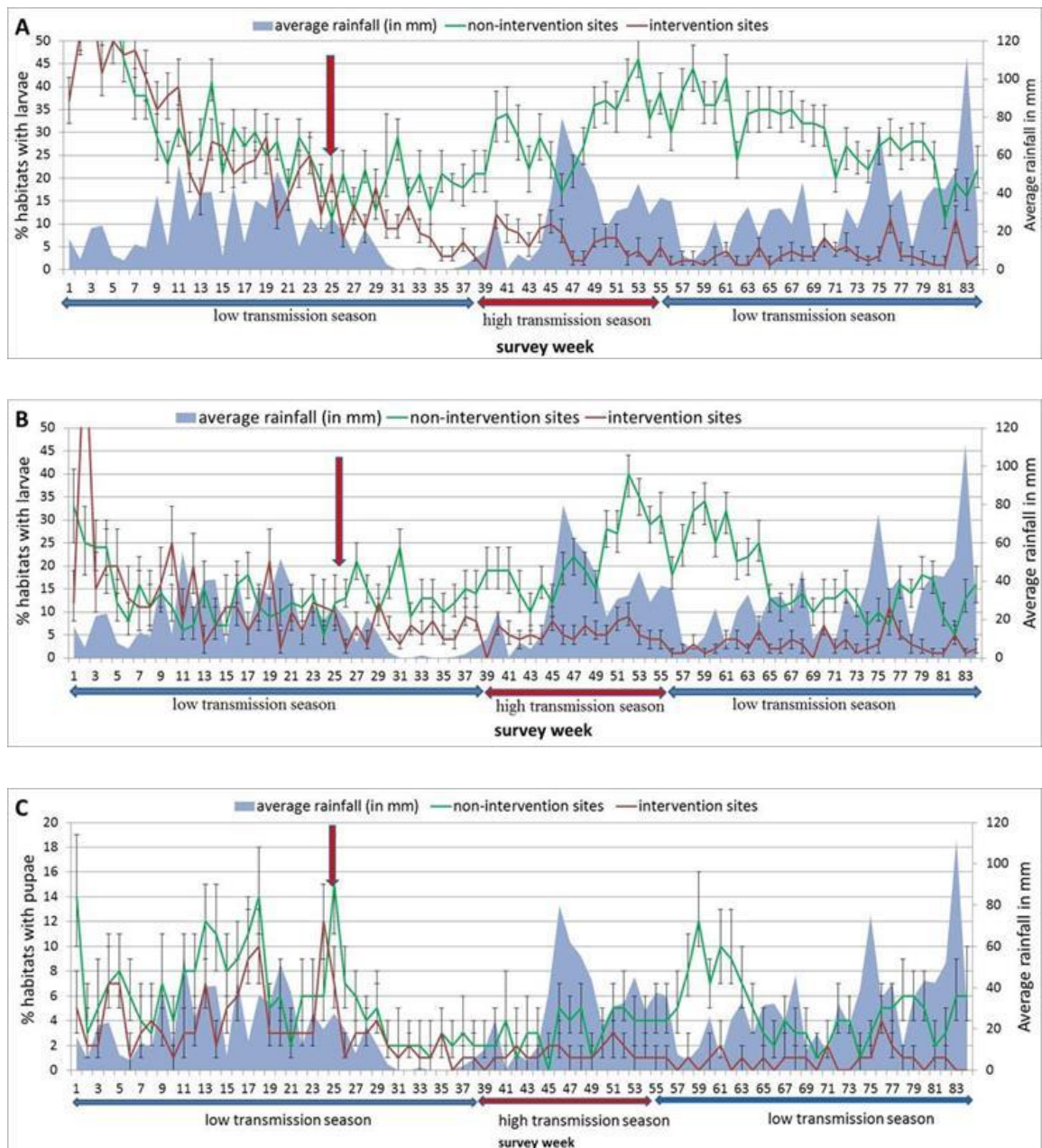
Figure 4.11: Mean proportion of aquatic habitats colonized by early (A) and late (B) instar *Anopheles* larvae and mosquito pupae (C) during the study period (error bars=95% confidence intervals). Red arrow indicates when the application of Sumilarv®0.5G to aquatic habitats in the intervention sites started

Figure 4.11 shows the seasonal habitat colonisation over 84 survey weeks. Whilst at baseline there is no difference, throughout the intervention the proportion of aquatic habitats colonized by *Anopheles* larvae and pupae was reduced in the intervention sites as compared to non-intervention sites. Greatest reductions in late instars and pupae occurred

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following the long rainy season (March-June) when the number of aquatic habitats colonized in the non-intervention sites increased greatly (Figure 4.11).

4.4.3   Mosquito immature abundance in sentinel aquatic habitats

Larval density of early instar *Anopheles* also divert in non-intervention and intervention areas with the beginning of the intervention with the most pronounced difference during and immediately after the long rainy season (high transmission season) that occurs between weeks 39 and 55. This trend is also reflected in the late *Anopheles* instar and pupae density although to a lesser extent (Figure 4.12). Since the latter two stages can serve as a proxy for productivity, it becomes clear that habitat productivity is highest during the long rains and consequently responsible for peak malaria transmission. Thus the impact of the intervention in reducing larval abundance in intervention sites is greater when comparisons are made during the high transmission seasons.
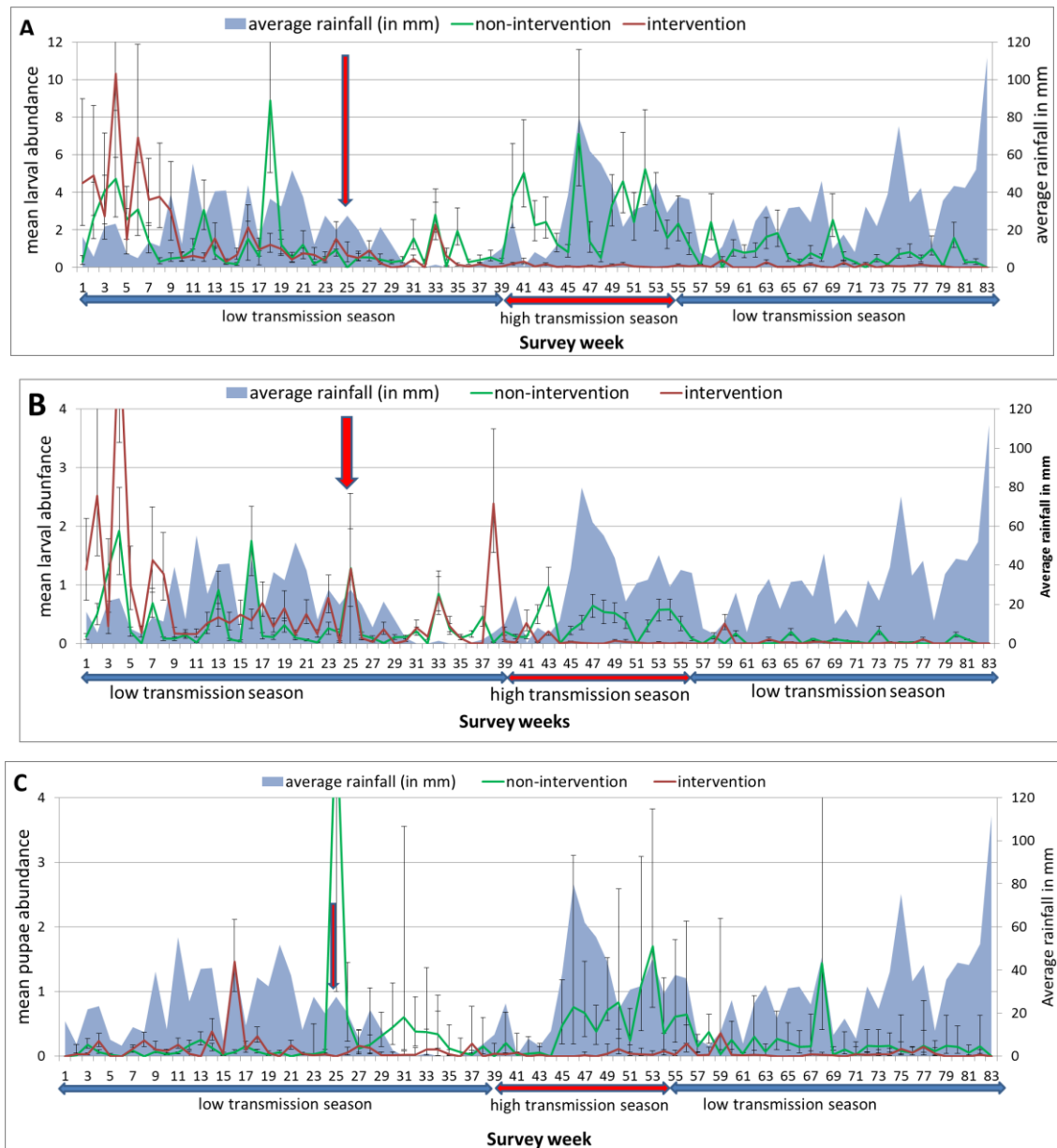
Figure 4.12: Mean abundance of immature stages per m^2 per survey week (error bars = 95% confidence intervals) in sentinel aquatic habitats during the study period. Red arrow indicates when the application of Sumilarv®0.5G to aquatic habitats in the intervention sites started. A-*Anopheles* early instars, B-*Anopheles* late instars, C-pupae

During the high transmission season, the probability of finding an early instar *Anopheles* was 30 times smaller in intervention sites than in non-intervention sites while it was five times smaller during the low transmission season. Similarly, the probability of finding a

late instar *Anopheles* in intervention sites was reduced 10-fold in intervention than non-intervention sites when high transmission seasons were compared while there was no impact seen during the low transmission season. This trend of greater reductions in abundance during the high than low transmission seasons was similarly observed for culicines. A pupa was 24-fold less likely to be found in the intervention sites during the high transmission season and four-fold less likely during the low transmission season (Table 4.7). The greater reductions in abundance during the high transmission in intervention sites can be explained by the significant increases in abundance of immatures in non-intervention sites during this time period.

Even when only habitats that actually have larvae are considered an increased abundance of immature stages occurred in non-intervention sites during the high transmission season as compared to the low transmission seasons (Table 4.7). This is probably due to the increased frequency in rains that improves water quality in habitats and consequently oviposition by gravid females and enhance larval survival at this time (Koenraadt *et al.* 2004). The impact of the intervention on larval density was apparent even when only habitats with larvae were considered (Table 4.7). This shows that the overall reduction in the previous analyses was not only due to fewer habitats being colonized but also due to lower numbers of eggs being laid in habitats. The impact of the intervention on pupae is difficult to interpret especially in the western Kenya highlands where aquatic habitats are extensive in size and pupae density per m^2 low (Ndenga *et al.* 2011). Moreover since it is difficult to distinguish between pupae of *Culex* and *Anopheles* in the field (Service 1971), we suggest that the impact of the intervention be evaluated on early instars as proxy for oviposition, late instars as proxy for survival of larvae and the monitoring tools discussed below as proxy of impact on adult emergence.

Table 4.7: Abundance of mosquito immatures per water surface area in sentinel aquatic habitats during intervention period (excluding baseline data)

Mosquito genera	Treatment arm	Malaria Season	Mean abundance per m^2 (95% CI)	Rate ratio (95% CI)	p-value
			Inclusive of all sentinel aquatic habitats		
	non-intervention	Low	0.909 (0.580-1.426)	1	
Anopheles early instar	non-intervention	High	2.445 (1.632-3.664)	2.69 (1.69-4.28)	<0.001
	Intervention	low	0.174 (0.105-0.287)	0.19 (0.10-0.36)	<0.001
	Intervention	High	0.008 (0.003-0.014)	0.09 (0.04-0.18)	<0.001
	non-intervention	Low	0.155 (0.086-0.280)	1	
Anopheles late instar	non-intervention	High	0.422 (0.244-0.733)	2.72 (1.48-5.02)	0.001
	Intervention	low	0.011 (0.048-0.257)	0.70 (0.26-1.91)	0.492
	Intervention	High	0.041 (0.017-0.098)	0.26 (0.09-0.74)	0.011
	non-intervention	Low	0.449 (0.260-0.775)	1	
Culicine early instar	non-intervention	High	1.114 (0.582-2.249)	2.55 (1.61-4.03)	<0.001
	Intervention	low	0.329 (0.135-0.801)	0.73 (0.28-1.88)	0.518
	Intervention	High	0.084 (0.041-0.172)	0.19 (0.08-0.42)	<0.001
	non-intervention	low	0.344 (0.201-0.588)	1	
Culicine late instar	non-intervention	high	0.750 (0.278-2.023)	2.18 (0.88-5.40)	0.092
	Intervention	low	0.156 (0.097-0.251)	0.45 (0.24-0.84)	0.013
	Intervention	high	0.135 (0.070-0.261)	0.39 (0.19-0.83)	0.014
	non-intervention	low	0.124 (0.053-0.291)	1	
	non-intervention	high	0.207 (0.109-0.394)	1.67 (0.59-4.77)	0.337
	Intervention	low	0.026 (0.012-0.057)	0.21 (0.07-0.65)	0.21
Pupae	Intervention	high	0.009 (0.003-0.020)	0.07 (0.02-0.28)	0.075
			Only sentinel aquatic habitats that contained mosquito immatures		
	non-intervention	low	4.361 (3.024-6.288)	1	
Anopheles early instar	non-intervention	high	7.763 (5.572-10.82)	1.78 (1.17-2.70)	0.007
	Intervention	low	2.414 (1.501-3.884)	0.55 (0.31-0.99)	0.046
	Intervention	high	0.902 (0.062-1.315)	0.21 (0.12-0.34)	<0.001
	non-intervention	low	2.520 (1.767-3.593)	1	
Anopheles late instar	non-intervention	high	3.813 (2.642-5.502)	1.51 (1.02-2.23)	0.037
	Intervention	low	3.004 (1.323-6.822)	1.19 (0.49-2.87)	0.696
	Intervention	high	1.058 (0.546-2.053)	0.42 (0.20-0.89)	0.023

Culicine early instar	non-intervention	low	4.606 (3.279-6.469)	1	
	non-intervention	high	10.567 (5.99-18.63)	2.29 (1.31-4.02)	0.004
	Intervention	low	5.706 (1.812-17.97)	1.24 (0.39-3.95)	0.717
	Intervention	high	1.043 (0.604-1.800)	0.23 (0.12-0.44)	<0.001
Culicine late instar	non-intervention	low	4.269 (2.729-6.678)	1	
	non-intervention	high	8.472 (4.546-15.79)	1.98 (1.01-3.89)	0.046
	Intervention	low	2.015 (1.380-2.942)	0.47 (0.26-0.85)	0.012
	Intervention	high	1.767 (1.071-2.916)	0.41 (0.21-0.81)	0.010
	non-intervention	low	3.192 (1.431-7.120)	1	
	non-intervention	high	3.625 (2.226-5.906)	0.21 (0.08-0.53)	0.791
	Intervention	low	1.163 (0.526-2.572)	0.36 (0.12-1.14)	0.083
Pupae	Intervention	high	0.065 (0.039-1.093)	0.21 (0.08-0.53)	0.001

4.4.4 Abundance of non-target aquatic insects in sentinel habitats

The impact of Sumilarv®0.5G application on the abundance of non-target aquatic insects such as odonata nymphs, coleoptera adults and hemiptera nymphs and adults (not separated) was examined.

Table 4.8 summarizes the analyses of the impact of Sumilarv®0.5G application on the abundances of the non-target organisms. Overall Sumilarv®0.5G application did not show any significant effect on coleopterans and hemipterans during both the high and low transmission seasons. However while an increased abundance of hemipterans was observed during the high transmission season in non-intervention study sites a decline in abundance occurred in the intervention sites during this time period as compared to the low season. This probably suggests an impact of Sumilarv®0.5G against hemipterans. The abundance of odonata in treated aquatic habitats was significantly reduced in both transmission seasons when both overall abundance and abundance in only aquatic habitats with these organisms were considered. Overall it was five times less likely to find an odonata in intervention sites than in non-intervention sites during the high transmission season and two times less likely in the low transmission season.

Table 4.8: Impact of Sumilarv®0.5G application on non-target aquatic organisms

Nontarget organism	Treatment arm	Malaria season	Mean abundance per m2 (95% CI)	Rate ratio (95% CI)	p-value
			**Inclusive of all sentinel aquatic habitats**		
	non-intervention	low	0.465 (0.316-0.685)	1	
	non-intervention	high	0.567 (0.381-0.785)	1.17 (0.72-1.92)	0.521
	Intervention	low	0.244 (0.170-0.350)	0.52 (0.31-0.87)	0.013
Odonata	Intervention	high	0.103 (0.072-0.148)	0.22 (0.13-0.38)	<0.001
	non-intervention	low	0.459 (0.233-0.904)	1	
	non-intervention	high	0.296 (0.159-0.553)	0.65 (0.40-1.04)	0.070
	Intervention	low	0.342 (0.201-0.768)	0.83 (0.55-1.11)	0.138
Coleoptera	Intervention	high	0.656 (0.398-0.889)	1.23 (0.10-0.57)	0.435
	non-intervention	low	0.435 (0.291-0.650)	1	
	non-intervention	high	0.536 (0.351-0.819)	1.23 (0.75-2.02)	0.406
	Intervention	low	0.395 (0.266-0.601)	0.96 (0.72-1.23)	0.333
Hemiptera	Intervention	high	0.359 (0.224-0.577)	0.83 (0.45-1.52)	0.539
			Only sentinel aquatic habitats that contained mosquito immatures		
	non-intervention	low	2.489 (1.786-3.467)	1	
	non-intervention	high	3.379 (2.661-4.292)	1.36 (0.91-2.02)	0.129
	Intervention	low	1.433 (1.067-1.925)	0.58 (0.37-0.89)	0.012
Odonata	Intervention	high	0.763 (0.566-1.029)	0.31 (0.20-0.47)	<0.001
	non-intervention	low	3.636 (1.968-6.750)	1	
	non-intervention	high	2.665 (1.842-3.856)	0.73 (0.43-1.24)	0.250
	Intervention	low	2.986 (1.991-4.879)	0.82 (0.56-1.45)	0.557
Coleoptera	Intervention	high	2.974 (1.685-4.391)	0.89 (0.13-0.54)	0.319
	non-intervention	low	0.435 (0.291-0.650)	1	
	non-intervention	high	0.536 (0.351-0.819)	1.36 (1.06-1.74)	0.014
	Intervention	low	0.335 (0.166-0.531)	0.84 (0.44-1.43)	0.467
Hemiptera	Intervention	high	0.359 (0.224-0.577)	0.69 (0.44-1.06)	0.092

4.4.5 Vector productivity of aquatic habitats

Immature collections: A total of 635 pupae were collected from sentinel aquatic habitats in non-intervention sites while 200 pupae were collected from intervention sites during weekly surveys in the intervention period. This corresponds to a 68% reduction in pupae collections from intervention sites compared to the collections from non-intervention sites. A total of 688 late instar *Anopheles* larvae were collected from non-intervention sites while only 75 late instar larvae were collected from aquatic habitats in intervention sites. Notably, reduced adult emergence of mosquito pupae was recorded during the high transmission season compared to the low transmission season even in sites without intervention (Table 4.9). This can possibly be attributed to stress on pupae caused by increased rains falling on the aquatic habitat during the high transmission season or density-dependent effects in aquatic habitats due to crowding (Lyimo *et al.* 1992; Romoser and Lucas 1999; Paaijmans *et al.* 2007). Importantly, it was 55 times less likely for a pupa collected from intervention sites to develop into an adult during the low transmission season and 472 times less likely during the high transmission season when compared to emergence rates in non-intervention sites (Table 4.9). Similar results were seen for late instar *Anopheles* larvae collected from intervention sites which were 28 times less likely to develop into adults during the low transmission season and 81 times less likely during the high transmission season when compared to emergence rates from non-intervention sites (Table 4.9). The greater reduction in adult emergence rates from collected pupae than from collected larvae is an indication that prolonged exposure of mosquito immatures to the insecticide is needed to enhance the impact on adult emergence inhibition.

Table 4.9: Adult emergence rate of late instar *Anopheles* and mosquito pupae collected from sentinel aquatic habitats

Treatment arm	Malaria Season	Mean proportion emerged adults (95% CI)	Odds ratio (95% CI)	p-value
Proportion of late instar *Anopheles* that emerged into adults				
non-intervention	Low	0.87 (0.83-0.90)	1	
non-intervention	High	0.83 (0.78-0.87)	0.731 (0.479-1.114)	0.140
Intervention	Low	0.19 (0.09-0.33)	0.035 (0.014-0.077)	<0.001
Intervention	High	0.06 (0.02-0.21)	0.009 (0.001-0.033)	<0.001
Proportion of mosquito pupae that emerged into adults				
non-intervention	Low	0.91 (0.88-0.94)	1	
non-intervention	High	0.83 (0.78-0.87)	0.472 (0.287-0.766)	0.003
Intervention	Low	0.16 (0.11-0.24)	0.018 (0.009-0.033)	<0.001
Intervention	High	0.01 (0.002-0.09)	0.001 (0.0001-0.006)	<0.001

Over the three-week survey period over 80% of larvae and pupae collected from habitats in non-intervention sites emerged as adults compared to only 9-18% (95% CI 6-21%) of larvae and 7-16% (95% 4-26%) of pupae collected from intervention sites that emerged as adults (Figure 4.13). Significant differences in adult emergence rates of larvae and pupae collected over the three-week survey period were not observed.
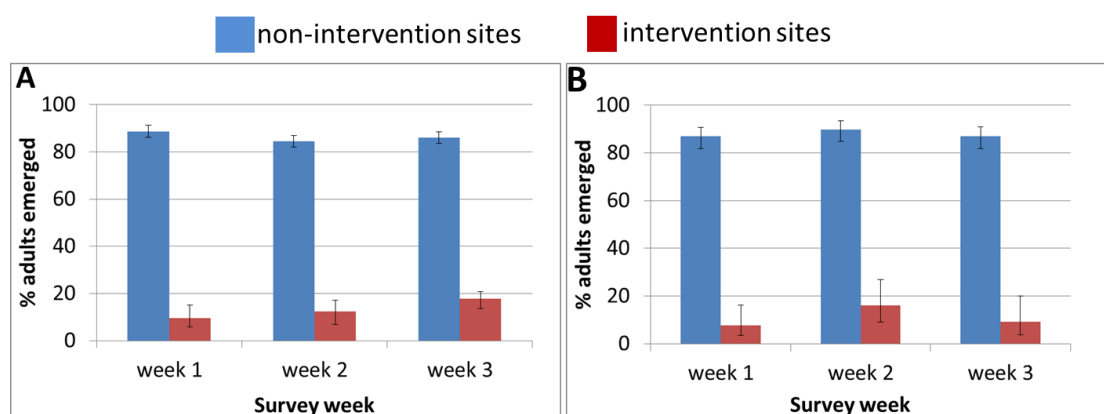
Figure 4.13: Mean percentage (%) of adults emerged from late instar *Anopheles* larvae (A) and mosquito pupae (B) collected from sentinel aquatic habitats in non-intervention and intervention sites. Error bars = 95% confidence intervals.

Water collections: Bioassays with insectary-reared *An. gambiae s.s.* larvae introduced into water samples from study sites showed in contrast to the previous method of immature collections increasing adult emergence rates over the three-week survey period (Figure 4.14).
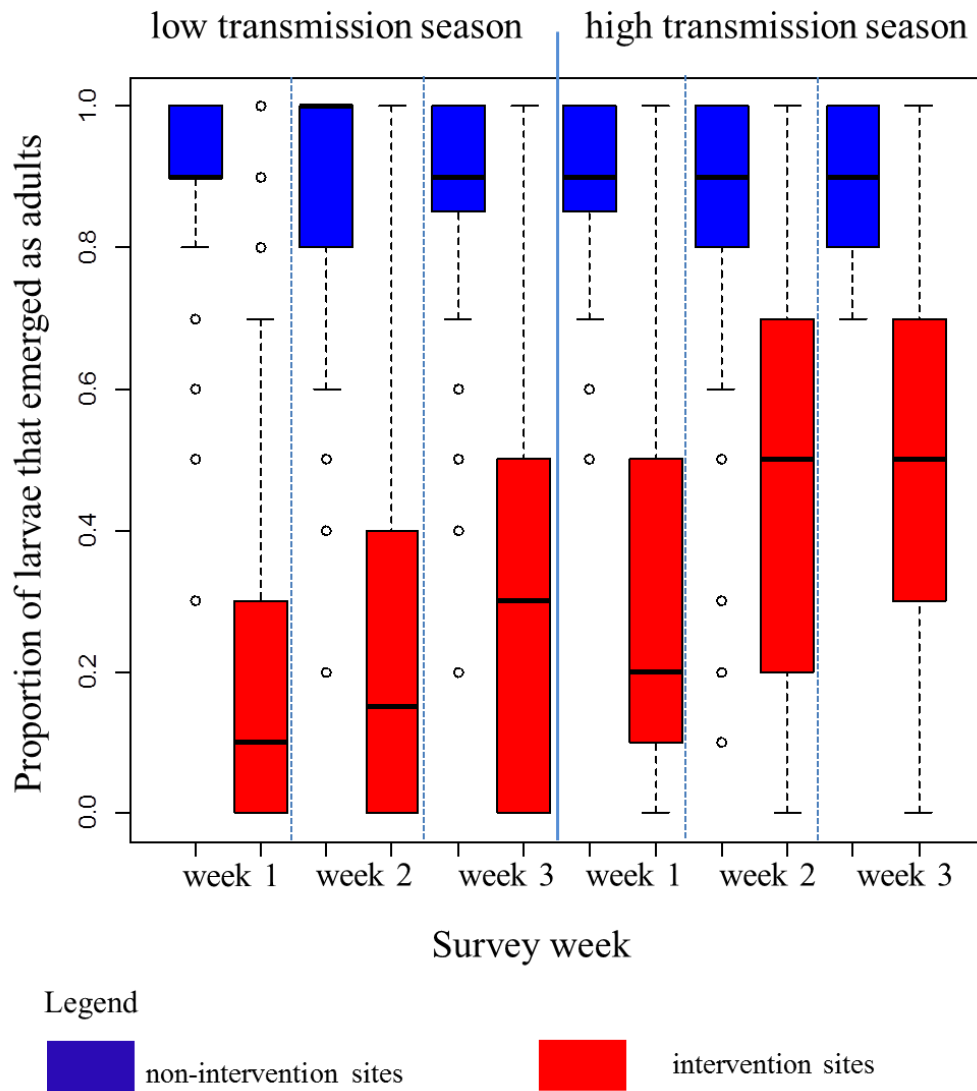
Figure 4.14: Box and whisker plots showing the median proportion and interquartile range of proportion of late instar larvae introduced into water samples collected from study sites during intervention period that emerged into adults

Overall it was 55 times less likely for a larvae introduced into water samples collected from Sumilarv®0.5G-treated aquatic habitats to develop into an adult compared to emergence rates of larvae introduced into untreated aquatic habitats during the low

transmission season. The impact of Sumilarv®0.5G was however slightly lower during the high transmission season. It was 29 times less likely for a larvae introduced into water samples collected from treated aquatic habitats in Sumilarv®0.5G-treated habitats to develop into an adult compared to adult emergence rates of larvae introduced into untreated water samples during the high transmission season (Table 4.10). The reduced impact of Sumilarv®0.5G during the high transmission season is likely to be an indication of high wash out effect of the insecticide in running water in the habitats due to the heavy rains that fall during the high malaria transmission season. Overall there were no main effects of survey week on adult emergence but interactions were identified between the treatment of Sumilarv®0.5G and survey week. The impact of the interactions can be calculated by multiplication of the odds ratios (Katz 2006). Thus during both the low and high transmission seasons it was twice as likely for a larvae introduced into water samples collected from intervention sites in the second and third weeks to develop into an adult as compared to emergence rates when larvae were introduced into water samples collected in the first week of survey (Table 4.10).

Table 4.10: Adult emergence of insectary-reared larvae introduced into water samples collected from the aquatic habitats in the field

	Odds ratio (95% CI)	p-value
low transmission season		
non-intervention sites	1	
intervention sites	0.018 (0.013-0.024)	<0.001
week 1	1	
week 2	0.839 (0.686-1.027)	0.088
week 3	0.932 (0.770-1.129)	0.473
intervention sites* week 1	1	
intervention sites* week 2	1.810 (1.355-2.420)	<0.001
intervention sites* week 3	2.463 (1.848-3.284)	<0.001
high transmission season		
non-intervention sites	1	
intervention sites	0.034 (0.025-0.046)	<0.001
week 1	1	
week 2	0.758 (0.584-0.983)	0.037
week 3	0.844 (0.665-1.072)	0.164
intervention sites* week 1	1	
intervention sites* week 2	2.484 (1.627-3.791)	<0.001
intervention sites* week 3	2.449 (1.673-3.585)	<0.001

*symbol for interaction

The adult emergence rates of larvae introduced into water samples from untreated water samples was above 88% over the three-week survey period during both the low and high transmission seasons. The adult emergence rates of larvae introduced into water samples from treated habitats were 16% (95% CI 11-21%) in week 1, 22% (95% CI 14-33%) in week 2 and 29% in week 3 after Sumilarv®0.5G application during the low transmission season. During the high transmission season the adult emergence rates of larvae introduced into treated water samples were 29% (95% CI 21-35%) in week 1, 44% (95% CI 34-53%) in week 2 and 46% (95% CI 41-59%) in week 3 after application of the insecticide.

Floating cup comparisons: The comparison of emergence rates from the floating cup experiment with the emergence rate from immature stages (larvae and pupae) collected from the habitat confirmed that the water collection method underestimates the impact of the intervention (Figure 4.15). Adult emergence of larvae and pupae from non-intervention sites were above 80% with all monitoring tools. On average, 42-58% (95% CI 36-63%) of larvae introduced into water samples collected from aquatic habitats in intervention sites developed into adults. Bioassays with floating cups for exposing insectary-reared late instar *An. gambiae s.s.* larvae directly into aquatic habitats showed much higher impact of Sumilarv®0.5G at inhibiting adult emergence than the water collection method. Over the three-week survey the adult emergence rates of larvae exposed directly to treated habitat water in floating cups was 16-22% (95% CI 7-30%). Bioassays with late instar *Anopheles* larvae and pupae collected from treated habitats showed a higher impact of Sumilarv®0.5G at inhibiting adult emergence. While on average 7-13% (95% CI 2-29%) of larvae collected over the three-week survey period from intervention sites developed into adults, adult emergence in pupae collected from intervention sites only occurred during week 2 of Sumilarv®0.5G application (Figure 4.15). These results clearly demonstrates the best tool to assess the impact of Sumilarv®0.5G and other insect growth regulators is to assess pupae collected from treated habitats for emergence.
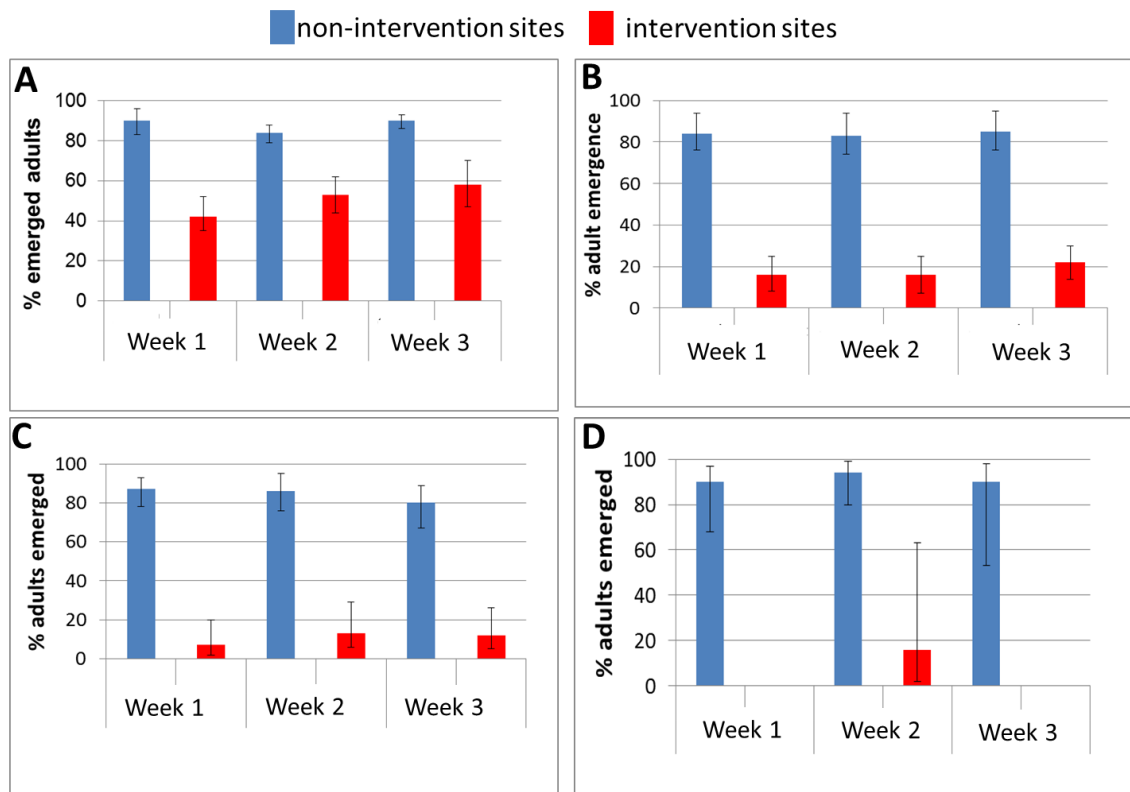
Figure 4.15: Percent adult emergence of (A) insectary-reared late instar *Anopheles* larvae introduced into water samples collected from aquatic habitats in field (B) insectary-reared late instar *Anopheles* larvae exposed directly in treated and untreated habitats in the field in floating cups (C) late instar *Anopheles* larvae and (D) pupae collected from aquatic habitats in the field

4.4.6   Persistence of Sumilarv®0.5G in habitats over dry periods

Some aquatic habitats in the intervention arm of the study dried after treatment for between seven and 313 days before they refilled with water (Table 4.11).

Table 4.11: Duration when treated aquatic habitats in intervention sites remained dry before refilling with water

Number of days habitats remained dry	Number of habitats
7-14	83
15-28	92
29-42	89
43-56	40
57-70	24
71-84	25
85-98	1
99-112	4
113-126	12
127-140	6
141-154	1
155-168	92
169-182	6
188	1
313	1

Bioassays with late instar *Anopheles* larvae showed Sumilarv®0.5G persisted in habitats that had been dry up to 56 days before refilling with water. Less than 20% adult emergence was observed in larvae collected from habitats that were dry for 56 days or less (Figure 4.16). However, still less than 50% adult emergence was recorded in larvae collected from treated habitats that remained dry for up to 100 days. Thereafter adult emergence rates was over 60% when habitats remained dry for more than 100 days before refilling with water (Figure 4.16).
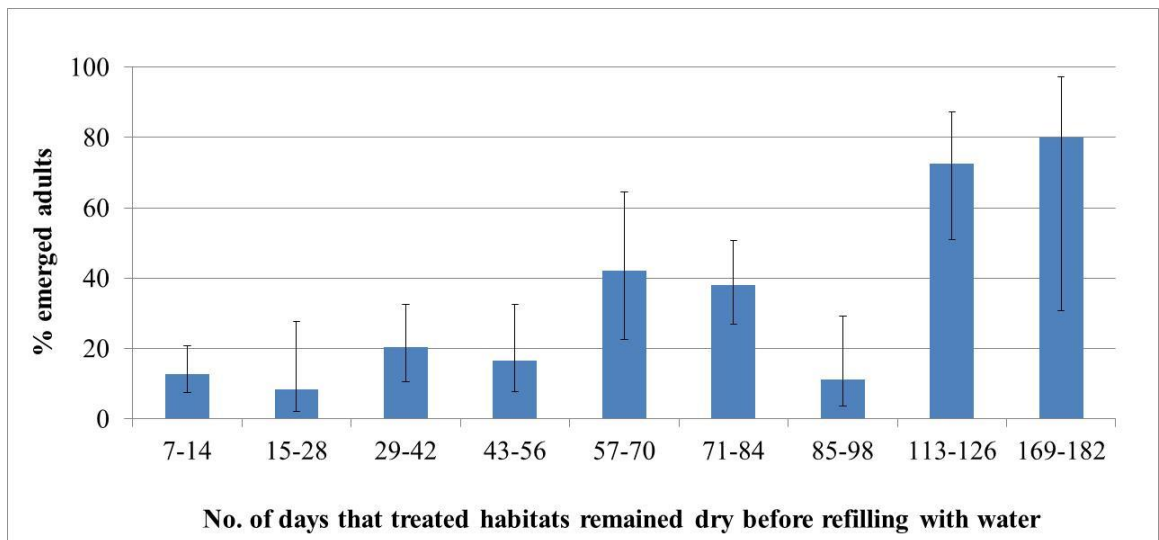
Figure 4.16: Adult emergence of late instar *Anopheles* larvae collected from treated habitats that temporarily fall dry

Since there were no untreated habitats for this test to serve as controls for comparison, we compared the adult emergence rates of larvae collected in these habitats to the expected minimum adult emergence from untreated habitats of 80% based on our results from the bioassays above (Figure 4.16). Our analysis revealed 2-12 fold reduction the proportion of adults emerging from larvae collected from treated habitats that remained dry for a maximum of 100 days before refilling with water (Table 4.12).

Table 4.12: Results of analysis comparing adult emergence of larvae collected from treated habitats that temporarily fall dry to an expected 80% adult emergence in the untreated habitats

Number of days habitats remained dry	Rate ratio (95% CI)	p-value
control (expected 80% adult emergence)	1	
7-14	0.127 (0.072-0.212)	<0.001
15-28	0.083 (0.014-0.285)	<0.001
29-42	0.205 (0.099-0.369)	<0.001
43-56	0.167 (0.070-0.335)	<0.001
57-70	0.421 (0.211-0.660)	<0.001
71-84	0.381 (0.264-0.512)	<0.001
85-98	0.111 (0.029-0.303)	<0.001
113-126	0.678 (0.456-1.041)	0.051
169-182	0.994 (0.878-1.110)	0.987

However, this effect could not be shown when water samples were taken from temporarily dried up habitats (Figure 4.17) suggesting that the active ingredient is slowly released from the organic matter (Schaefer *et al.* 1991). Greater than 50% adult emergence was recorded in larvae introduced into water samples collected from habitats that had been dry for only 7-14 days (Figure 4.17).
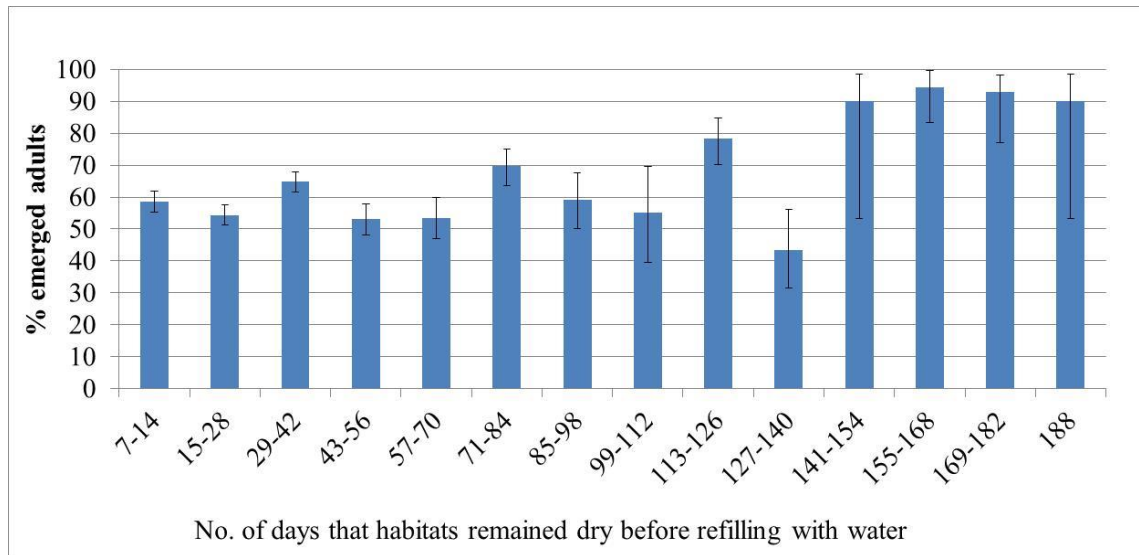
Figure 4.17: Adult emergence of insectary-reared late instar *An. gambiae s.s.* larvae introduced into water samples collected from habitats that temporary fall dry

Comparison of the emergence rates of larvae introduced into water samples to the expected minimum emergence rates of 80% from untreated water revealed however that the moderate reductions were significant up to 112 days (Table 4.13). No pupae were collected from habitats before the next treatment round.

Table 4.13: Results of analysis comparing adult emergence of larvae introduced into water samples collected from the field to a hypothetical adult emergence of 80% in the untreated habitats

Number of days habitat were dry	Odds ratio (95% CI)	p-value
control (minimum 80% expected adult emergence)	1	
7-14	0.587 (0.552-0.620)	<0.001
15-28	0.544 (0.512-0.577)	<0.001
29-42	0.649 (0.617-0.681)	<0.001
43-56	0.530 (0.480-0.580)	<0.001
57-70	0.533 (0.468-0.597)	<0.001
71-84	0.696 (0.633-0.752)	0.043
85-98	0.592 (0.498-0.679)	<0.001
99-112	0.550 (0.386-0.704)	<0.001
113-126	0.783 (0.697-1.101)	0.732
127-140	0.433 (0.308-0.567)	<0.001
141-154	0.900 (0.541-0.994)	0.693
155-168	0.956 (0.798-1.323)	0.908
169-182	0.934 (0.878-1.123)	0.567
188	0.967 (0.809-0.998)	0.991

4.4.7 Risk of vector production from untreated habitats

A total of 43 new aquatic habitats were created in-between successive Sumilarv®0.5G application cycles in the intervention study sites. The highest number of new habitats appeared in the first round of Sumilarv®0.5G application. In this first round of insecticide application the new habitats were mostly created in Mudabala (8) where burrow pits were filled with water for fish farming. In subsequent rounds only few new habitats were created (Figure 4.18), a common phenomenon in the western Kenya highlands where aquatic habitats are stable (Himeidan *et al.* 2009). Only four pupae were collected from the new habitats during the period when they remained untreated. All four pupae successfully developed into adults. In addition a total of 100 late instar anopheline larvae were collected from these new habitats. Of these, 83 successfully developed into adults. The fact that only four pupae were collected from untreated aquatic habitats that appeared in-between insecticide application cycles throughout the one-year intervention period suggest that the 3 weekly application of Sumilarv®0.5G is sufficient to inhibit adult vector production in the western Kenya highlands.
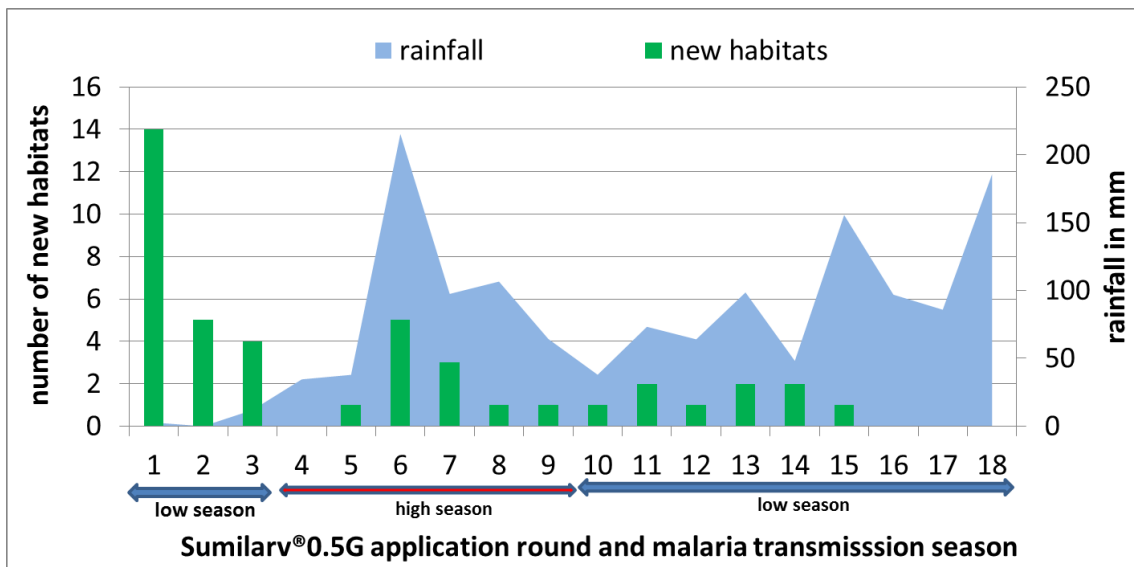
Figure 4.18: New aquatic habitats created during Sumilarv®0.5G application rounds

4.4.8 Liquid chromatography-mass spectrometry (LC-MS) analysis of treated water samples

A total of 10 jars containing water samples were broken during transportation. Thus the number of water samples collected from intervention sites that were extracted for analysis included eight samples collected in first and second weeks and seven water samples collected during the third week after application of Sumilarv®.5G. The number of water samples from non-intervention sites analysed for PPF presence included three samples collected during the first week, five samples collected during the second week and four water samples collected during the third week after application of the insecticide in aquatic habitats in the intervention sites.

The peak identified to be PPF when the standard (Sumilarv®0.5G) was run in the LC-MS had a retention time of 2.018-2.896 minutes while the retention time of the internal standard (4-benzylbiphenyl) was 0.638-1.401 minutes.

These retention times of PPF were used to confirm the presence of the insecticide in water samples by identifying ions with similar masses as that of PPF. On the other hand the retention time of 4-benzylbiphenyl (internal standard) was used to identify its peak and corresponding area to assist in quantification of the PPF detected in the sample.

Similarly the peak of the internal standard at the retention time was identified by the molecular mass of associated ions.

PPF was not detected in any of the water samples collected from aquatic habitats in the non-intervention site. PPF was also not detected in six water samples collected from the intervention site. These included three water samples collected in first week, one water sample collected in second week and two water samples collected in third week after application of the insecticide to aquatic habitats. It is likely that PPF concentrations in these water samples were below the detection limit. PPF was detected in 17 water samples collected from the intervention site. These included five water samples collected during the first and third weeks and seven water samples collected during the second week after application of Sumilarv®0.5G. Thus the number of water samples in which PPF was detected were used to estimate the weekly average concentrations of PPF in water in the field habitats. The average concentration of PPF detected in water samples from intervention sites were 0.0012 ppm (95% CI 0.0002-0.0024 ppm) in water samples collected in first week, 0.0022 ppm (95% CI 0.0008-0.0043 ppm) collected in second week and 0.0006 ppm (95% CI 0.0001-0.0012) in water collected in third week after application of Sumilarv®0.5G to aquatic habitats (Figure 4.19). It is likely that these averages are an overestimate given that PPF was not detected in some of the water samples. Nevertheless, the estimated concentration of PPF detected in third week after Sumilarv®0.5G application was almost half that estimated in first week (Figure 4.19). However, large variability was detected between samples and differences were not statistically significant. Data pooled for all three weeks suggest a mean concentration of 0.0013 ppm (95% CI 0.0001-0.0048).
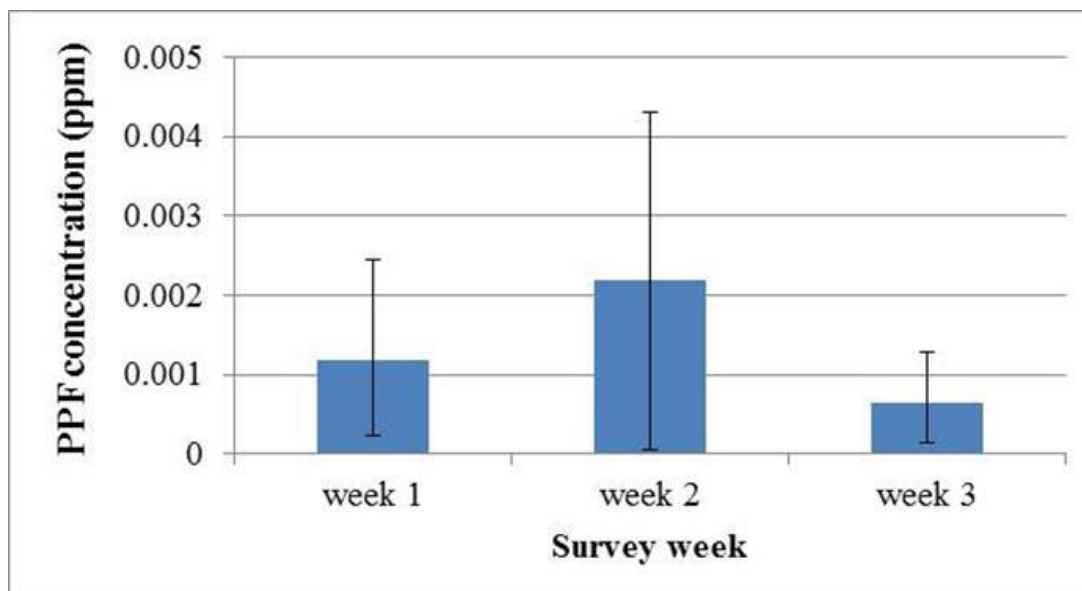
Figure 4.19: Average weekly concentrations of PPF detected in water samples collected in intervention sites

4.4.9   Species composition of *Anopheles* mosquitoes collected in the field

A total of 138 *Anopheles* mosquitoes that emerged from field collected larvae and pupae were processed for species identification. Fifty-five of these were however in very bad shape and could not be identified. Of the 83 *Anopheles* mosquitoes identified morphologically 78.3% (65) were *An. gambiae s.l.* The remaining were *An. coustani* (7) and *An. funestus* (11). PCR further identified the *An. gambiae s.l.* to be made of 92.3% (60) *An. gambiae s.s.* and 7.7% (5) *An. arabiensis* (Figure 4.20).
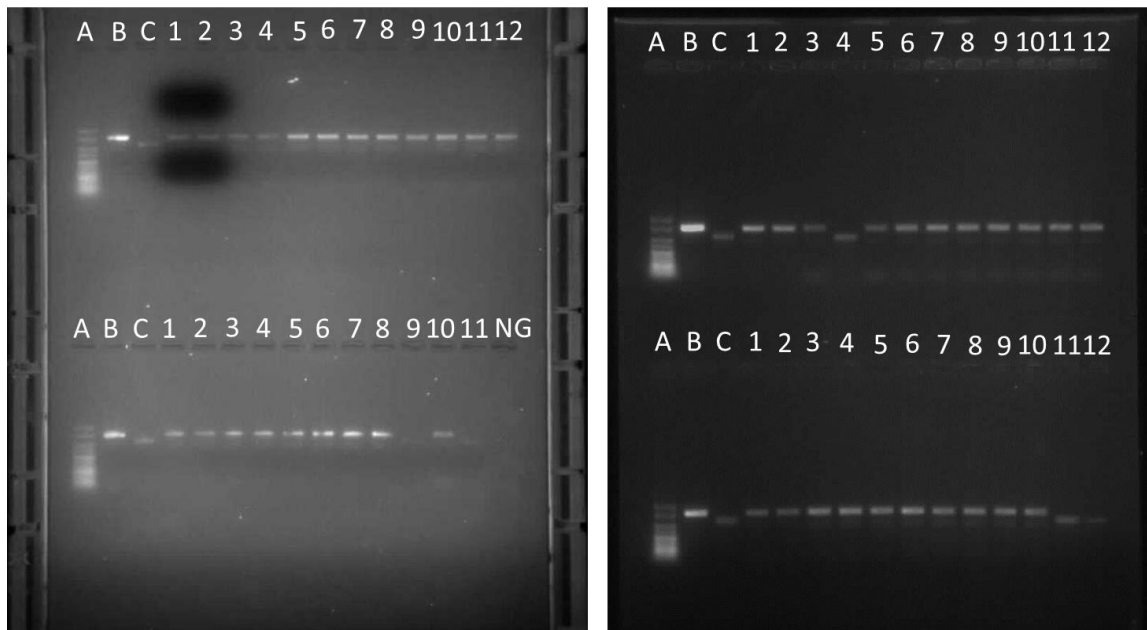
Figure 4.20: Electrophoresis gels showing molecular identification of *An. gambiae s.l.* A-ladder, B-positive control *An. gambiae s.s.*, C-positive control *An. arabiensis*, NG-negative control, nos. 1-12- mosquito samples

4.5 Discussion

In this study the operational application of the insect growth regulator Sumilarv®0.5G was effective at inhibiting adult production of the major Afrotropical malaria vectors from treated aquatic habitats by over 80% compared to emergence in untreated habitats. The effectiveness of the three-weekly application at suppressing adult vector production from treated habitats highlights the potential of this insecticide for control of malaria vectors in the western Kenya highlands. Overall the three-weekly application of Sumilarv®0.5G to aquatic habitats in intervention sites was effective at: (1) reducing the colonization and abundance of aquatic habitats with mosquito larvae and pupae, (2) inhibiting adult emergence of larvae and pupae collected from treated habitats, and (3) preventing adult emergence from untreated aquatic habitats created in-between successive treatment rounds. Furthermore the persistence of PPF in habitats during periods of dryness was confirmed in this study.

The finding of reduced colonization and abundance of aquatic habitats by mosquitoes in intervention sites during the intervention period was unexpected since larvae are not directly affected by the low dose application. This can only be attributed to a quickly

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reduced overall adult vector population in the intervention sites due to the intervention as supported by our data on pupae development and emergence and supported by published work (Suzuki *et al.* 1989; Okazawa *et al.* 1991). Reduced adult populations consequently lead to reduced oviposition and therefore less habitats are colonised and in lower numbers. This finding is highly encouraging not only for the impact but also for the potential of monitoring the impact through larval surveys.

It likely that much higher concentration of Sumilarv®0.5G is needed for controlling culicine mosquitoes. This reduced susceptibility of culicine compared to *Anopheles* mosquitoes has been reported in previous studies (Kawada *et al.* 1988; Ansari *et al.* 1991). For instance studies conducted both in the laboratory and field by Ansari *et al.* (1991) found *Cx. quinquefasciatus* to be less susceptible to PPF than *An. stephensi*. Similarly Kawada *et al.* (1988) found *Cx. pipiens pallens* to be less susceptible to PPF than *An. stephensi*. Another possible explanation for the reduced impact on habitat positivity and abundance of culicines is the fact that in our study the application of Sumilarv®0.5G was focused on open aquatic habitats that are often less colonized by culicines compared to closed aquatic habitats such as pit latrines, soakage pits and septic tanks that our study did not consider. The latter are preferred breeding sites for culicines (Chavasse *et al.* 1995b; Impoinvil *et al.* 2008). It is therefore likely that there was continued re-colonization of aquatic habitats even in the intervention sites by adult culicines produced in higher densities in these closed pits that did not receive Sumilarv®0.5G.

To our knowledge our study is the first to evaluate the effectiveness of PPF formulations for the control of Afro-tropical malaria vectors under operational field conditions. In this study the three-weekly application of Sumilarv®0.5G was effective at inhibiting adult emergence from larvae and pupae collected from treated aquatic habitats by over 80%. Thus this study indicates the high susceptibility of malaria vectors in the western Kenya highlands to PPF. Previous studies have shown PPF to completely inhibit adult emergence of different mosquito genera in treated aquatic habitats in the field for three weeks up to six months (Kamimura and Arakawa 1991; Okazawa *et al.* 1991; Yapabandara and Curtis 2002; Seng *et al.* 2008). The finding that significantly greater reductions in adult emergence from treated aquatic habitats were achieved during the high transmission season as compared to the low transmission season strongly suggests that larviciding targeted at the high transmission season would be highly effective at

suppressing overall vector densities and reducing malaria transmission during its peak transmission time. Targeted interventions for malaria control have often been identified to be more cost-effective approaches than non-targeted interventions (Woolhouse *et al.* 1997; Carter *et al.* 2000; Bousema *et al.* 2012), however frequently it is suggested to target interventions in space (Mutuku *et al.* 2006; Gu *et al.* 2008), but here we suggest the value of targeting in time.

It is evident from this study that Sumilarv®0.5G has negative effects against some of the non-target aquatic organisms in the larval breeding habitats of malaria vectors in western Kenya highlands. Minor morphogenetic aberrations of PPF against odonata has previously been reported (Schaefer and Miura 1990). Despite the effects of Sumilarv®0.5G on odonata, the insecticide had minimal or no impact on other non-target organisms common in the mosquito larval habitats in our study area. Thus evaluations of impact of Sumilarv®0.5G on odonata at lower concentrations are necessary. Nevertheless, Sumilarv®0.5G appears safe against other non-target insects evaluated in this study. Targeting Sumilarv®0.5G application to habitats in time can be used to reduce the impact of the insecticide against non-target aquatic insects.

Although to date there is no report of development of mosquito resistance to PPF, the possibility of this happening cannot be ruled out (Schaefer and Mulligan 1991). It would be interesting to understand whether the progeny of mosquitoes that survive exposure to PPF in the field during their immature stages become less susceptible to the insecticide than unexposed mosquitoes. If this is so resistance to PPF may spread. This is especially due to reports of mosquito resistance to other insect growth regulators that share the same mechanism of action as PPF (Brown *et al.* 1978; Dame *et al.* 1998). It is suggested that increased metabolic detoxification of methoprene is responsible for development of resistance of mosquitoes to this juvenile hormone mimic (Brown and Hooper 1979). Moreover cross resistance to juvenile hormone mimics such as methoprene and hydropene and a chitin synthesis inhibitor has been reported (Brown *et al.* 1978). We suggest that targeted application of Sumilarv®0.5G in time or use of the insecticide in rotation or in combination with other larvicides with other classes of insecticides with different modes of action can be used to manage insecticide resistance development.

It is clear from our study that bioassays with water samples collected from aquatic habitats in the field underestimates the impact of Sumilarv®0.5G at inhibiting adult

vector emergence from treated habitats. Our findings are in agreement with those of previous studies that show reduced impact of PPF when larvae were introduced into collected water samples (Kawada *et al.* 1988; Yapabandara *et al.* 2001). In fact one of these studies reported the effectiveness of PPF to be three times less when larvae were exposed in water collected from the field than when larvae were exposed directly in treated water in floating cups (Kawada *et al.* 1988). Previous studies indicate that PPF actively adsorbs onto organic material and mud in treated aquatic habitats leaving minimal amounts of the insecticide dissolved in water at any given time (Mulligan III and Schaefer 1990; Schaefer and Miura 1990; Schaefer *et al.* 1991). The granular PPF formulation used in this study was a slow release formulation that slowly releases PPF into the water. Thus removal of water from the habitat removes it from its source which can explain the reduced emergent inhibition of insectary-reared larvae introduced into the water samples. Nevertheless, our chromatographic analysis detected PPF in some of the water samples collected from treated aquatic habitats. It is important to mention here that the water samples were collected from as close to the base of the aquatic habitats as possible increasing the amount of solid materials collected from the habitats. This is because the PPF granule sinks to the bottom of habitats thus likely creates a heterogeneous concentration of the chemical in the water with greater concentrations closer to the base of the habitat. Nevertheless our chromatographic analysis indicates declining amount of PPF in the water samples over the three-week survey period. This is likely due to slower degradation of the insecticide overtime in aquatic habitats in the field due to exposure to sunlight (Hemingway *et al.* 1988; Schaefer *et al.* 1988).

It is likely that PPF persists in mud and organic material during periods of dryness and is slowly released when habitats become wet again. This phenomenon has been reported in other studies (Okazawa *et al.* 1991; Yapabandara and Curtis 2002). Other than persistence during dry periods, PPF has also been shown to retain its emergence inhibition effects even after dilution of treated water or replacement of treated with untreated water (Okazawa *et al.* 1991; Vythilingam *et al.* 2005). This is clearly shown in our study of reduced adult emergence of pupae and larvae collected from treated aquatic habitats during the high transmission season. This is indicative of the potential of Sumilarv®0.5G for mosquito control even during the rainy season when dilution of applied larvicide would normally be expected.

The three-weekly application of Sumilarv®0.5G in the study area was short enough to prevent adult mosquito production from untreated aquatic habitats newly created in-between successive treatment rounds. If this would be so in areas with higher water temperatures (Bayoh and Lindsay 2003; Lyons *et al.* 2013) where larval development is faster would have to be confirmed.

4.6   Conclusion

During peak malaria transmission PPF reduced the abundance of mature anopheline larvae by more than 80% in the intervention sites. The findings of this study suggest that Sumilarv®0.5G would have to be re-applied at least once every month and 12-15 times in a year. Despite showing impact on odonata, Sumilarv®0.5G appears safe against the other non-target aquatic insects. The unique mode of action of PPF can be used to manage development of insecticide especially if the insecticide is targeted in time or its application done rotationally or in combination with other insecticides with different modes of action. Our findings recommend 3-4 weekly application of Sumilarv®0.5G in areas where mosquito larval habitats are focal and well defined as in the western Kenya highlands.

5 Pyriproxyfen for mosquito control: female sterilization or horizontal transfer to oviposition substrates by *Anopheles gambiae sensu stricto* and *Culex quinquefasciatus*

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5.1 Abstract

Background: The use of gravid mosquitoes as vehicles to auto-disseminate larvicides was recently demonstrated for the transfer of pyriproxyfen (PPF) by container-breeding *Aedes* mosquitoes and presents an appealing idea to explore for other disease vectors. The success of such an approach depends on the female's behaviour, the appropriate time of exposure and the amount of PPF that can be carried by an individual. Here we explore the effect of PPF exposure at seven time points around blood feeding on individual *Anopheles gambiae sensu stricto* and *Culex quinquefasciatus* fecundity and ability to transfer in laboratory assays.

Method: Mosquitoes were exposed to 2.6 mg PPF per m^2 at 48, 24 and 0.5 hours before and after blood meal and on the day of egg-laying. The proportion of exposed females (N=80-100) laying eggs, the number of eggs laid and hatched was studied. Transfer of PPF to the oviposition cups was assessed by introducing 10 late instar insectary-reared *An. gambiae s.s.* larvae into all the oviposition cups and monitored for adult emergence inhibition.

Results: Exposure to PPF between 24 hours before and after a blood meal had significant sterilizing effects: females of both species were 6 times less likely (Odds ratio (OR) 0.16, 95% confidence interval (CI) 0.10-0.26) to lay eggs than unexposed females. Of the few eggs laid, the odds of an egg hatching was 17 times reduced (OR 0.06, 95% CI 0.04-0.08) in *Anopheles* but only 1.2 times (OR 0.82, 95% CI 0.73-0.93) in *Culex*. Adult emergence inhibition from larvae introduced in the oviposition cups was observed only from cups in which eggs were laid. When females were exposed to PPF close to egg laying they transferred enough PPF to reduce emergence by 65-71% (95% CI 62-74%).

Conclusion: PPF exposure within a day before and after blood feeding affects egg-development in *An. gambiae s.s.* and *Cx. quinquefasciatus* and presents a promising opportunity for integrated control of vectors and nuisance mosquitoes. However, sterilized females are unlikely to visit an oviposition site and therefore do not transfer lethal concentrations of PPF to aquatic habitats. This suggests that for successful auto-dissemination the optimum time for contamination is close to oviposition.

5.2   Background

Mosquito larval source management is an effective method for controlling mosquito-borne diseases (Soper 1966; Killeen *et al.* 2002b; Walker and Lynch 2007; White *et al.* 2011a; Tusting *et al.* 2013). However, application of larvicides requires labour intensive programmes that are complex to organize and expensive to run (Vanek *et al.* 2006; Fillinger *et al.* 2008; Chaki *et al.* 2009). Thus novel strategies for larvicide application need to be explored to minimize efforts and costs (Devine and Killeen 2010; Fillinger and Lindsay 2011). Using the gravid female mosquito as a vehicle to auto-disseminate larvicides has been demonstrated recently for the transfer of pyriproxyfen (PPF) by container-breeding *Aedes* mosquitoes (Itoh *et al.* 1994; Devine *et al.* 2009; Caputo *et al.* 2012) and presents an appealing idea to explore for the control of other mosquito genera.

PPF is a juvenile hormone mimic and affects immature and adult mosquito stages in different ways (Yapabandara and Curtis 2004; Sihuincha *et al.* 2005; Harris *et al.* 2013; Mbare *et al.* 2013). The major effect of PPF on mosquitoes is the inhibition of metamorphosis to prevent emergence of adults from pupae (Mulligan III and Schaefer 1990; Yapabandara *et al.* 2001). PPF has extremely low toxicity to humans (WHO 2008a), is effective at controlling mosquito larvae at very low doses (Yapabandara and Curtis 2002; Mbare *et al.* 2013) and can persist for up to six months in a variety of aquatic habitat types (Chavasse *et al.* 1995a; Yapabandara *et al.* 2001; Sihuincha *et al.* 2005; Vythilingam *et al.* 2005). In addition, exposure of larvae to sub-lethal doses of PPF affects the adults' egg development, egg production and reduces the hatching of eggs (Loh and Yap 1989; Kamal and Khater 2010). Exposure to PPF has been studied extensively in *Aedes* mosquitoes (Itoh *et al.* 1994; Dell Chism and Apperson 2003; Sihuincha *et al.* 2005; Caputo *et al.* 2012; Ohba *et al.* 2013) and it has been shown that topical application can also reduce the reproductive capacity of adults (Itoh *et al.* 1994; Sihuincha *et al.* 2005; Ohba *et al.* 2013) depending on dosage and time of exposure in relation to the blood meal (Itoh *et al.* 1994), which signals the start of egg development (Lounibos *et al.* 1998). However, inconsistent information on the effect of PPF exposure on egg-laying and hatching of eggs laid can be found for various species requiring more research in this subject area (Miller 1993; Itoh *et al.* 1994; Sihuincha *et al.* 2005; Aiku *et al.* 2006).

To date only three studies have evaluated the impact of topical contact of PPF on *Anopheles gambiae sensu lato*, the major African malaria vector (Ohashi *et al.* 2012; Harris *et al.* 2013; Ngufor *et al.* 2014). Ohashi *et al.* (2012) exposed *An. gambiae s.s.* to treated nets immediately before or after a blood meal and reported complete sterilization in females exposed to nets that retained an approximate dose of 35 mg/m^2 PPF and 3.5 mg/m^2 PPF. However, at a 10 times lower dosage the proportion of females laying eggs was reduced by less than 50% compared to the control when exposed just before the blood meal and not at all when exposed after the blood meal. A more recent study by Ngufor *et al.* (2014) confirmed complete sterilization in wild *An. gambiae s.s.* exposed to PPF-treated nets in experimental hut trials. Harris *et al.* (2013) however, observed complete sterilization of female *An. arabiensis* only 24 hours after the blood meal (exposed to 3mg/m^2 PPF) but not when exposed 24 hours before a blood meal, challenging the idea that treating bed nets would be a successful intervention for this species.

Culex quinquefasciatus is another important disease vector responsible for the transmission of *Wucheria bancrofti* (lymphatic filariasis), and arboviruses like Western equine encephalitis virus, St Louis encephalitis virus and West Nile virus (Ramaiah *et al.* 1989; Ramaiah *et al.* 1994). It is also an abundant nuisance mosquito in many tropical and subtropical areas (Dossou-yovo *et al.* 1995; David *et al.* 2012). Conflicting reports arise from two studies that evaluate the impact of PPF on exposed *Cx. quinquefasciatus*. Whilst Mosqueira *et al.* (2010) reported both a reduction in the number of eggs laid and hatchings in *Cx. quinquefasciatus* exposed 24-36 hours before blood meal to an insecticidal paint formulation that contained PPF, Ngufor *et al.* (2014) found that exposure of *Cx. quinquefasciatus* to PPF-treated nets while seeking a blood meal had no effect on the reproductive capacity.

Whilst the sterilizing effect of PPF on adult mosquito vectors is by itself an important aspect to study for developing novel vector control strategies, it is also likely that it affects the potential of a female to transfer the insecticide to a larval habitat. The major challenge in the development of such an auto-dissemination approach is therefore to find the best timing and strategy to expose female mosquitoes to PPF to ensure that a large quantity of the insecticide gets picked up and transferred to an aquatic habitat. The best knowledge we have of the behaviour of *An. gambiae s.s.* is its indoor host-seeking and resting behaviour associated with the need for a blood meal (Smith *et al.* 1966; Boreman

and Port 1982; de Jong and Knols 1995; Mukabana *et al.* 2002). Consequently, contaminating females during this time period would be the easiest e.g. exposing females to treated resting sites (Harris *et al.* 2013) or bed nets (Aiku *et al.* 2006), however this timing might coincide with sterilizing effects and affect the ability to transfer PPF. Another challenge of the auto-dissemination approach for malaria control is the low density of adult anophelines in comparison to the surface area of the aquatic habitats (Fillinger *et al.* 2004). To increase the amount of PPF transferred to *An. gambiae s.l.* larval habitats, other co-habiting mosquito species i.e. *Culex* mosquitoes (Robert *et al.* 1998; Muturi *et al.* 2008; Dejenie *et al.* 2011) might also be targeted for transfer, especially since their immature stages are frequently of a greater density (Fillinger *et al.* 2004; Munga *et al.* 2006; Ndenga *et al.* 2012).

Here we explored the effect of PPF exposure at different points in time before and after a blood meal on the egg-laying and hatching of eggs in *An. gambiae s.s.* and *Cx. quiquefasciatus* and how this affects their ability to transfer PPF to a breeding site. We had the following hypotheses: (1) PPF exposure of adult *An. gambiae s.s.* and *Cx. quinquefasciatus* affects their ability to lay eggs and the number of offspring hatched from eggs laid, (2) the impact is largest when exposure takes place around blood feeding time (3) the concentration of PPF transferred by a single female is very low requiring a large number of females to transfer lethal concentrations (LC_{99}) (4) sterile females do not transfer PPF and (5) for auto-dissemination of PPF females need to be exposed not more than 24 hours prior to oviposition.

5.3   Methods

5.3.1   Mosquitoes

The study was carried at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (*icipe*-TOC) located in Mbita, along the shores of Lake Victoria, Western Kenya (geographic coordinates 0º 26' 06.19" S, 34º 12' 53.13"E; altitude 1,137m above sea level) with larvae and pupae of *An. gambiae s.s.* and *Cx. quinquefasciatus* obtained from the *icipe*-TOC's insectary. Larvae were reared in round plastic tubs (diameter 0.6m) filled with 5 litres of water (height approximately 5 cm) from Lake Victoria filtered through a charcoal-sand filter. Mosquito larvae were fed with

Tetramin© Baby Fish food twice daily. Mosquito larvae were reared at ambient climate and light conditions in a netting-screened greenhouse with temperature of 25-28ºC, relative humidity of 68-75% and a natural 12 hours of dark and 12 hours of light cycle. Pupae were collected from tubs and transferred into holding cages measuring 30x30x30 cm covered with mosquito netting. Adults were provided with 6% glucose solution *ad libitum*. Mosquitoes of both species were provided with a single blood meal when they were three days old; *An. gambiae s.s.* fed on a human arm for 20 minutes whilst *Cx. quinquefasciatus* were fed on a rabbit for 20 minutes. The females of either species were maintained in cages with equal numbers of males of the same species at all times to increase the chances of insemination.

5.3.2 Test insecticide

An experimental formulation of Sumilarv® dust containing 2% of PPF was provided by the manufacturer, Sumitomo Chemicals, Japan. Dust particles measured approximately12 μm diameter. Sumilarv® is a registered trademark of Sumitomo Chemical Company.

5.3.3 Exposing female mosquitoes to PPF

A suspension was prepared by mixing 0.25 g of the insecticide with 10 ml of acetone in a 100 ml glass beaker and vortexing on a shaker for 20 minutes. The inner surfaces of plastic jars (7.8 cm diameter, 9.2 cm height, 350 ml capacity) were coated by pipetting 150 μl of the suspension (0.075 mg active ingredient) into the jar. To ensure uniform coating of the base and side surfaces an additional 100 μl of acetone was added to the jar. The jar was then rolled several times on its base and side. The total surface area coated was approximately 0.028 m^2 to give a concentration of 2.6 mg/m^2 of active ingredient. A control jar of similar measurements was treated in a similar manner with acetone. Jars were left to air-dry for 30 minutes. New suspensions and jars were used for every treatment and replicate round.

Female mosquitoes originating from the same batch of pupae per round were exposed to PPF at the following times in relation to when they were blood fed (Figure 5.1): (1) 48 hours before a blood meal (2) 24 hours before a blood meal (3) 0.5 hours before blood meal; and (4) 0.5 hours after a blood meal (5) 24 hours after a blood meal (6) 48 hours

after a blood meal, and (7) on the day of egg-laying (72 hours after a blood meal in *An. gambiae s.s.* and 144 hours after a blood meal in *Cx. quinquefasciatus*). Control females were exposed to acetone-only contaminated jars 0.5 hours before a blood meal.
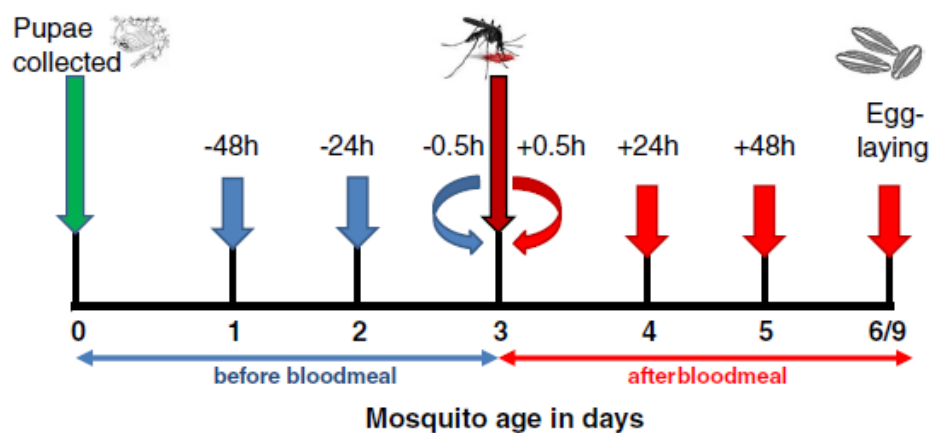
Figure 5.1: Schematic diagram of the PPF-exposure times for *An. gambiae s.s.* and *Cx. quinquefasciatus*. Blue arrows show treatment show treatment groups exposed before a blood meal, red arrows show treatment groups exposed after a blood meal. Control females were exposed to acetone at 0.5 hours before blood meal. Time of egg-laying was in *An. gambiae s.s.* 72 hours after a blood meal (6 day old females) and in *Cx. quinquefasciatus* 144 hours after a blood meal (9 day old females). All treatment groups and control were tested in parallel, 20 individual females at a time, repeated 4-5 times (rounds).

Groups of 150 females per treatment per round were transferred to a PPF-contaminated jar covered with non-contaminated mosquito netting for 30 minutes. Most of the females rested on the plastic, but when a mosquito attempted to rest on the mosquito netting it was gently disturbed to rest on the contaminated surfaces of the jar. After exposure they were transferred into 30x30x30 cm cages per treatment group and an equal number of males added to maximize the chance of females mated at the time of experiment. Glucose solution (6%) was provided *ad libitum*. On the day of experiment (see below) 20 gravid females per treatment were selected from their holding cages.

5.3.4 Measuring the effect of PPF exposure on females' ability to lay eggs and the eggs' ability to hatch

Oviposition experiments were implemented 72 hours after a blood meal with *An. gambiae s.s.* and 144 hours after a blood meal with *Cx. quinquefasciatus* based on the different egg maturation times. For each experimental round and treatment 20 gravid females were selected individually from their holding cage and transferred to netting covered cages of 15x15x15 cm at 18:00 h. A single female was introduced into a cage that contained a glass cup (diameter 7 cm) filled with 100 ml of non-chlorinated tap water for oviposition. *Anopheles gambiae s.s.* females exposed to PPF 72 hours after a blood meal and *Cx. quinquefasciatus* exposed to PPF 144 hours after a blood meal were transferred directly from the exposure jar into the experimental cages containing an oviposition cup. Mosquitoes were left to lay eggs overnight. The following morning the presence of eggs or egg rafts were recorded, and in the case of *An. gambiae s.s.* the number of eggs counted, and then transferred into separate 300 ml plastic cups filled with 100 ml non-chlorinated tap water. The number of larvae that hatched from eggs laid by individual females was recorded.

Five rounds of the experiment were carried out with *An. gambiae s.s.* (5 x 20 replicates/treatment) and four rounds with *Cx. quinquefasciatus* (4x 20 replicates/treatment) on separate dates. Therefore, in total 100 individual *An. gambiae s.s.* and 80 individual *Cx. quinquefasciatus* females were tested in each treatment arm.

5.3.5 Assessment of delayed egg-laying in PPF-exposed *An. gambiae s.s.*

To assess whether PPF exposure caused delayed egg-laying in female *An. gambiae s.s.*, tests were conducted with females exposed to PPF: (1) 24 hours before a blood meal, (2) 0.5 hours before a blood meal, (3) 0.5 hours after a blood meal and (4) 24 hours after a blood meal. These were compared to a control group of females that were exposed to acetone 0.5 hours before a blood meal. Females were prepared as described above and provided with oviposition cups 72 hours after a blood meal and left to lay eggs overnight. The following morning the presence and number of eggs by each female was recorded. Thereafter fresh oviposition cups were provided in all cages with the same mosquitoes maintained in the cages with 6% glucose solution *ad libitum*. The oviposition cup was left in the cage for a further two days to determine if mosquitoes would lay eggs. These tests

were conducted in three rounds on separate dates with each round containing 20 replicates of each treatment and the control group. Thus in total 60 individual mosquitoes per treatment and control group were tested.

5.3.6 Analyses of transfer of PPF by adult *An. gambiae s.s.* and *Cx. quinquefasciatus* to the water in the oviposition cups

To evaluate whether *An. gambiae s.s.* and *Cx. quinquefasciatus* transferred PPF to the water, 10 insectary-reared late instar *An. gambiae s.s.* larvae were introduced into all the oviposition cups in the morning after the removal of the eggs. For that, larvae were randomly collected from rearing tubs in the larval insectary to ensure that equal sizes of larvae were used in the experimental cups (Araujo *et al.* 2012). The larvae were monitored daily for mortality or pupation. During the monitoring period mosquito larvae were fed on fish food (Tetramin© Baby) daily. This was done by wetting a blunt toothpick in non-chlorinated tap water followed by dipping less than 1 mm of toothpick into the larval food. The toothpick was then dipped onto the surface of the test water. Pupae were transferred into a separate glass cup with approximately 50 ml of water non-chlorinated tap water and the cup covered with mosquito netting to prevent any escape of emerged adults. Pupae were monitored for adult emergence.

5.3.7 Statistical analyses

Generalized estimating equations (GEE) were used to analyse the data. The experimental round was included as repeated measure. Proportions were analysed by fitting a binomial distribution with logit link function and counts analysed by fitting a negative binomial distribution with log link function. An exchangeable correlation matrix was assumed. Treatment group was included as the fixed factor in the models with the control group as reference. All means (proportion or counts) per treatment and their 95% confidence intervals (CIs) were modelled as the exponential of the parameter estimates for models with no intercept included. Multiple comparisons of treatments were also calculated based on the model parameter estimates. Abbott's formula (Abbott 1987) was used to calculate proportion reductions in egg-laying responses, egg-hatching success and emergence of adults from larvae introduced in the different treatment groups taking the natural response/mortality of the control group into account.

5.3.8 Ethical considerations

Ethical approval for this study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol no. 422).

5.4 Results

5.4.1 Effect of PPF exposure on females' ability to lay eggs and the eggs' ability to hatch

PPF exposure affected *An. gambiae s.s.* and *Cx. quinquefasciatus* egg-laying as early as 48 hours prior and up to 24 hours after a blood meal in *An. gambiae s.s.* and 48 hours after a blood meal in *Cx. quinquefasciatus* (Table 5.1). However, the proportion of females laying was only reduced by approximately one third when exposed to PPF 48 hours before a blood meal compared to the control group (Table 5.1). The highest reduction due to the treatments in both species was roughly 60%, which was achieved by PPF exposure between 24 hours before and 24 hours after a blood meal in *An. gambiae s.s.* and between 24 hours before and 0.5 hours after a blood meal in *Cx. quinquefasciatus*. In *An. gambiae s.s.*, the odds of laying as compared to not laying in the control was 3.3:1 whilst the odds of laying versus not laying was on average 0.45:1 in females exposed to PPF 24 hours before until 24 hours after a blood meal. Hence compared to the control the odds of laying was 7-8 times reduced (OR 0.12-0.15) when *An. gambiae s.s.* were exposed to PPF 24 hours before and up to 24 hours after a blood meal. Similarly, the odds of laying in *Cx. quinquefasciatus* was 4-9 times reduced (OR 0.11-0.25) when females were exposed to PPF between 24 hours before and 24 hours after a blood meal. Late contamination of *An. gambiae s.s.* with PPF at 48 hours and 72 hours after a blood meal and of *Cx. quinquefasciatus* at 144 hours after a blood meal did not affect the proportion of females laying eggs (Table 5.1).

Table 5.1: Effect of PPF exposure on the proportion of females laying eggs

Exposure time to PPF in relation to blood meal	Proportion that laid eggs (95% CI)	Proportion reduction in laying (95% CI)	Odds ratio (95% CI)	p-value
*Anopheles gambiae s.s.**				
72 hours after	0.80 (0.73-0.87)	0	1.20 (0.68-2.14)	0.460
48 hours after	0.80 (0.75-0.85)	0	1.21 (0.78-1.88)	0.390
24 hours after	0.33 (0.24-0.43)	0.56 (0.48-0.66)	0.15 (0.08-0.29)	<0.001
0.5 hours after	0.31 (0.23-0.41)	0.59 (0.50-0.68)	0.14 (0.05-0.34)	<0.001
0.5 hours before	0.33 (0.24-0.43)	0.57 (0.48-0.66)	0.15 (0.11-0.20)	<0.001
24 hours before	0.29 (0.21-0.39)	0.62 (0.52-0.70)	0.12 (0.07-0.21)	<0.001
48 hours before	0.52 (0.42-0.62)	0.32 (0.24-0.41)	0.32 (0.18-0.60)	<0.001
Control	0.76 (0.71-0.82)	-	1	
*Culex quinquefasciatus***				
144 hours after	0.68 (0.58-0.76)	0.05 (0.02-0.10)	0.87 (0.67-1.12)	0.450
48 hours after	0.58 (0.47-0.78)	0.19 (0.14-0.27)	0.48 (0.34-0.68)	0.020
24 hours after	0.41 (0.31-0.52)	0.43 (0.33-0.52)	0.25 (0.14-0.43)	<0.001
0.5 hours after	0.24 (0.16-0.34)	0.66 (0.56-0.75)	0.11 (0.08-0.16)	<0.001
0.5 hours before	0.29 (0.20-0.40)	0.59 (0.48-0.69)	0.14 (0.09-0.20)	<0.001
24 hours before	0.31 (0.22-0.42)	0.56 (0.46-0.66)	0.16 (0.10-0.27)	<0.001
48 hours before	0.46 (0.36-0.57)	0.36 (0.26-0.44)	0.31 (0.18-0.51)	<0.001
Control	0.72 (0.65-0.78)	-	1	

* Egg-laying took place 72 hours after blood meal

** Egg-laying took place 144 hours after blood meal

Of those few *An. gambiae s.s.* that laid eggs, the mean number of eggs laid per female was reduced by 21-36% compared to the control females if exposure to PPF occurred between 24 hours before and 24 hours after a blood meal whilst the numbers were similar to the control when exposure occurred 48 hours and 72 hours after a blood meal (Table 5.2).

Table 5.2: Mean number of eggs laid by unexposed and PPF-exposed *An. gambiae s.s.*

Exposure time to PPF in relation to blood meal	Mean no. of eggs* (95% CI)	Odds ratio (95% CI)	p-value
72 hours after	49.4 (45.5-53.6)[a,c]	0.97 (0.86-1.09)	0.580
48 hours after	49.4 (46.4-52.6)[a,c]	0.97 (0.88-1.07)	0.520
24 hours after	37.8 (32.3-44.2)[b,c]	0.74 (0.62-0.90)	0.002
0.5 hours after	32.9 (27.9-38.7)[b]	0.64 (0.53-0.79)	<0.001
0.5 hours before	40.0 (34.2-46.8)[a,b]	0.78 (0.65-0.95)	0.010
24 hours before	40.3 (34.1-47.6)[a,b]	0.79 (0.65-0.97)	0.019
48 hours before	45.0 (39.8-51.0)[a,b]	0.88 (0.76-1.03)	0.110
Control	51.1 (47.9-54.4)[a]	1	

* Only females that laid eggs were included in analysis

 Values without letters in common differ significantly (p<0.05) in mean number of eggs laid.

It was 13-20 times less likely for an *An. gambiae s.s.* egg to hatch into a larva (OR 0.05-0.08) when the mother was exposed to PPF between 24 hours before and 24 hours after blood feeding (Table 5.3). However, there was no difference in egg hatchings in eggs laid by *An. gambiae s.s.* exposed close to oviposition time with those laid by control females (Tables 5.3). The impact of PPF exposure on the mean number of larvae that successfully hatched from an egg raft of *Cx. quinquefasciatus* was only moderately reduced by 1.3-1.7 times compared to egg hatching in the control (Table 5.3).

Table 5.3: Effect of PPF exposure of female mosquito on hatching of her eggs

Anopheles gambiae s.s.*

Exposure time to PPF in relation to blood meal	Mean proportion eggs hatched* (95% CI)	Proportion reduction in hatched larvae (95% CI)	Odds ratio (95% CI)	p-value
72 hours after	0.86 (0.85-0.87)	0	0.99 (0.80-1.23)	0.910
48 hours after	0.84 (0.82-0.85)	0	0.99 (0.82-1.19)	0.910
24 hours after	0.22 (0.19-0.24)	0.73 (0.71-0.77)	0.06 (0.05-0.09)	<0.001
0.5 hours after	0.19 (0.17-0.23)	0.77 (0.73-0.80)	0.05 (0.03-0.07)	<0.001
0.5 hours before	0.21 (0.18-0.23)	0.75 (0.72-0.78)	0.06 (0.05-0.08)	<0.001
24 hours before	0.24 (0.22-0.27)	0.71 (0.68-0.73)	0.08 (0.06-0.11)	<0.001
48 hours before	0.54 (0.51-0.56)	0.35 (0.34-0.38)	0.27 (0.22-0.34)	<0.001
Control	0.84 (0.83-0.85)	-	1	

Culex quinquefasciatus**

Exposure time to PPF in relation to blood meal	Mean no. of larvae hatched per egg raft (95% CI)	Proportion reduction in hatched larvae (95% CI)	Odds ratio (95% CI)	p-value
144 hours after	76.4 (75.3-77.5)	6.9 (5.2-8.6)	0.94 (0.89-0.98)	0.008
48 hours after	66.0 (60.6-72.0)	18.5 (15.8-21.3)	0.81 (0.77-0.86)	<0.001
24 hours after	66.4 (63.7-69.2)	18.0 (13.9-21.8)	0.82 (0.77-0.87)	<0.001
0.5 hours after	67.8 (59.9-76.8)	16.6 (9.8-22.8)	0.83 (0.70-0.99)	0.035
0.5 hours before	61.9 (56.2-68.2)	23.6 (18.3-28.5)	0.76 (0.69-0.83)	<0.001
24 hours before	72.9 (65.0-81.7)	10.4 (4.8-15.8)	0.90 (0.76-1.03)	0.130
48 hours before	51.2 (49.4-53.0)	36.9 (33.9-39.8)	0.63 (0.60-0.66)	<0.001
Control	81.4 (76.6-86.6)	-	1	

*Eggs were counted for *An. gambiae s.s.* and the proportion that hatched calculated.

**The number of eggs per egg raft was not counted. Comparisons are made between mean numbers of larvae per egg raft.

PPF exposure did not induce any significant delays in egg-laying. The exposure either sterilized the female so that she did not lay at all, or she laid 72 hours after the last blood meal like unexposed control females (Table 5.4).

Table 5.4: Evaluation of delayed egg-laying in *An. gambiae s.s.* due to PPF exposure

Exposure time to PPF in relation to blood meal	Number of females exposed	Females laying eggs 72 hrs after blood meal	Females laying eggs later than 72 hrs after blood meal	Had the female laid eggs before?
24 hours after	60	14	0	_
0.5 hours after	60	23	1	No
0.5 hours before	60	24	0	_
24 hours before	60	17	1	No
Control	60	49	2	No

5.4.2 Transfer of PPF by adult *An. gambiae s.s.* and *Cx. quinquefasciatus* to the water in the oviposition cups

Transfer of PPF to the oviposition substrate and consequent emergence inhibition of introduced late instar *An. gambiae s.s.* larvae was assessed separately for the following two groups: (1) oviposition substrates originating from females that laid eggs; and (2) oviposition substrates originating from females that did not lay eggs.

Emergence was inhibited from all treatments compared to the control when females laid eggs. However, the reduction was very low with 13-28% emergence inhibition from cups that were visited by *An. gambiae s.s.* females exposed to PPF between 48 hours before to 24 hours after a blood meal and 6-19% emergence inhibition from cups that were visited by *Cx. quinquefasciatus* females that were exposed between 48 hours before to 48 hours after a blood meal (Table 5.5, Figure 5.2). Biologically significant emergence inhibition was only achieved when females were exposed to PPF very close to oviposition time i.e. 52-65% from treatments with *An. gambiae s.s.* exposed 48 hours to 72 hours after a blood meal and 71% from treatments with *Cx. quinquefasciatus* exposed 144 hours after a blood meal.

Table 5.5: Adult emergence from late instar larvae introduced into oviposition substrates

Exposure time to PPF in relation to blood meal	Mean adults emerged (95% CI)	Proportion emergence inhibition (95% CI)	Odds ratio (95% CI)	p-value
Anopheles gambiae s.s.				
Females that laid eggs				
72 hours after	0.32 (0.29-0.35)	0.65 (0.62-0.68)	0.04 (0.03-0.05)	<0.001
48 hours after	0.44 (0.41-0.46)	0.52 (0.51-0.54)	0.07 (0.05-0.09)	<0.001
24 hours after	0.66 (0.60-0.71)	0.28 (0.24-0.33)	0.18 (0.14-0.25)	<0.001
0.5 hours after	0.75 (0.70-0.80)	0.18 (0.14-0.22)	0.31 (0.19-0.51)	<0.001
0.5 hours before	0.78 (0.73-0.82)	0.15 (0.21-0.19)	0.36 (0.26-0.50)	<0.001
24 hours before	0.79 (0.74-0.83)	0.14 (0.11-0.18)	0.38 (0.25-0.58)	<0.001
48 hours before	0.80 (0.76-0.83)	0.13 (0.10-0.16)	0.38 (0.17-0.83)	0.015
Control	0.92 (0.90-0.93)		1	
Females that did not lay eggs				
72 hours after	0.88 (0.84-0.92)	-	0.80 (0.48-1.33)	0.380
48 hours after	0.87 (0.84-0.90)	-	0.69 (0.47-1.03)	0.070
24 hours after	0.86 (0.83-0.88)	-	0.62 (0.41-0.94)	0.020
0.5 hours after	0.86 (0.84-0.89)	-	0.67 (0.38-1.17)	0.160
0.5 hours before	0.88 (0.86-0.90)	-	0.79 (0.50-1.23)	0.290
24 hours before	0.90 (0.88-0.92)	-	0.96 (0.57-1.61)	0.870
48 hours before	0.89 (0.86-0.91)	-	0.82 (0.56-1.21)	0.320
Control	0.90 (0.88-0.93)	-	1	
Culex quinquefasciatus				
Females that laid eggs				
144 hours after	0.25 (0.22-0.29)	0.71 (0.67-0.74)	0.07 (0.06-0.09)	<0.001
48 hours after	0.70 (0.66-0.74)	0.19 (0.16-0.21)	0.28 (0.15-0.53)	<0.001
24 hours after	0.78 (0.73-0.82)	0.09 (0.07-0.13)	0.39 (0.29-0.54)	<0.001
0.5 hours after	0.76 (0.70-0.82)	0.12 (0.07-0.16)	0.37 (0.30-0.46)	<0.001
0.5 hours before	0.74 (0.68-0.79)	0.14 (0.10-0.19)	0.32 (0.16-0.65)	0.002
24 hours before	0.71 (0.65-0.76)	0.17 (0.14-0.23)	0.28 (0.23-0.36)	<0.001
48 hours before	0.81 (0.76-0.84)	0.06 (0.04-0.10)	0.46 (0.34-0.62)	<0.001
Control	0.86 (0.84-0.88)		1	
Females that did not lay eggs				
144 hours after	0.84 (0.79-0.87)	-	1.17 (0.94-1.45)	0.170
48 hours after	0.78 (0.73-0.82)	-	0.78 (0.61-0.99)	0.038
24 hours after	0.83 (0.80-0.86)	-	1.08 (0.60-1.96)	0.790
0.5 hours after	0.84 (0.81-0.87)	-	1.14 (0.90-1.45)	0.270
0.5 hours before	0.86 (0.83-0.88)	-	1.31 (0.88-1.95)	0.180
24 hours before	0.84 (0.80-0.87)	-	1.13 (0.69-1.83)	0.640

48 hours before	0.84 (0.80-0.87)	-	1.14 (0.83-1.57)	0.410
Control	0.82 (0.78-0.85)		1	

Conversely, when females did not lay eggs in the provided oviposition cup, emergence of introduced larvae was the same as in the control for all treatments and both species (Table 5.5, Figure 5.2).
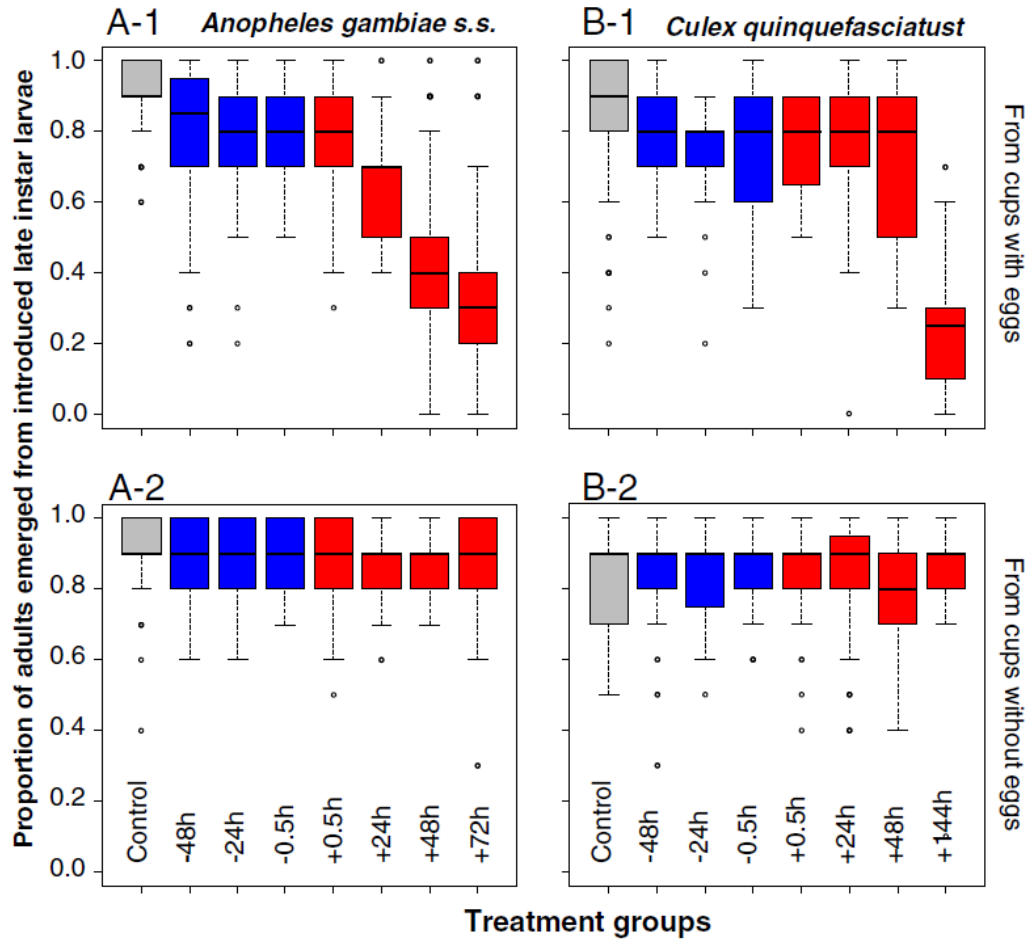
Figure 5.2: Box and whisker plots showing the median adult emergence rates from late instar larvae introduced into oviposition cups. Results for PPF-exposed *An. gambiae s.s.* (A) and *Culex quinquefasciatus* (B) from cups in which eggs were laid (1) and for cups in which eggs were not laid (2). Blue box plots show treatment groups exposed before a blood meal, red box plots show treatment groups exposed after a blood meal.

5.5   Discussion

Our study confirms a strong sterilizing effect of PPF on both *An. gambiae s.s.* and *Cx. quinquefasciatus* when females were exposed within 24 hours before or after a blood meal. Moreover, in our simple system we demonstrated that gravid females can transfer lethal concentrations of PPF to oviposition sites. However, our results suggest that for the use in an auto-dissemination approach females of both species would need to be exposed to PPF when already gravid so that sufficient PPF can be delivered to aquatic habitats.

The effect of PPF exposure on *An. gambiae s.s.* was three fold as it reduced the proportion of females laying eggs, the number of eggs laid and the number of eggs that successfully hatched into larvae when females were exposed to 2.6 mg/m^2 PPF between 24 hours before and 24 hours after a blood meal. However, the main effect of PPF exposure on *Cx. quinquefasciatus* during the same time interval was only in reducing the number of females laying eggs.

Overall, the number of offspring produced by females exposed to PPF 24 hours before to 24 hours after a blood meal was reduced between 91-94% in *An. gambiae s.s.* and 60-75% in *Cx. quinquefasciatus* compared to control females. The differences in sterilization between the two mosquito species might be explained by the larger size of *Cx. quinquefasciatus* relative to *An. gambiae s.s.* and their different ability to metabolize insecticides (Huestis *et al.* 2011; Kweka *et al.* 2012). Thus it is likely that larger concentrations of PPF are required to increase the impact of topical application on *Culex* mosquitoes.

The dependence of exposure time to PPF on reducing egg laying and hatching in mosquitoes has been shown in other studies (Itoh *et al.* 1994; Ohashi *et al.* 2012; Harris *et al.* 2013), however reported results are not consistent. For instance while Itoh *et al.* (1994) observed a reduction in number of eggs laid by *Ae. aegypti* exposed to PPF on the same day of blood meal, Sihuincha *et al.* (2005) reported that exposure of the same mosquito species at similar point in time did not affect the number of eggs laid. Only few studies have been done on the effect of PPF on egg-laying and hatching in *Anopheles* mosquitoes with contrasting findings. Aiku *et al.* (2006) reported that *An. stephensi* exposed to bed nets treated with 2% PPF at 24 hours after blood meal were as likely to

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lay and laid similar numbers of eggs as control mosquitoes but eggs were less likely to hatch. However Miller (1993) found that exposure of the same mosquito species to bed nets treated with 0.5 mg PPF/m^2 at the time of blood meal caused a reduction in number of eggs laid. These differences on the effect of PPF might be explained by the variations in PPF dosages used in the separate studies and the characteristics of surfaces onto which PPF is applied (Mosqueira *et al.* 2010; Ohashi *et al.* 2012).

Our study confirms the observation of Ohashi *et al.* (2012) that exposure of laboratory reared *An. gambiae s.s.* females to PPF at comparable dosage before and after a blood meal significantly reduces the number of offspring produced from these females. A recent study by Ngufor *et al.* (2014) also found complete sterilization in wild pyrethroid-resistant *An. gambiae s.s.* that came into contact with PPF treated nets while seeking a blood meal. Our observations extend their evidence by showing that the sterilizing effect can be achieved during a relatively large window of time between 24 hours before to 24 hours after a blood meal and at a relatively low concentration. Our results contrast however with those of Harris *et al.* (2013) that showed for the sibling species *An. arabiensis* a sterilizing effect when exposure took place 24 hours after the blood meal but not 24 hours before the blood meal. Further studies might be warranted to explore the individual susceptibility of these closely related species further when aiming at developing intervention strategies targeting both sibling species by topical application at the same time.

Our study provides strong evidence that exposure of adult vectors, both anophelines and culicines to PPF can contribute significantly to reduce their population density. The sensitivity of both *An. gambiae s.s.* and *Cx. quinquefasciatus* to sterilization by PPF close to a blood meal presents an excellent opportunity to integrate PPF in insecticide-treated bed nets, include PPF in indoor spays or wall paints to apply on inner surfaces of houses to reduce mosquitoes' reproductive capacity as females seek a blood meal or as they rest indoors after taking a blood meal. This impact would be greatly enhanced when sterilization occurs in successive gonotrophic cycles in addition to reduced lifespan as previously shown for *An. gambiae s.s.* exposed to PPF-treated nets (Ohashi *et al.* 2012). However, if both species should be targeted by the intervention, more research might be required to find the optimum dosages. Our findings on the sterilizing effect of PPF on *Cx. quinquefasciatus* confirm previous findings from a study on insecticidal paint containing

PPF (Mosqueira *et al.* 2010). Yet, a recent experimental hut trial with wild *Cx. quinquefasciatus* could not demonstrate any impact of exposure to treated nets on this species (Ngufor *et al.* 2014). Unfortunately, this study does not report the PPF dosage and one can only speculate that the larger size of the mosquito combined with a lower resting time on contaminated surfaces might be responsible for the differences between studies.

We were able to demonstrate in principle that female *An. gambiae s.s.* and *Cx. quinquefasciatus* can transfer PPF from contaminated resting surfaces to aquatic substrates. This study demonstrated that the greatest adult emergence inhibition occurred when *Cx. quinquefasciatus* females were exposed to PPF immediately prior to oviposition. Thus targeting gravid *Culex* species at their resting sites would increase the amount of PPF transferred to aquatic habitats in which immature stages of *An. gambiae s.l.* develop. However, the longer period in the gonotrophic cycle of *Culex* relative to that of *An. gambiae s.s.* presents a challenge in using *Cx. quinquefasciatus* or other *Culex* species for auto-dissemination. Whilst *An. gambiae s.s.* took 72 hours (3 days) after blood meal to lay eggs, *Cx. quinquefasciatus* females laid eggs only 144 hours (6 days) after blood meal. Studies have described the gonotrophic cycle in *An. gambiae s.s.* to last 2-3 days (Gillies and Wilkes 1965; Quinones *et al.* 1997) while that of *Cx. quinquefasciatus* and other *Culex* species lasts 3-6 days (Subra 1981; Begum *et al.* 1985; McHugh 1990; Garcia-Rejon *et al.* 2008). As shown in our study, this extended period increases the amount of PPF that this mosquito species will lose if exposure to the chemical is not done close to oviposition time. The loss of PPF overtime from body surfaces of mosquitoes has been explored in other studies (Itoh *et al.* 1994; Gaugler *et al.* 2012).

The auto-dissemination technique has been successfully explored with *Aedes* mosquitoes in both laboratory and field settings (Itoh 1993; Itoh *et al.* 1994; Dell Chism and Apperson 2003; Sihuincha *et al.* 2005; Devine *et al.* 2009; Caputo *et al.* 2012; Gaugler *et al.* 2012). Field studies have shown that *Ae. aegypti* and *Ae. albopictus* females can transfer PPF from limited contaminated resting sites to larval habitats to reduce adult emergence rates of developing larvae by 42-100% (Devine *et al.* 2009; Caputo *et al.* 2012). Three factors that are related to the oviposition behaviour of targeted *Aedes* mosquitoes contribute to the success of this strategy in the control of this mosquito species. First, *Aedes* mosquitoes utilize containers that hold small volumes of water as breeding habitats (Burkot *et al.* 2007; Vezzani 2007; Bartlett-Healy *et al.* 2012). Second,

laboratory assays indicate that 94% of *Ae. aegypti* distribute their eggs in up to seven oviposition cups in a single gonotrophic cycle, a phenomenon termed as skip-oviposition (Chadee 2010), and field studies have shown that a relatively large number of females lay their eggs in a small oviposition container (Colton *et al.* 2003; Rapley *et al.* 2009; Barrera *et al.* 2013; Mackay *et al.* 2013). Third, PPF contamination in successful trials took place close to oviposition time (Itoh *et al.* 1994; Dell Chism and Apperson 2003; Gaugler *et al.* 2012). These factors permit *Aedes* mosquitoes to accomplish several transfer events of PPF between contaminated surfaces and aquatic habitats to amplify adult emergence inhibition. *Aedes'* behaviour is in sharp contrast to that of *An. gambiae s.l. Anopheles gambiae s.l.* colonizes natural habitats of varying size and stability (Gimnig *et al.* 2001; Fillinger *et al.* 2004; Majambere *et al.* 2008) and is frequently found in extensive water bodies (Majambere *et al.* 2010) with low larval densities per surface area (Mala and Irungu 2011; Ndenga *et al.* 2011). Furthermore molecular evidence of sibling relationships suggest that few females (average of 2-10 females) lay eggs in a typical larval habitat (Chen *et al.* 2008b). Although *An. gambiae s.l.* does skip-oviposit occasionally (Chen *et al.* 2006; Herrera-Varela *et al.* 2014), it is not the norm in this species. A recent study (Herrera-Varela *et al.* 2014) showed that approximately 20-30% of gravid females might choose more than one habitat to lay her eggs.

To our knowledge this is the first report of the potential use of the disease vectors, *An. gambiae s.s.* and *Cx. quinquefasciatus* for use in auto-dissemination of PPF to aquatic substrates to inhibit adult emergence. A recent study by Lwetoijera and others (Lwetoijera *et al.* 2014) provided proof of principle that another member of *An. arabiensis*, a member of the *An. gambiae s.l.*, can transfer lethal doses of PPF from contaminated resting surfaces to oviposition water to effectively inhibit successful development of eggs laid in the water into adult vectors. In the present study significantly higher emergence inhibition rates were recorded in oviposition cups where PPF-exposed female mosquitoes laid eggs compared to the controls. However sterilized females that were exposed to PPF between 24 hours before and after a blood meal did not transfer sufficient PPF to water to cause biologically important emergent inhibition rates. There are two possible explanations for this phenomenon. First, sterile females have less or no mature eggs to lay (Judson and de Lumen 1976; Bai *et al.* 2011) and therefore have little urge to visit aquatic substrates. Secondly, chemical analysis by high performance liquid

chromatography (HPLC) reveal that early exposure of mosquitoes to PPF results in loss of greater amounts of the chemical before oviposition time (Itoh *et al.* 1994).

Our study suggests that for *An. gambiae s.s.* and *Cx. quinquefasciatus* to optimally auto-disseminate PPF exposure must take place close to oviposition. However, even when both species were exposed that late only 65% and 71% emergence inhibition was achieved in oviposition substrates in which *An. gambiae s.s.* and *Cx. quinquefasciatus* laid eggs, respectively. Considering the small volume of water (100 ml) in a small oviposition cup of $0.004m^2$ used here, it is estimated that two females of either species exposed to PPF immediately prior to oviposition would be required to transfer sufficient PPF to cause complete emergence inhibition in such a small habitat. This suggests that hundreds of mosquitoes would be required to transfer lethal concentrations to $1m^2$ of habitat and the majority of natural habitats exceed this size (Fillinger *et al.* 2004). This suggests that the auto-dissemination is less likely to be effective for control of *Anopheles* mosquitoes in the more difficult field situations than it is for *Aedes* control or would at least require PPF formulations with much higher percentage of the active ingredient than the 2% tested here. Further studies are needed to understand the behaviour of gravid mosquitoes as they leave the houses (or other feeding and resting locations) to lay eggs. This would help to gain knowledge of the outdoor resting surfaces of gravid *An. gambiae s.l.* to serve as potential auto-dissemination stations. Species-specific oviposition attractants might be used to lure gravid females to the auto-dissemination stations to pick up lethal doses of PPF for transfer to uncontaminated aquatic habitats (Matowo *et al.* 2013; Snetselaar *et al.* 2014).

5.6   Conclusion

Anopheles gambiae s.s. and *Cx. quinquefasciatus* are highly affected by topical application of PPF reducing their viable offspring by 90% and 70%, respectively, when exposed to $2.6mg/m^2$ one day before to one day after a blood meal. The time interval of greatest susceptibility is excellent for use on PPF treated materials and indoor sprays and paints on resting surfaces and could provide a significant contribution to malaria control by suppressing the vector population. Importantly, it presents a promising opportunity for integrated control of different vectors and nuisance mosquitoes. It is considered that the integration of PPF in available insecticides would help in the management of resistance to

pyrethroids (WHO 2012c; Ngufor *et al.* 2014). However, sterilized females are unlikely to visit an oviposition site and therefore do not transfer lethal concentrations of PPF to aquatic habitats. This suggests that for successful auto-dissemination the optimum time for contamination is close to oviposition which requires further studies of the species' resting behaviour after blood meals.

6 Attract to a habitat – kill with a larvicide: Evaluation of a potential new attract and kill strategy for the control of mosquitoes

Oscar Mbare, Ulrike Fillinger

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6.1 Abstract

Background: Larvicides that persist in water bodies over extended time periods can only efficiently control mosquitoes if their applications do not produce a repellent effect on gravid females. Furthermore, addition of an attractive oviposition semiochemical to a larvicide might turn an aquatic habitat into a 'reproductive sink' for mosquitoes. In this study, we explored (1) the oviposition response of gravid *Anopheles gambiae sensu stricto* to water treated with the insect growth regulator Sumilarv®0.5G or the silicone-based surface film Aquatain® Mosquito Formulation (AMF) in semi-field systems and (2) the potential of combining these larvicides with a recently discovered oviposition attractant (cedrol) for the development of a novel 'attract and kill' strategy.

Method: Squares of electrocuting nets powered by a 12 V battery via a spark box and surrounding an artificial pond were used to evaluate the orientation of gravid *An. gambiae s.s.* towards test or control pond in a semi-field system. First, the orientation of gravid females towards test pond treated with either Sumilarv®0.5G or AMF was compared to control pond containing untreated water. Then the attractiveness of ponds treated with 20 ppm cedrol was compared to untreated pond. The potential of an 'attract and kill' strategy was assessed by evaluating the orientation of females to test pond containing water treated with Sumilarv®0.5G or AMF plus cedrol compared to untreated water. Experiments were conducted with 200 gravid females released into the semi-field system. Each experiment was replicated over 12 nights.

Results: Equal proportions of gravid *An. gambiae s.s.* were collected approaching untreated and larvicide-treated ponds indicating that neither attractive nor repellent cues from the larvicides were received by females from a distance. Similarly, neither 20 ppm cedrol treated ponds nor ponds treated with both cedrol and a larvicide did lead to an increased response of gravid females.

Conclusion: Unexpectedly, in this study we could not confirm that cedrol treated water attracts gravid *An. gambiae s.s.* as previously published. This is likely based on the poor release of this not very volatile compound when used in a pond without any air currents. Consequently, the study was not in a position to confirm the possibility of attracting gravid females to an aquatic habitat and then killing them with a larvicide applied to the habitat. For development of such a strategy more work needs to be invested to develop improved mechanisms to release cedrol and other oviposition attractants from water.

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6.2 Background

Targeting larval habitats that are most productive for adult vectors has been proposed by some authors to be an appealing strategy for control of immature stages of *Anopheles gambiae sensu lato* (Gu *et al.* 2008; Smith *et al.* 2013). The identification of productive habitats is however not always straightforward (Shililu *et al.* 2003a; Sattler *et al.* 2005; Killeen *et al.* 2006; Ndenga *et al.* 2011). In fact the productivity of larval habitats for malaria vector in the field has been variably described by different authors based on the presence or absence (Gimnig *et al.* 2001; Mwangangi *et al.* 2007) or abundance of larvae and pupae (Mutuku *et al.* 2006; Ndenga *et al.* 2011) or adult vector production of habitats (Munga *et al.* 2006; Fillinger *et al.* 2009b; Kweka *et al.* 2011). Some authors however suggest that gravid mosquitoes can be lured to oviposition sites by addition of substances that act as oviposition attractants (Bentley and Day 1989). Furthermore the incorporation of both oviposition attractants and an insecticide (adulticide) or sticky material has culminated in the development of oviposition traps (lethal ovi-traps) used in attract and kill strategies that target gravid *Aedes* females (Chadee and Ritchie 2010; Eiras *et al.* 2014). Another strategy for 'attract and kill' would be to target the immature stages of mosquitoes in which case gravid females would be lured to lay eggs in water treated with larvicides to kill immatures that develop from laid eggs (Ong and Jaal 2015).

There are two prerequisites for a successful attract and kill technique to control immature stages of malaria vectors. These are an effective substance to serve as attractant to lure gravid females to oviposit in treated water and a persistent larvicide that effectively kills over extended period of times (avoiding that the females is attracted but the larvicide does not kill anymore). Recently, the sesquiterpene alcohol cedrol was identified as an oviposition attractant for gravid *An. gambiae s.l.* under both laboratory and field conditions (Lindh *et al.* 2015) providing for the first time the opportunity to test 'attract and kill' strategies for this species. The insect growth regulator, Sumilarv®0.5G and the silicone-based monomolecular film Aquatain Mosquito Formulation (AMF) have demonstrated persistent effect against immature stages of mosquitoes in the field (Chavasse *et al.* 1995a; Yapabandara and Curtis 2002; Webb and Russell 2012). Furthermore, our own studies under standardized field conditions found both Sumilarv®0.5G and AMF to provide effective control of immature stages of *An. gambiae*

s.l. up to 6 weeks post-application (Mbare *et al.* 2013; Mbare *et al.* 2014a). It is however important that larvicides used in 'attract and kill' strategies do not elicit a repellent effect against target gravid mosquitoes since the repellency would be counterproductive as the gravid mosquito would ultimately search for alternative habitats to oviposit (Bukhari and Knols 2009). Furthermore, the combination of a semiochemical with a larvicide might affect either of the two components. Consequently, this study aimed to explore the oviposition response of gravid *An. gambiae s.s.* to: (1) water in a small pond treated with Sumilarv®0.5G or AMF; (2) water treated with cedrol, and (3) water treated with a combination of either Sumilarv®0.5G or AMF and cedrol.

6.3 Methods

6.3.1 Study area

All experiments were carried out in a semi-field system at the International Centre of Insect Physiology and Ecology (icipe-TOC), Mbita located on the shores of Lake Victoria in Homabay County in Western Kenya (geographic coordinates 0^0 26' 06.19" S, 34^0 12' 53.13"E; altitude 1,137 m above sea level). The semi-field system (Figure 6.1) was made of a greenhouse-like building 11.4 m long, 7.1 m wide and 2.8 m high at the wall and 4.0 m high at the highest point of the roof. Walls were screened by fibre-glass netting gauze (1.7x1.5 mm) and the roof covered by glass panels. The floor of the building was filled with sand to a depth of approximately 30 cm to enable digging down of artificial ponds into the ground.

Figure 6.1: Semi-field system at icipe-TOC, Mbita, Kenya

6.3.2   Artificial ponds

On any given experimental night two artificial ponds were set-up in opposite corners of the semi-field system by sinking black, round plastic tubs (diameter 0.42 m, depth 10 cm) into the ground. The tubs were filled with 7 l of non-chlorinated tap water directly pumped from Lake Victoria. Water in the control pond was left untreated while water in the test pond received the appropriate application of larvicide, cedrol, or combination of larvicide and cedrol (see below).

6.3.3   Test insecticides

Sumilarv®0.5G was provided by the manufacturer Sumitomo Chemical, Japan. It is a granular formulation containing 0.5% pyriproxyfen (PPF) (weight: weight), the active ingredient. PPF is an insect growth regulator that acts by inhibiting adult emergence in exposed immature stages (Invest and Lucas 2008).

Aquatain® Mosquito Formulation (AMF) was provided by the manufacturer, Aquatain Products Pty Ltd., Australia. AMF contains 78% polydimethylsiloxane (silicone), the active ingredient. AMF is a monomolecular silicone-based surface film that spreads spontaneously and rapidly over the water surface to form a uniform ultrathin film about one molecule in thickness-a monolayer. It physically kills mosquito larvae and pupae by

lowering the water surface tension that subsequently suffocates the immature stages (Corbet *et al.* 2000).

The doses of these two larvicides used in the experiments were those found to be effective at inhibiting adult emergence of *An. gambiae s.s.* and *An. arabiensis* in standardized field tests (Mbare *et al.* 2013; Mbare *et al.* 2014a). Thus in all these experiments Sumilarv®0.5G was applied at 5 mg ai/m^2 and AMF was applied at 1 ml/m^2. Thus based on the surface area of water in the artificial test pond (0.14 m^2) assuming a standard depth of 10 cm (WHO 2005a), the amount of Sumilarv®0.5G applied into test ponds was 140 mg (0.14g) and the volume of AMF applied was 0.14 ml (140µl). The two larvicides were evaluated separately on different dates with different batches of mosquitoes.

6.3.4 Oviposition attractant

Cedrol ≥99.0% (sum of enantiomers, GC, Sigma-Aldrich, Steinheim, USA) was used as the oviposition attractant in all experiments in this study (Lindh *et al.* 2015). Cedrol was previously identified as an oviposition attractant of gravid *An. gambiae s.l.* from volatile collections from soil infusion (Lindh *et al.* 2015).

In this study ponds were treated with 20 ppm cedrol with the aim to attract gravid *An. gambiae s.s*. First, stock solutions of 10,000 ppm cedrol in ethanol were prepared by adding 150 mg of cedrol to 15 ml of absolute ethanol (puriss. Pa, absolute, ≥99.8% (GC), Sigma-Aldrich). To prepare 20 ppm cedrol, 14 ml of stock solution was added into 7 l of lake water in the test pond.

6.3.5 Mosquitoes

All experiments in this study were done with insectary-reared gravid *An. gambiae s.s.* (Mbita strain) obtained from the icipe-TOC insectary. Detailed information on mosquito maintenance is provided elsewhere (Das *et al.* 2007). Gravid mosquitoes were prepared as follows: 300 unfed female and 300 male mosquitoes, two to three days old were selected from netting-covered 30x30x30 cm insectary holding cages at midday and transferred into a similar cage and held at ambient conditions (25-28°C and relative humidity of 68-

75%). To prevent desiccation of mosquitoes, cotton towels (50x25 cm) saturated with water were placed on top of the cage netting. Mosquitoes were starved of 6% glucose solution seven hours prior to receiving a blood meal on human arm at 19:00 h. Immediately after the blood meal the 6% glucose solution in a glass vial with a paper wick was returned in the cage. Mosquitoes were blood fed on two consecutive days. Gravid females were selected from the cage two days after the last blood meal for experiments. Selection was made by visually inspecting the abdomen.

6.3.6 Squares of electrocuting nets to measure odour-oriented behaviour of gravid females

Squares of electrocuting nets (E-nets) were recently developed for analysing the pre-oviposition behaviour of gravid *An. gambiae s.s.* in semi-field systems (Dugassa *et al.* 2012) (Figure 6.2). An electrocuting net was made of an aluminium frame (1.0 m high x 0.5 m wide) with aluminium rods fixed to the two shorter opposite sides of the frame with wooden joints. Four nets were joined in a square to surround an artificial pond. A 12 V 50 Ah lead acid battery (Chloride Exide Ltd., Kenya) connected to a spark box (Alan Cullis, South Africa) set at 50% spark energy was used to charge two electrocuting nets. The settings were chosen based on previous work; 50% spark energy is the highest energy that does not produce sparks on the net while the spark box is switched on (Dugassa *et al.* 2012). Electric current flows across copper wires (diameter 0.2 mm) fixed to an aluminium bar at one end of the shorter frame with springs that served as conductors and to the other aluminium bar at the other shorter end of the frame with loops made of fish lines (Damyl® fishing lines) to serve as insulators. To allow for flow of opposite charges in opposite directions the ends of the wires with the springs and those with the loops of fish lines attached to the two aluminium bars were alternated in successive copper wires. Two successive copper wires were held 8 mm apart. The electric current generates differentials of >2.5kV between two adjacent wires that electrocutes mosquitoes approaching the electrocuting net when functional (Vale 1974; Knols *et al.* 1998).The collecting device for electrocuted mosquitoes that fall to the ground was made of a sticky film (yellow roller trap; Oecos, UK), which was mounted on aluminium boards (60x50 cm) and placed beneath the complete square of E-nets. The sticky boards were placed both on the outside and inside of the E-nets. The board of sticky material placed inside was made by first making a circular hole in which the tub fits and the remaining parts

covered with sticky material. The number of mosquitoes approaching a given pond was calculated by summing the total mosquitoes found stuck on the sticky boards both on the inside and outside of the square of electrocuting as well as those found dead in the pond.

Figure 6.2: Square of electrocuting nets for analysing pre-oviposition behaviour of gravid mosquitoes. (A) Overview of the set-up: (1) artificial pond created by sinking a plastic tub filled with water into the ground, (2) sticky boards for collection and preservation of electrocuted mosquitoes, (3) 12 V battery, (4) spark box, (5) clamp and stand. (B) Close-up of electrocuting net: (1) aluminium frame (2) fish line wire (3) spring (4) aluminium bar.

6.3.7   Experimental design

Artificial ponds surrounded by squares of E-nets were set-up as described above. The corner where ponds were placed per night and control and test ponds were determined randomly using a paper lottery system. Each experiment was carried out using a complete

randomized block design (CRBD) with 12 replicate nights with different batches of mosquitoes. All experiments started at 18:00 h when 200 gravid *An. gambiae s.s.* were released at the centre of the semi-field system and stopped at 08:00 h the following morning when mosquitoes orienting towards either pond in the semi-field system were counted.

6.3.7.1 Analysing the response of gravid *An. gambiae s.s.* to water treated with Sumilarv®0.5G or AMF

In the first experiment the orientation of gravid females towards test pond treated with either Sumilarv®0.5G or AMF was compared with the orientation towards an untreated pond that served as control. The objective was to evaluate whether ponds treated with these larvicides affect the pre-oviposition behaviour by either repelling or attracting gravid *An. gambiae s.s.*

6.3.7.2 Analysing the response of gravid *An. gambiae s.s.* to water treated with 20 ppm cedrol

The objective of this second experiment was to assess whether and to what extent a synthetic attractant identified for *An. gambiae s.s.* could be used to lure gravid mosquitoes from a distance to ponds in which the chemical was applied. Water in the test pond was treated with cedrol to have a final concentration of 20 ppm cedrol as described above while 14 ml of ethanol (solvent used to dissolve cedrol) was added to tap water in control pond.

6.3.7.3 Analysing the response of gravid *An. gambiae s.s.* to water treated with Sumilarv®0.5G or AMF plus 20 ppm cedrol

The third experiment explored the possibility of attracting gravid females to lay eggs to a test pond treated with larvicide that would consequently kill her offspring. Thus here cedrol was added into test pond already treated with either Sumilarv®0.5G or AMF while the water in the control pond was left untreated. The larvicide was applied first into the pond and then cedrol added with a pipette in the test pond. The content of the test pond was then stirred by use of a metallic rod.

6.3.8 Statistical analyses

Data analyses were done using generalized linear mixed effects models. R statistical software package version 2.14.2 including packages MASS, lme4, glht and multicomp were used for analysis (RTeam 2011).Experiments with the two larvicides were analysed separately. The proportion of mosquitoes that were trapped around the test pond (fixed factor) in each experiment were modelled. Since the first experiment showed that larvicide treatment did not affect the choice of the gravid females, this experiment was used as the reference for analysing the effect of cedrol treated tests in consequent experiments. Experimental night and corner of the semi-field system where the ponds were set were included in the models as random factors. The models were fitted using a binomial distribution with a logit link function. The excess variation (over dispersion) in factors was adjusted by creating a random factor with a differential level for each row of the data set. The parameter estimates of the models were used to predict the mean proportions of females per treatment and their 95% confidence intervals (CIs) by removing the intercept from the models (Seavy *et al.* 2005).

6.3.9 Ethical review

Ethical approval for this study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol no. 422).

6.4   Results

On average 57% (95% CI 51-63%) of all released mosquitoes (n=200) in any experimental night responded and were captured on the sticky boards.
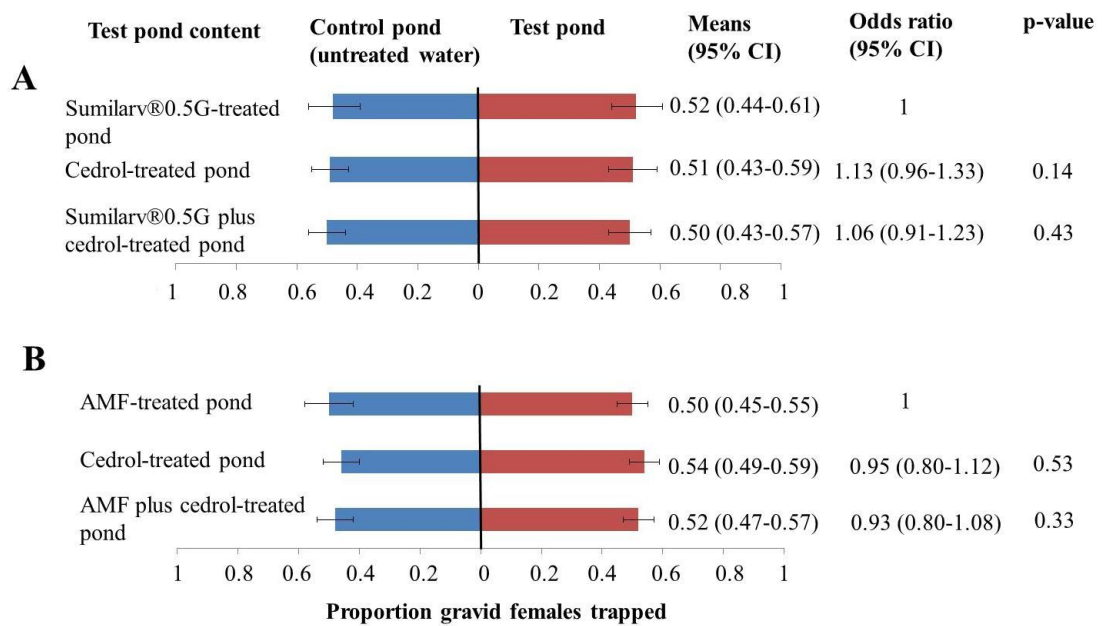
Figure 6.3: Response of gravid *An. gambiae s.s.* towards treatments in a pond. Error bars=95% CI. A- Response of gravid females to Surmilarv®0.5G, cedrol, Sumilarv®0.5G and cedrol versus untreated water. **B-** Response of females to AMF, cedrol, AMF and cedrol-treated versus untreated water

Gravid *An. gambiae s.s.* responded in equal proportion to ponds treated with insecticide and ponds with lake water only (Figure 6.3). Unexpectedly, there were no differences in the proportional distribution from this reference when the test ponds were treated with cedrol alone or with cedrol and insecticide (Figure 6.3). This means that the addition of larvicides only or larvicide plus cedrol to water in the test pond did not elicit an attractive or repellent effect on gravid *An. gambiae s.s.*

6.5   Discussion

This study failed to establish the proof of principle that the recently discovered oviposition attractant cedrol combined with a residual larvicide can attract gravid vectors and kill their offspring in aquatic habitats. This came as a surprise since it has been shown recently that 5 ppm of cedrol in lake water attracted twice as many gravid females to modified BG-sentinel traps than lake water alone (Lindh et al. 2015). However, BG-sentinel traps produce counter flow air currents that likely increase the release rate of

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cedrol from the water and lead to a constant odour plume released from the trap. These traps also do not provide any visual cues (e.g. reflection from a water surface). Here we already increased the amount of cedrol applied to water in anticipation of these difficulties and based on some preliminary experiments in which we found that 20 ppm increased the response rate of gravid females. However, these preliminary experiments were done in a different greenhouse environment and mosquitoes were not collected with E-nets but with sticky screens over the water surface, both might have contributed to the different observations. This study therefore illustrates the need to focus research in the development of efficient slow release mechanisms to enhance the attractiveness of the semiochemical to gravid *An. gambiae s.l.* especially if it would need to be used in natural habitats or traps that do not provide an enhanced air flow. This would also entail development of better formulations of cedrol that would ensure constant high release rates of the attractive odours over the same period of time that the larvicides remain effective for mosquito control. Furthermore, the difference to the previous work could be affected by the visual stimulus from the water surface in our experiments. Again, to develop effective attract and kill strategies it is important to investigate interactions between visual and chemical cues in the pre-oviposition behaviour of malaria vectors (McCrae 1984; Sumba *et al.* 2004; Huang *et al.* 2005). It is equally important to understand the interactions between larvicide and attractant when applied in the same water body. This is more so for surface films such as AMF that form a uniform film over water surface (Corbet *et al.* 2000) which is likely to reduce the amount of attractant released from the water. The silicone film of AMF created on water surface reduces water evaporation (Bukhari *et al.* 2011) and it is therefore likely that it also reduces release of volatile chemicals such as attractants added to the water. An insight into this interactions could be obtained by carrying out headspace collections of volatiles emitted from treated water (Lindh *et al.* 2015). This might also be overcome by developing release mechanisms in which the attractant is not applied directly in water, although this might be more complicated and might be useful for larger and more permanent habitats.

Sumilarv®0.5G and AMF did not by themselves affect the pre-oviposition behaviour of gravid malaria mosquitoes. This finding is important in the context of mosquito control since their application in the field will not divert gravid mosquitoes to water bodies that are untreated. A previous AMF formulation that contained 2% eucalyptus was shown to repel gravid *An. gambiae s.s.* and *An. stephensi* in the laboratory (Bukhari and Knols

2009). This non-repellent effect of the larvicides shown in our study provides an increased chance of females visiting treated habitats subsequently drowning when attempting to lay eggs as AMF reduces the water surface tension (Bukhari and Knols 2009) or killing larvae that hatch from eggs laid in treated water (Ong and Jaal 2015). In this context it would be useful to make the larval habitats more attractive to gravid females by addition of highly attractive semiochemicals as this would not only kill the offspring but also adult female by drowning (Bukhari and Knols 2009). That larvicide applications might affect the oviposition behaviour of gravid females has been shown before. The oviposition response of *Aedes* to larvicide-treated water has been shown with conflicting reports. For instance, while Marina *et al*. (2011) found *Bacillus thuringiensis israelensis* (*Bti*) and spinosad to have non-repellent effect against *Ae. aegypti* and *Ae. albopictus* based on similar egg numbers laid in larvicide-treated and untreated water, Perez *et al*. (2007) reported increased egg-laying by *Ae. aegypti* in water treated with spinosad than untreated water. Similarly, Stoops *et al*. (2005) and Carrieri *et al*. (2009) observed increased egg-laying by *Ae. albopictus* in *Bti*-treated than untreated water. Moreover, while Carroll (1979) observed increased egg-laying by *Ae. aegypti* in water treated with the insect growth regulator methoprene, Ritchie and Long (2003) did not find differences in the number of eggs laid by *Ae. aegypti* in methoprene-treated and untreated water. It is likely that for some larvicides the response of gravid mosquitoes to larvicide-treated water is dose-dependent as shown by Perez *et al*. (2007) who observed increasingly more gravid *Ae. aegypti* to visit water treated at a higher dose of spinosad (20 ppm ai) than water treated at lower dose (5 ppm ai) or untreated water. These authors hypothesized that the increased attractiveness of spinosad at higher dosages to gravid females was due to the increased earthly oduor of the insecticide. Similarly Romi *et al*. (2006) found the effect of spinosad on *Ae. aegpyti*, *An. stephensi* and *Cx. pipiens* to be dependent on mosquito species and application dosage.

6.6   Conclusion

The aim of this study is to serve as the proof of principle that attractive oviposition semiochemicals can be combined with persistent larvicides for an 'attract and kill' strategy in integrated vector control. However more research to develop improved mechanisms for slow release of attractive odours from the source and development of long-lasting baits for release of semiochemicals (Mukabana *et al*. 2012; Mweresa *et al*.

2014; Mweresa *et al.* 2015) for use in this strategy are warranted. Moreover, it is equally important to prioritize research aimed at identification of additional oviposition attractants of *An. gambiae s.l.* for use in attract and kill strategies.

7 Development of an auto-dissemination station for gravid *Anopheles gambiae sensu stricto* for use in attract and kill strategies

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7.1 Abstract

Background: The difficulty to identify a high proportion of larval habitats of Afrotropical malaria vectors present a major limitation for the control of their immature stages. This study explored the potential of using an attract and kill strategy that exploits the oviposition behaviour of adult *Anopheles gambiae sensu stricto* by contaminating the female with a 10% pyriproxyfen (PPF) dust formulation for transfer to larval habitats.

Methods: Preliminary studies in cages and under semi-field systems were conducted to develop a baiting station that was made of an artificial pond containing water treated with 20 ppm cedrol, an oviposition attractant. The pond was covered with fibre-glass netting treated with PPF (1.6 g PPF/m^2). Three identical semi-field systems were used to assess the potential of gravid *An. gambiae s.s.* to transfer PPF from the baiting station to three open ponds constructed in each semi-field system. Gravid females were released in the test and one of the control semi-field systems that had its baiting station covered with untreated netting. No mosquitoes were released in the other control semi-field system that had its baiting station covered with PPF-treated netting. Transfer of PPF to open ponds was assessed by monitoring emergence of late instar insectary-reared *An. gambiae s.s.* larvae introduced into open ponds. Liquid chromatography-mass spectrometry (LC-MS) was performed to quantify the amount of PPF that a single female can pick up from baiting station and the amount of PPF transferred by a female to water.

Results: On average 86% (95% CI 81-89%) of larvae introduced into the open ponds in the two control semi-field systems developed into adults. Transfer of PPF in the test semi-field system was dependent on the distance of open ponds from baiting station. While only 25% (95% CI 22-29%) adult emergence was observed in larvae introduced into open ponds closest to the baiting station, the emergence rates increased to 92% (95% CI 89-94%) in larvae introduced in the ponds furthest away. The average PPF picked up by a single female from a baiting station was 112 μg (95% CI 93-123 μg) while the average concentration of PPF transferred by a single female to 100 ml of water was 230 ng/l (95% CI 180-290 ng/l).

Conclusion: This study is proof of the principle that PPF can be auto-disseminated by gravid females with help of attractive baiting stations. However, it also clearly shows that females only transfer PPF to the nearest habitats which present a major limitation of this approach for malaria vector control. An individual female carries approximately 112 μg

PPF which requires approximately 500 females to visit a 1 m^2 and 10 cm deep habitat to transfer the 100% lethal concentration determined in the laboratory. Additional studies are needed to determine the optimum number of dissemination stations that would be needed to effectively control malaria vectors under natural field conditions.

7.2   Background

A novel approach of insecticide application termed auto-dissemination that exploits the adult insect as a 'vehicle' to deliver the insecticide has been successfully evaluated for control of social insects such as ants and termites as well as cockroaches (Soeprono and Rust 2004; Buczkowski *et al.* 2008; Choe and Rust 2008; Gautam *et al.* 2012; Neoh *et al.* 2012). This technique is greatly dependent on the insect's behaviour to deliver lethal doses of the insecticide to the other target insects with minimal human labour. There has been increasing interest in the exploration of this strategy for mosquito control (Itoh *et al.* 1994; Sihuincha *et al.* 2005; Devine *et al.* 2009). An important requirement for the success of the auto-dissemination technique for mosquito control is the use of an insecticide that works at extremely low concentrations and persists in the larval habitat at low doses against mosquitoes. The juvenile hormone analogue, pyriproxyfen (PPF), has been shown to provide persistent control of immature stages of mosquitoes at very low application dosages (Yapabandara and Curtis 2002; Seng *et al.* 2008; Mbare *et al.* 2013) and is therefore an excellent candidate molecule for this approach.

Laboratory and field studies have demonstrated the potential of container breeding *Aedes* mosquitoes to transfer PPF from contaminated surfaces to larval habitats to completely inhibit adult emergence (Devine *et al.* 2009; Caputo *et al.* 2012). In fact, field studies showed that contamination of only limited resting surfaces of *Aedes* with PPF can cause 42-100% adult emergence inhibition of larvae in a large number of larval habitats of this mosquito genera (Devine *et al.* 2009; Caputo *et al.* 2012). The skip oviposition behaviour (Colton *et al.* 2003; Chadee 2010) and preference of the targeted *Aedes* species to lay eggs in containers that hold small volumes of water (Burkot *et al.* 2007; Vezzani 2007) have been identified as factors that contribute to the success of the auto-dissemination technique (Devine *et al.* 2009; Caputo *et al.* 2012). These successes have recently led to proposals to evaluate the technique for the control of Afrotropical malaria vectors (Devine and Killeen 2010). Auto-dissemination is an approach where oviposition site-

seeking females deliver the insecticide to their preferred egg-laying sites. The amount of insecticide delivered is highly dependent on the number of females visiting this site. Recent studies conducted in semi-field systems in Tanzania provide evidence of the potential of *An. arabiensis* to transfer PPF from resting surfaces to larval habitats consequently inhibiting larval development (Lwetoijera *et al.* 2014). However, in their study Lwetoijera *et al.* (2014) released 1500-5000 host seeking females into the semi-field systems and therefore it is likely that most became sterilized on contact with PPF contaminated surfaces before or after blood meal (Harris *et al.* 2013; Mbare *et al.* 2014b). However, recently we showed that the optimum contamination time of female malaria vectors for use in auto-dissemination is when they are gravid and close to oviposition (Mbare *et al.* 2014b). Thus, a more effective approach for auto-dissemination is to target the gravid female not the host-seeking female. The aim of this study was to design a baiting station for gravid females and to test the transfer of PPF from this station to open ponds under semi-field conditions.

7.3   Methods

7.3.1   Study site

The experiments were carried out in semi-field systems located within the compound of the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (icipe-TOC) located on the shore of Lake Victoria in Mbita, Homabay county, western Kenya (geographic coordinates 0^0 26' 06.19" S, 34^0 12' 53.13"E; altitude 1,137 m above sea level). Mbita is characterized by tropical climate with a minimum temperature of 16 ºC and maximum temperature 29ºC. The area experiences two rainy seasons, the long rains between March and June while the short rains are experienced between October and December.

7.3.2   Test insecticide

An experimental formulation of Sumilarv® dust containing 10% of PPF was used in all experiments. Sumilarv® is a registered trademark of Sumitomo Chemical Company. The insecticide was provided by the manufacturer. Dust particles measured approximately 12μm diameter.

7.3.3 Preliminary experiments to develop a baiting station

7.3.3.1 Contamination of adult *Anopheles gambiae sensu stricto* with PPF

It has been previously shown that water vapour is a general attractant for malaria vector (Okal *et al.* 2013). Therefore, water in an artificial site was considered a necessity to attract gravid females. For the water vapour to be released but females to be prevented from accessing the water to lay eggs, fly gauze (black fibre-glass netting gauze (1.7x1.5 mm) treated with PPF was used to prevent PPF-contaminated gravid mosquitoes approaching the breeding site. Two methods of applying the PPF to the netting material were tested in cage experiments. For the first method netting gauze (diameter 7 cm) was treated with 1 g of PPF dust applied with a soft brush to ensure uniform spreading of PPF over the netting surface. The amount of PPF on netting gauze was 1.3 g/m^2 after weighing. In the second method PPF was formulated with cooking oil and then applied to the netting. Here 1 g of PPF dust was mixed in 2 ml of oil and this formulation applied to the netting with a brush. The netting treated with PPF served as the dissemination station. The control netting gauze was left uncontaminated and was used in control cages.

Experiments in cages were conducted to determine which of the two methods of treating the netting gauze enabled gravid *An. gambiae s.s.* to pick up and transfer sufficient PPF to oviposition water to inhibit development of larvae into adults. Each cage was provided with two glass cups (Pyrex®, 100 ml, diameter 7 cm). The first cup in each cage was filled with 100 ml non-chlorinated tap water from Lake Victoria while the second cup that served as the baiting station was filled with 100 ml of six-day old soil infusion that has been shown previously to attract gravid females in cages (Herrera-Varela *et al.* 2014) to lure gravid females. The top of the cup that served as the baiting station in the control cages was covered with untreated netting while in the test cages it was covered with netting gauze treated with either PPF dust or PPF dust formulated in oil. The top of the other cup filled with water was left open in all cages to allow for egg-laying by gravid females.

In each cage five gravid *An. gambiae s.s.* were released at 18:00 h and left overnight. The following morning presence of eggs in the open cups was assessed. Then to confirm the transfer of PPF in test cages, 10 insectary-reared late instar *An. gambiae s.s.* larvae were introduced into all open cups with water in all cages and monitored for adult emergence. Larvae were fed daily on Tetramin®Baby fish food. Because PPF does not produce acute toxicity on mosquito larvae but prevents emergence of adults from exposed pupae (Invest and Lucas 2008), any pupae that developed were transferred into plastic cups (diameter 7 cm) and monitored for emergence. It took 6-7 days for all larvae introduced into the cups to develop into adults or die. These experiments were conducted in three rounds on separate dates. There were five replicate cages per treatment in each experimental round (thus in total there were 15 cages with untreated netting gauze, 15 cages with netting gauze treated with PPF dust and 15 cages with netting treated with PPF dust formulated in oil). The position of the two cups in a cage was randomly allocated to one of the four corners in the first cage. The positions of the cups in subsequent cages were rotated in the next possible corners in a clockwise direction relative to the positions in the previous cage.

The soil infusion was prepared by incubating 15 l of non-chlorinated tap water with 2 kg of soil collected from a known breeding site of *An. gambiae s.l.* larvae. Infusions were prepared in round plastic tubs (diameter 0.42 m) and left for six days before use in experiments. During the six days incubation period tubs were covered with mosquito netting and kept in sheds that protected them from rains.

7.3.3.2 Evaluation if gravid *An. gambiae s.s.* can be lured to a pond

These experiments were conducted in a semi-field system (10.8 m long × 6.7 m wide × 2.4 m high) at icipe-TOC (Figure 7.1). Four artificial ponds were created by digging down round enamel tubs (diameter 0.42 m, depth 8 cm) at the four corners of the semi-field system. The tubs were dug 1 m away from the nearest wall of the semi-field system. During each experimental round three of the ponds were filled with 7 l of non-chlorinated tap water while the fourth pond was filled with a test substrate to attract gravid females.

Two test substrates were tested based on previous published work that showed their potential in attracting gravid female *An. gambiae s.s.*: a six-day old soil infusion (Herrera-

Varela *et al.* 2014) and the sesquiterpene alcohol cedrol (Cedrol ≥99.0% (sum of enantiomers, GC, Sigma-Aldrich, Steinheim, USA) (Lindh *et al.* 2015). The two substrates were evaluated separately on different dates. Thus at any time of the experiments the test pond was filled with either 7 l of six-day old soil infusion or 7 l of non-chlorinated tap water treated with cedrol. Two concentrations of cedrol were tested sequentially: 5 ppm and 20 ppm. Cedrol was prepared in ethanol by first preparing a stock solution of 10,000 ppm by dissolving 150 mg of cedrol to 15 ml of absolute ethanol (≥99.8% (GC), Sigma Aldrich). Dilutions were made by adding the appropriate volume of stock solution to water in the pond. For instance, 5 ppm cedrol was prepared by adding 3.5 ml of stock solution into 7 l of water in the dug down tub. Similarly 20 ppm cedrol was prepared by adding 14 ml of stock solution into 7 l of water in tub.

To simulate the natural environment during experiments, gravid females were released inside a small wooden hut (1.78 m long x 1.73 wide x1.80 m high) that was set up in the centre of the semi-field system (Figure 7.1).

Figure 7.1: Semi-field system showing artificial hut constructed at the centre of the semi-field system

The hut had a door and two windows that were shut when the experiment was in progress. The hut had two open eaves (0.84 m x 0.18 m) located at opposite sides which served as exit points for the gravid mosquitoes released in the hut. In each experimental round 200 gravid *An. gambiae s.s.* were released in the centre of the hut. To measure the number of mosquitoes visiting a pond, the top of each pond was covered by a black fibre-glass netting gauze cut to size (diameter 0.42 m) on which a fine film of insect glue was sprayed (Oeco insect spray, Oecos, UK) to trap the mosquitoes as they searched for oviposition substrate to lay eggs. Gravid mosquitoes were released in the semi-field system at 18:00 h and left overnight. The following morning the number of mosquitoes trapped on the sticky screens placed on top of each pond was counted. Each of the test substrates were evaluated during 12 nights with fresh batches of mosquitoes. The four ponds were randomly allocated in all four corners of the semi-field system using a randomized complete block design.

7.3.3.3 Evaluation of the auto-dissemination of PPF by gravid *An. gambiae s.s.* from a baiting station to larval habitats

These experiments were conducted in three identical semi-field systems which included a small wooden hut at the centre and four ponds in the corners (Figure 7.2).
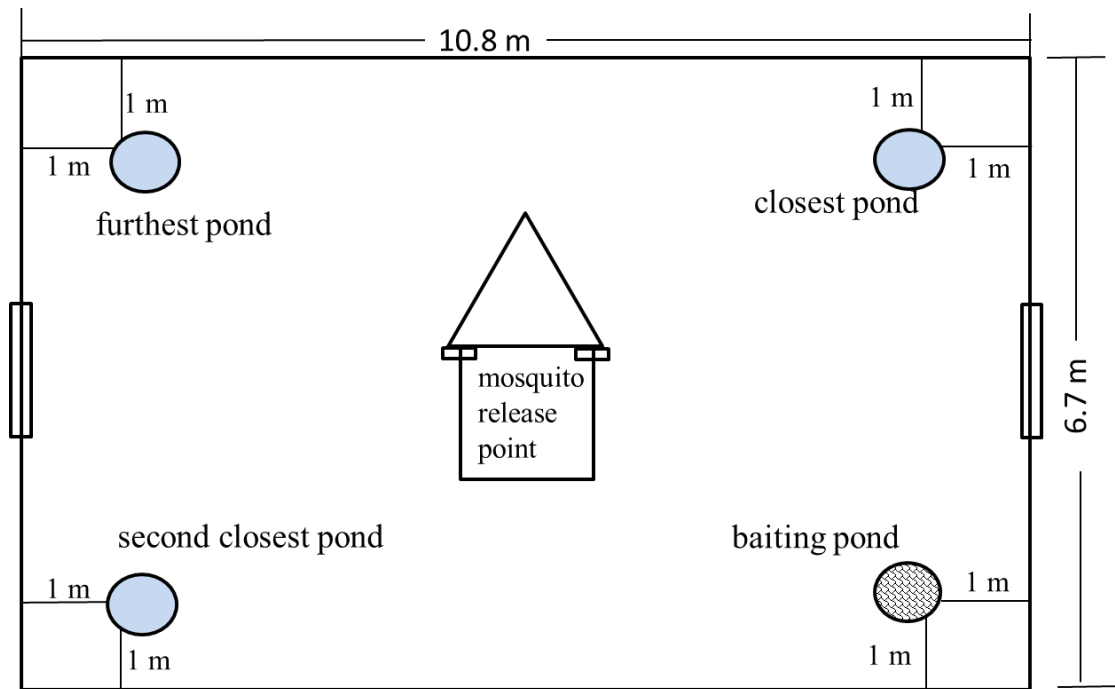
Figure 7.2: Schematic representation of semi-field system showing location of ponds and the artificial hut that serve as release point of gravid mosquitoes

In the first system, three ponds were filled with 7 l of non-chlorinated tap water, whilst the fourth pond served as the baiting station which consisted of 7 l of water treated with 20 ppm cedrol as described above. On top of the cedrol-treated pond a netting gauze of diameter 0.42 m was placed and treated with 3.5 g PPF (1.6 g PPF/m^2 after weighing amount retained on netting). This PPF-treated netting on top of baiting station served as the dissemination station. The three open ponds were recorded in reference to their distance to the baiting station, the closest was approximately 4.4 m away, the second closest approximately 8.4 m away and the furthest approximately 10.3 m away from the baiting station on the opposite site of the hut in the centre (Figure 7.2). Two hundred gravid *An. gambiae s.s.* were released at 18:00 h per experimental night in the centre of the hut. The second semi-field system contained exactly the same set up as the first, with the only difference that no mosquitoes were released in the system. The aim here was to investigate if PPF might be distributed by wind to neighbouring ponds rather than mosquitoes. In the third semi-field system, mosquitoes were released but the netting gauze of the baiting station was not treated with PPF. This last set up served to investigate

natural emergence rates from ponds when no insecticide was present in the system. The second and third semi-field systems thus served as controls.

The following morning the open ponds were assessed for presence of eggs laid to confirm the visit of the ponds by the gravid females. To ensure sufficient replication of the experiment the impact of PPF was not assessed by monitoring the development of eggs that were laid by the exposed females which would have taken about two weeks to complete one experiment, but instead the possible transfer of PPF by females to the ponds was assessed by monitoring the adult emergence of 50 insectary-reared late instar *An. gambiae s.s.* larvae that were introduced into the open ponds in all three set-ups in the morning after gravid females were released. Introduced larvae were fed daily with a pinch of Tetramin®Baby Fish food. Any pupae that developed in the three ponds were transferred into 200 ml plastic cups (diameter 7 cm) and monitored for emergence. It took 6-7 days for all introduced larvae to develop into adults or die. Thereafter the ponds and hut were cleaned and set afresh and all remaining alive adult mosquitoes aspirated using a motorized backpack aspirator (John W. Hock Company, USA). A new set of experiments was set-up with fresh batches of adult gravid mosquitoes and mosquito larvae. The experiments were conducted for 12 rounds with each round lasting seven days. The four ponds were randomly allocated in all four corners of the three semi-field systems in a randomized complete block design. To avoid contamination the semi-field systems in which the test and the two control experiments were not conduced were not changed.

7.3.3.4 Liquid-chromatography-mass spectrometry quantification of the amount of PPF carried by an individual mosquito and transferred to a water sample

An enamel bowl (diameter 0.42 m) filled with 7 l of non-chlorinated tap water was introduced into a 60x 60x60 cm cage (BugDorm-2120F; MegaView Science Taiwan) (Figure 7.3).

Figure 7.3: BugDorm insect tent with bowl filled with water. The top of the bowl was covered with netting contaminated with PPF dust

The top of the bowl was covered with netting contaminated with 3.5 g (1.6 g PPF/m^2 after weighing amount retained on netting) PPF dust as described above. Two gravid *An. gambiae s.s.* were introduced at a time into the cage and observed. Females that made contact with the netting were aspirated from the cage and used in experiments. The first experiment aimed at determining the amount of PPF that a single mosquito picks up from the treated surface. Here the females that made contact with the PPF-treated netting were transferred into epperdorf tubes (Greiner Bio-One) and frozen at -70°C until they were brought to the laboratories at icipe-Nairobi for quantification of PPF on their bodies. The aim of the second experiment was to determine the amount of PPF that a single mosquito transfers to water. Bioassays were conducted by introducing individual gravid females that made contact with the PPF-treated material into 15x15x15 cm cages containing a glass cup (diameter 7 cm) filled with 100 ml of non-chlorinated tap water. The females were left overnight to lay eggs. The following morning individual cups were assessed for the presence and number of eggs. To confirm the transfer of PPF into the water in the cup late instar *An. gambiae s.s.* larvae were introduced and monitored for adult emergence as described above. Comparisons were made to a control group of gravid females that were unexposed to PPF. Thirty replicates of test and control cages were done. When all larvae had died or emerged as adults, the water from the cups was transferred into 50 ml glass jars. Water samples were frozen in an ultra-low temperature freezer (New Brunswick

Scientific). The frozen samples were transported in a cool box to the Chemical Ecology Laboratory at icipe-Nairobi in a cool box for chromatographic quantification of PPF.

PPF was washed off the body of an individual mosquito in an eppendorf tube using 1.5 ml methanol (Sigma Aldrich, 99.9% HPLC grade). The content of the eppendorf tubes were agitated in a sonicator (Branson 2510 Ultrasonic cleaner, Eagle Road, Danbury) at 25 ºC for 5 minutes. It was then then centrifuged at 13,000 revolutions per minute (rpm) for 5 minutes in a centrifuge (PRISM^{TM} Microcentrifuge). The supernatant was transferred into 2 ml glass vials and used for detection of PPF.

Water samples were first pooled into groups of 10 before extraction (10x50 ml). Thus there were six pools of water samples in which females that contacted PPF laid eggs and another six pools of water samples in which females unexposed to PPF laid eggs. Each pool of water samples were extracted separately. Approximately 500 ml of water samples was extracted in 200 ml chloroform (Sigma Aldrich, 99.9% HPLC grade) to separate the aqueous and organic layers. The organic layer where PPF was expected to dissolve was concentrated by evaporating it to dryness in a rotary evaporator (HEIDOLPH INSTRUMENTS, Germany). The residue was dissolved in1 ml methanol (Sigma Aldrich) and stored at 4 °C awaiting analysis. To assist in quantification of PPF a known concentration (0.00002 μg) of 4-benzyliphenyl (Sigma Aldrich) was added into each extracted water sample as internal standard just before the liquid chromatography-mass spectrometry electron using electron spray ionization (LC/ESI-MS) was run. First the standards of pure 10% PPF and 4-benzylbiphenyl was initially run separately in the LC-MS system to confirm the retention times of PPF and the internal standard. PPF used as standard was prepared by dissolving 40 mg of PPF (10%) in 1.5 ml ethanol in a 2 ml glass vial. This was agitated in a sonicator at 25 ºC for 5 minutes. The mixture centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred into 2 ml glass vials and used for detection of PPF. The peaks of PPF and 4-benzylbiphenyl at the retention times were identified based on the molecular masses of their individual ions (molecular masses of pyriproxyfen-322 and 4-benzylbiphenyl-247).

The LC/ESI-MS used consisted of a quaternary LC pump (Model 1200) coupled to Agilent MSD 6120-Single quadruple MS with electrospray source (Palo Alto, CA). The MS component of the system was used to verify the peak assigned to PPF or 4-benzylbiphenyl as the active ingredients based on their identification on molecular masses

of the ions. The system was controlled using ChemStation software (Hewlett-Packard). Reverse-phase liquid chromatography was performed using an Agilent Technologies 1200 infinite series LC, equipped with a Zorbax Eclipse Plus C_{18} column, 4.6 x 100 mm x 3.5 µm (Phenomenex, Torrance, CA). The following gradient using A (5% formic acid in LC-grade ultra pure H_2O) and B (LC-grade methanol) (Sigma, St. Louis, MO) was used; 0-5 min, 95-100% B; 5-10 min, 100% B; 100-5 min. The mobile phase liquid was acetonitrile (Sigma Aldrich). The flow rate was held constant at 0.7 mL min^{-1}. The sample injection volume was 100 µl, and data were acquired in a full-scan positive-ion mode using a 100 to 500*m/z* scan range. The dwell time for each ion was 50 ms. Other parameters of the mass spectrometer were as follows: capillary voltage, 3.0 kV; cone voltage, 70 V; extract voltage, 5 V; RF voltage, 0.5 V; source temperature, 110ºC; nitrogen gas temperature for desolvation, 350ºC; and nitrogen gas flow for desolvation, 400 L/h.

7.3.4 Data analysis

Data were analysed in R statistical software package version 2.13. Generalized estimating equations were used to analyse all data with experimental round included as repeated measure in the models. Data collected in cage and semi-field experiments that determine the transfer of PPF to water were analysed as proportions. Proportions were analysed by fitting a binomial distribution with a logit function and an exchangeable correlation matrix assumed. In analysing data performed in cage experiments to determine if mosquito can pick up PPF dust or PPF dust formulated in oil from treated netting, the cage (control or test) was included as fixed factor with the control cage used as the reference. In semi-field experiments to evaluate the potential of gravid female to transfer PPF to open ponds, the open pond ID identified by its distance from the baiting station was used as the fixed factors with the pond closest to the baiting station used as the reference.

Count data collected in experiment evaluating the number of mosquitoes visiting ponds treated with six-day old soil infusion or cedrol were fitted to a Poisson distribution with a log link function. Here the ponds were included in the model as fixed factors with the pond serving as the baiting station used the reference. All means (proportions or counts)

per treatment and their corresponding 95% confidence intervals (CIs) were modelled as the exponential of the parameter estimated for the individual models with no intercept included.

7.4   Results

7.4.1   Gravid *An. gambiae s.s.* pick up more PPF when only dusted on netting than when formulated in oil

Both application methods of PPF on the nettings of the baiting stations lead to the transfer of PPF to the open cup and significant reduction in the emergence of adults from introduced larvae (Figure 7.4 and Table 7.1) However, emergence inhibition was 5 times higher when the netting of the baiting station was dusted with PPF than formulated in oil indicating that larger amounts of PPF were picked and transferred.
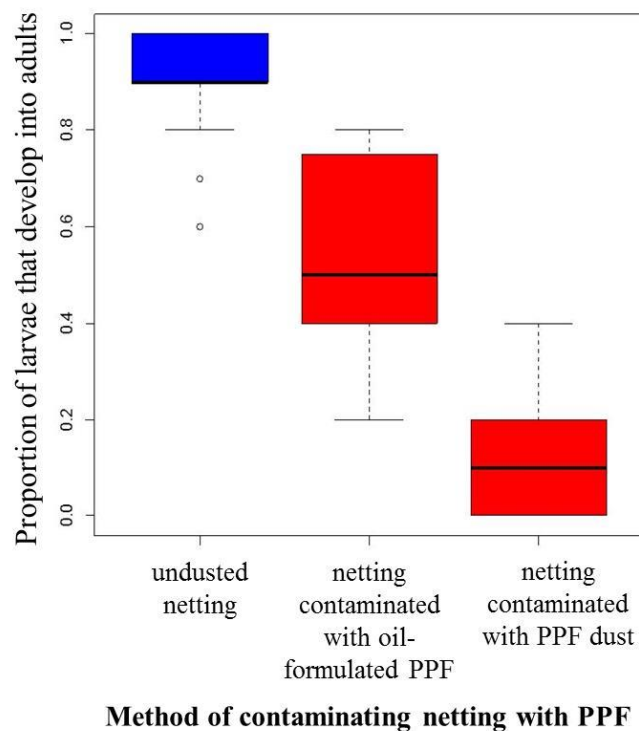
Figure 7.4: Box and whisker plots showing the median proportion and interquartile range of adult emerged in cage experiments to determine the best method to treat netting with PPF for pick-up with mosquitoes

Table 7.1: Adult emergence rates of larvae introduced into oviposition cups in cage experiments

Method of contaminating netting gauze	Proportion adults (95%CI)	Odds ratio (95% CI)	p-value
non-contaminated	0.89 (0.83-0.93)	1	
oil-formulated PPF	0.55 (0.35-0.62)	0.144 (0.073-0.282)	<0.001
PPF powder	0.11 (0.07-0.17)	0.015 (0.006-0.036)	<0.001

7.4.2 Oviposition attractants can lure gravid *An. gambiae s.s.* to a baiting station

The number of mosquitoes trapped on the sticky screens over ponds containing six-day old soil infusion or treated with cedrol at 5 or 20 ppm was higher than the number trapped on sticky screens over ponds with untreated water (Table 7.2). The attractiveness of six-day old soil infusion and water treated with 5 ppm cedrol was similar and not very strong; a female was only approximately 1.3 times more likely to land on the test treatment than controls (Table 7.2). When the water was treated with 20 ppm of cedrol however, it was twice as likely for a female to be trapped as compared to ponds with untreated water (Table 7.2).

Table 7.2: Results of the statistical analyses of the three experiments to evaluate the attractiveness six-day old soil infusion and water treated with cedrol

Pond	Mean catches (95% CI)	Rate ratio (95% CI)	p-value
	Attraction to six-day old infusion		
treatment	38.7 (33.2-45.0)	1	
control 1	28.7 (24.9-33.1)	0.744 (0.591-0.935)	0.011
control 2	25.7 (21.7-30.5)	0.666 (0.544-0.816)	<0.001
control 3	27.0 (23.3-31.3)	0.698 (0.555-0.878)	0.002
	Attraction to 5 ppm cedrol		
treatment	32.5 (30.2-35.0)	1	
control 1	24.7 (21.4-28.5)	0.759 (0.626-0.921)	0.005
control 2	25.9 (23.1-29.1)	0.797 (0.698-0.911)	0.001
control 3	26.4 (23.2-30.1)	0.813 (0.703-0.940)	0.005
	Attraction to 20 ppm cedrol		
treatment	52.3 (45.5-60.0)	1	
control 1	28.0 (24.0-32.7)	0.536 (0.430-0.668)	<0.001
control 2	32.4 (27.6-38.1)	0.620 (0.484-0.795)	<0.001
control 3	27.2 (21.0-35.1)	0.520 (0.378-0.715)	<0.001

7.4.3 Transfer of PPF by gravid *An. gambiae s.s.* is dependent on the distance of the habitat from the dissemination station

In all semi-field systems where gravid females were released inside the hut, eggs were observed the following morning in all three open ponds at any experimental night, however, egg numbers were not further quantified or larvae followed up. The potential transfer of PPF was evaluated based on the adult emergence rate from introduced third instar larvae. Here, although the best approach would be to observe the laid eggs for adult emergence/emergence inhibition, insectary-reared late instar larvae were introduced to reduce the number of days that a single test round would take.

In the absence of PPF on the baiting station as well as in the absence of gravid females in the system, emergence rates of introduced larvae were over 80% (Table 7.3); on average for both experiments 86% (81-89%). For some unexplained reason the emergence rate in the control experiment 2 where no mosquitoes were released but PPF was present on the baiting station was consistently higher than in control experiment 1 where mosquitoes were released but no PPF was present in the system (Table 7.3). This might be due to some small microclimate differences in the two systems used, or might be due to some unexplained interaction between the early instars originating from the oviposition and the introduced larvae in control 1. Importantly, in both control experiments, emergence rates were similar in all three open ponds in the systems (Table 7.3). Wind did not transfer PPF from the baiting station to the open ponds.

Table 7.3: Adult emergence rates of larvae introduced into open ponds in the three experiments to evaluate transfer of PPF in semi-field systems

Ponds	Mean proportion (95% CI)	Odds ratio (95% CI)	p-value
Control 1- Mosquitoes released in semi-field system & untreated netting gauze placed on top of baiting station			
closest to baiting station	0.85 (0.82-0.87)	1	
medium to baiting station	0.83 (0.80-0.86)	0.87 (0.62-1.23)	0.443
furthest to baiting station	0.84 (0.81-0.87)	0.99 (0.71-1.37)	0.944
Control 2- No mosquitoes released in semi-field system & netting gauze treated with PPF dust placed on top of baiting station			
closest to baiting station	0.89 (0.86-0.91)	1	
medium to baiting station	0.89 (0.87-0.92)	1.03 (0.72-1.49)	0.854
furthest to baiting station	0.88 (0.85-0.91)	0.94 (0.65-1.34)	0.721
Test-mosquitoes released in semi-field system & netting gauze contaminated with PPF dust on top of baiting station			
closest to baiting station	0.25 (0.22-0.29)	1	
medium to baiting station	0.58 (0.54-0.62)	4.07 (3.19-5.21)	<0.001
furthest to baiting station	0.92 (0.89-0.94)	33.89 (24.16-48.47)	<0.001
Emergence inhibition due to auto-dissemination – comparison of test with control 2			
control	0.89 (0.84-0.94)	1	
closest test	0.25 (0.20-0.33)	0.042 (0.023-0.077)	<0.001
medium test	0.58 (0.51-0.66)	0.173 (0.098-0.303)	<0.001
furthest test	0.92 (0.85-0.98)	1.437 (0.846-2.444)	0.180

The presence of a PPF treated baiting station when gravid females were released in the system significantly influenced the emergence of adults from the three open ponds (Table 7.3) confirming that PPF was transferred by gravid females. On average, only 25% (22-29%) of introduced larvae emerged from the pond closest to the baiting station. However, the further away from the baiting station the open pond was, the less likely was it that emergence was inhibited. When comparing the emergence rates from the ponds in the test experiment with the average emergence rate from control ponds, significant emergence inhibition was only observed for the two ponds closest to the baiting station. It was around 20 times less likely for an adult to emerge from the ponds closest to a baiting station (approximately 4.4 m) and 5 times less likely from the ponds that were

approximately twice (approximately 8.4 m) as far away from the baiting station as the closest pond than it was for an adult to emerge from any pond in the control experiments (Table 7.3). No emergence inhibition was recorded from the open pond that was furthest away from baiting station and located in the opposite corner of the baiting station on the other side of the hut suggesting that no or insufficient PPF was transferred to this pond.

7.4.4 LC-MS analysis of amount of PPF carried by carried by individual mosquito

Ninety percent (n= 30) of females that landed on PPF-treated netting laid eggs when provided with water in a glass cup in a cage. A similar number (n= 30) of unexposed (control) females laid eggs. There was no difference in the mean number of eggs laid by females that were exposed to PPF and those that were not (p=0.78). The average number of eggs laid by all females was 61 (95% CI 50-76). Significant differences were however observed in adult emergence rates from larvae that were introduced into the cups (Table 7.4). It was 17 times less likely for a larva to emerge when it was introduced into water in which PPF exposed female had laid eggs than when introduced into a cup in which unexposed female had laid eggs (Table 7.4).

Table 7.4: Adult emergence rate of late instar larvae introduced into water in which females laid eggs

	Mean proportion emergence (95% CI)	Odds ratio (95% CI)	p-value
unexposed females	0.93 (0.89-0.97)	1	
PPF-exposed females	0.45 (0.39-0.51)	0.06 (0.03-0.10)	0.007

Based on the control emergence of 93% (89-97%), the corrected percent emergence inhibition (Abbott 1987) observed was 52% (46-56%); in other words an individual female transferred to 100 ml of water the concentration that inhibited emergence of approximately 50% (EI_{50}).

The amount of PPF washed off a single female mosquito could not be detected in LC-MS. Thus samples from 20 females were pooled for analysis with the LC-MS system. In total PPF was washed off the body of 140 females that had made contact with PPF and a

similar number that did not make contact with PPF (controls). Thus there were seven pools of females that made contact with PPF and another seven pools that did not make contact with PPF. PPF was not detected in any of the washes from mosquitoes that did not make contact with PPF. PPF was below the detection limit in two of the pools of washes from mosquitoes that made contact with PPF. To determine the amount of PPF washed off a single female, the amount detected from a pool was divided by 20 (the number of females in a pool). Thus the estimated amount of PPF washed off an individual female from the five pools in which PPF was detected was 141 µg, 120 µg, 93 µg, 117 µg and 89 µg. Thus the average amount of PPF washed off an individual mosquito was found to be 112 µg (95% CI 103-123µg). This is however likely to be an overestimate considering that PPF levels were below detection limits in two water samples and were not included in calculating this average. Assuming that individual female transfers this amount of PPF to 100 ml water used in our cage bioassays subsequently provides a concentration of 1.12 mg PPF/l in water (1.12 ppm).

PPF was not detected in any of the water samples in which females that did not make contact with PPF laid eggs. However PPF was detected in three out of the six water samples in which females that made contact with PPF laid eggs. The estimated concentration of PPF detected in the individual water samples in the three pools were 330 ng/l, 160 ng/l and 190 ng/l. Thus the average estimated concentration of PPF in a single oviposition cup used in our bioassay was 230 ng/l (95% CI 180-290 ng/l). This is equivalent to 0.00023 mg/l (0.00023 ppm). This is similarly likely to be an overestimate since three of water samples in which PPF was below the detection limit were not included in estimating the average. This is the concentration that provided around 50% emergence inhibition of larvae introduced in water in our bioassays. Comparisons of the concentration detected in water samples and the expected amount that a single female can transfer to water after picking up PPF from treated surfaces reveals that an individual female transfers 4,869 times less PPF than it picks up from treated surface.

7.5   Discussion

This is the first study that developed a baiting station for gravid *Anopheles gambiae s.s.* for the auto-dissemination of PPF to aquatic habitats and shows in principle that gravid females can be lured to a target, be contaminated with PPF and transfer PPF from there to

an aquatic habitat while laying eggs. However, although 200 gravid females were released in a relatively small space of approximately 170 cubic metres adult emergence was inhibited by only 70% (corrected based on control emergence) from ponds that were no more than 5 metres from the baiting station, and emergence was not inhibited at all when the baiting station was only around 10 metres away and the distance between pond and baiting station obstructed by the presence of a hut. These results strongly suggest that even if females can be lured successfully to a baiting station, they are likely to transfer the PPF to the closest available and suitable oviposition sites requiring a large number of baiting stations should this approach be successful in targeting all *Anopheles* larval habitats in an area.

Our study highlights a number of challenges for developing the auto-dissemination approach for African malaria vectors that utilize a large number of habitats of variable size for oviposition (Fillinger *et al.* 2004; Majambere *et al.* 2008). *Anopheles* can only transfer PPF to an aquatic habitat when she is exposed to PPF whilst already gravid, otherwise she would be sterilized and not visit an aquatic habitat (Mbare *et al.* 2014b). Therefore, the gravid female must be targeted for picking up the PPF. The aim here was therefore to develop a baiting station especially attractive for the target species. However, to date, only water-vapour (Okal *et al.* 2013), a soil infusion made from a specific habitat found at icipe-TOC campus (Herrera-Varela *et al.* 2014) and the chemical cedrol, that was identified from this soil infusion (Lindh *et al.* 2015), has been with certainty shown to attract gravid females of *Anopheles gambiae s.s.* under experimental conditions. Consequently, those were utilized for designing a baiting station. Our study confirms the recent findings that six-day soil infusion made from soil from a specific location at icipe-TOC and cedrol-treated water attracts gravid *An. gambiae s.s.* under semi-field condition (Herrera-Varela *et al.* 2014; Lindh *et al.* 2015). Our study further highlights that these two oviposition attractants can be used in an attract and kill approach as recommended by the authors of these discoveries (Herrera-Varela *et al.* 2014; Lindh *et al.* 2015). However, contrary to recently published work by Lindh *et al*. (2015) who observed that water treated with 5 ppm of cedrol doubled the catch we only achieved the same result with 20 ppm cedrol compared to the untreated control. The reason for this finding may be the absence of any air current or reduced airflow generated by the baiting station in our semi-field set-up. Lindh *et al.* (2015) used modified BG-Sentinel traps that produce air circulation with help of a fan. It is likely that larger amounts of cedrol and water vapour

are released by the fan trap which provides a stronger signal for oviposition site-seeking females. This highlights the need for developing much improved release mechanisms for attractive odours from baiting stations targeting gravid females. In order to attract gravid females from a larger distance (more than 10 metres) the baiting station needs to be far more attractive than the one tested here. It is likely that the females that left the hut through the eaves on side without the baiting pond went in equal proportions to the two ponds located on this side of the eave that were the closest. These females are unlikely to have visited the pond that served as baiting station as it was far away and not visible from their exit point. This is probably an indication of the need to develop more attractive substrates to add to baiting stations to lure gravid females at greater distances.

Another challenge that would need to be addressed when developing a baiting station for auto-dissemination is the presentation of PPF to the approaching female for her to pick up the largest possible amount for transfer. Our cage tests showed that gravid females picked up more PPF from treated surfaces when PPF was just dusted on top than when formulated in oil. There are two possible explanations for this. First the oil might prevent mosquitoes from picking sufficient PPF as it adheres PPF more on the netting. Second it might also be that the oil contributed to a larger proportion of PPF remaining on the mosquito's body thus limiting the chance of PPF getting in contact with water. PPF in dust or powder form has been used previously in some of the successful studies that evaluated the potential of auto-dissemination for mosquito control (Devine *et al.* 2009; Caputo *et al.* 2012; Lwetoijera *et al.* 2014), however, for large scale application and cost-effective use of the active ingredient there is need to investigate strategies that use PPF more efficiently. Here clearly a lot of the active ingredient on the netting gauze was wasted since not all the material was taken up. The development of an efficient dissemination station for use in field conditions is critical for the success of auto-dissemination technique (Caputo *et al.* 2012; Snetselaar *et al.* 2014). The dissemination station in our study was made of netting gauze contaminated with PPF dust and placed on top of an artificial pond made of metallic tub. Cedrol, an oviposition attractant of *An. gambiae s.l.* (Lindh *et al.* 2015) was added to the pond to lure gravid *An. gambiae s.s.* to the pond. These are simple tools that are readily available and can thus be easily used in the field. It is however critical that better methods of releasing cedrol under field conditions are developed in addition to identification of more attractive semiochemicals that can be used in a more potent blend to lure gravid *An. gambiae s.s.* more strongly to

the baiting station. Furthermore, improved technologies of contaminating the female with PPF could include the electrostatic charging of the PPF particles to ensure a higher amount of PPF placed on an individual female (Huang *et al.* 2010).

The comparison of amount that a mosquito picks up from a surface on contact and the ultimate concentration in water as shown in our chromatographic analyses indicate that the gravid female transfers around 4,800 times less PPF to larval habitats than that picked up from treated surface. This is not surprising as the amount of PPF on the insect cuticle is likely to decrease with time due to loss during flight and penetration through the insect cuticle (Medina *et al.* 2002; Schneider *et al.* 2008). It is also possible that the female does not deliver all PPF on her cuticle to the water especially PPF on cuticular surface that do not make contact with water. The chromatography confirms our findings from the bioassay, that a single female transferred the concentration that inhibited the emergence of 50% (EI_{50}) of the larvae in 100 ml of water. The average concentration of PPF detected in water used in the bioassays was 0.00023 mg/l (95% CI 0.000180-0.000290 mg/l) PPF which correlates well with our previous findings from laboratory assays when the EI_{50} was found to be 0.000120 ng/l (95% CI 0.000090-0.000160 ng/l) (Mbare *et al.* 2013). The findings are also consistent with what we found when testing auto-dissemination in cage bioassays previously (Mbare *et al.* 2014b). Even though in our previous study females were contaminated in a plastic jar coated with PPF, a single female caused approximately 50% of the introduced larvae not to emerge (Mbare *et al.* 2014b). Taken together it appears that this is the maximum amount that a female *An. gambiae s.s.* can transfer to an aquatic habitat.

This study showed the transfer of PPF to a larval habitats is dependent on the distance of the pond from the dissemination station; the closer a pond is to the dissemination site the more PPF gets transferred and therefore the higher the emergence inhibition rates. Similar habitats further away are less likely to be visited and therefore less PPF gets transferred. This suggests that numerous dissemination stations would be required in the field for gravid *An. gambiae s.l.* to transfer sufficient lethal doses of PPF to their larval habitats. This is a substantial challenge considering the large number and extensive nature of the larval habitats of *An. gambiae s.l.* in some areas (Fillinger *et al.* 2004; Majambere *et al.* 2008). A recent model shows that the success of auto-dissemination for malaria vector control would be dependent on the abundance of adult vectors, the number and stability of larval habitats and persistence of the insecticide used (Devine and Killeen 2010). The

only other study to evaluate the potential of auto-dissemination for control of vectors of the *An. gambiae* species complex under semi-field settings reported that *An. arabiensis* could transfer PPF from contaminated resting pots to artificial larval habitats to cause 82% adult emergence inhibition of offspring of females that laid eggs in the habitats (Lwetoijera *et al.* 2014). Several factors might explain the greater impact in this study. Lwetoijera *et al.* (2014) placed eight dissemination stations (resting pots) treated with PPF and provided only two very small larval habitats (capacity 2.5 l) making a dissemination station to breeding habitat ratio of 4:1. This is in comparison to a ratio of 1:3 in our study. The capacity of the larval habitats in their study was three times smaller than in our study. Moreover the larval habitats in their study were much closer to the dissemination stations (1-8 m away) than in our study. The higher number of dissemination stations increased the chance of a mosquito resting on a PPF-contaminated surface which subsequently increased the number of mosquitoes that pick up PPF for transfer to the limited number of larval habitats. Furthermore, a total of 5000 females were released in the semi-field system in their study further increasing the likelihood of a mosquito visiting a dissemination station and the number of oviposition events in a single larval habitat. Thus the concentration of PPF in those small larval habitats was probably higher than in ours.

7.6   Conclusion

Our study carried out under controlled conditions highlight potential limitations of auto-dissemination strategy for control of Afrotropical malaria vectors. The finding that gravid *An. gambiae s.s.* from a baiting station are most likely to visit the pond closest to the station demonstrates the need to conduct further studies under similar conditions to explore the required ratio of baiting stations to larval habitats for adult gravid females to transfer sufficient PPF that effectively controls immature stages of malaria vectors in all habitats. Moreover investigations to assess if mosquitoes of other genera such as *Culex* can be used to amplify the amount of PPF transferred to larval habitats of *An. gambiae s.s.* under similar conditions are needed. This is because culicine and *Anopheles* larvae frequently occupy the same aquatic habitats in the field (Fillinger *et al.* 2004; Ndenga *et al.* 2011). Also of importance are studies aimed at improving the efficacy of this prototype baiting station to increase its attractiveness to gravid malaria vectors by

determining better mechanisms that ensure optimum release of the attractant from the baiting station, better materials that retain large amounts of PPF after treatment to serve as dissemination station as well as improve the physical components such as visual contrast of the baiting station and construct a protective barrier from rain. Furthermore field evaluations are necessary to assess performance of this baiting station in attracting gravid malaria vectors especially during both the dry and rainy seasons when the number of larval habitats increase.

8 Synthesis

Vector control is an essential component of the malaria elimination strategy and remains the most effective measure to prevent malaria transmission. It is one of the four main strategies of the Global Malaria Action Plan (WHO 2006a; RBM 2008). There is however increasing consensus that the current frontline vector control measures will not be sufficient to achieve the ultimate goal of malaria elimination in most of sub-Saharan Africa (Govella *et al.* 2013; Durnez and Coosemans 2014; Killeen 2014; WHO 2014a). Thus evidence-based integrated approaches that encompass utilization of multiple intervention tools are recommended to sustain the achievements in reducing malaria so far and further suppress malaria transmission (WHO 2004; WHO 2011). Research is required to develop and rationalize vector control strategies that can be implemented outside of houses to target both endophilic and exophilic vectors and/or use insecticides with a completely different mode of action than those used indoors for adult mosquito control to manage insecticide resistance. In this thesis two insecticides were investigated: the silicone-based surface film Aquatain Mosquito Formulation (AMF) and the insect-growth regulator pyriproxyfen (PPF).

8.1 Key findings

8.1.1 PPF and AMF provide persistent control of immature stages of *An. gambiae s.l.*

Laboratory tests showed that *An. gambiae s.s.* and *An. arabiensis*, were highly susceptible to PPF and AMF at low doses. The effective doses identified in laboratory tests were 10 times lower than those previously identified for microbials (Fillinger *et al.* 2003; Majambere *et al.* 2007). Under standardized field conditions both insecticides provided over 80% adult emergence inhibition over a six weeks survey period. Moreover, the standardized field tests provided evidence of sub-lethal effects of PPF and AMF in adult *An. gambaie s.s.* that survived exposure to the insecticides during larval development in treated water. Adults emerged from ponds treated with larvicides laid fewer eggs and had reduced egg-hatching rates as compared to adults that emerged from ponds with untreated water. The persistence of the insecticides in treated habitats and their sub-lethal impact augment their potential as malaria vector control tools.

Field studies conducted under operational conditions in the western Kenya highlands confirmed that monthly application of PPF to the natural larval breeding habitats of *An. gambiae s.l.* for one year effectively inhibited more than 80% emergence of adult vectors from treated aquatic habitats located in the intervention study sites. The field findings also confirmed the persistence of PPF for mosquito control including *Anopheles* shown in other studies (Chavasse *et al.* 1995a; Yapabandara *et al.* 2001; Seng *et al.* 2008) and further showed that the application interval of PPF was optimal to prevent adult vector production from habitats newly created in-between successive treatment cycles.

Larval source management (LSM) is increasingly being re-considered for integration into malaria control strategies in Africa (WHO 2011; Tusting *et al.* 2013; WHO 2013b). Addition of larviciding to indoor vector control interventions is a readily available tool that could be used by National Programmes for example in transmission hotspots or in areas targeted for malaria elimination (Fillinger and Lindsay 2011), however, the high demand on labour and costs for frequent application hamper the initiation of such integrated programmes. Cost for larviciding programmes using *Bacillus* products that need to be applied in weekly intervals are primarily driven by the costs of the product and labour costs for frequent application (Worrall and Fillinger 2011). Here evidence is provided, that suggests that the operational PPF application could achieve similar reductions in malaria vectors and transmission than previously shown with larvicides that require weekly application (Fillinger and Lindsay 2006; Fillinger *et al.* 2009a) at a third of the effort. Furthermore, at least at the transmission setting in the western Kenya highlands, vector population dynamics suggest that the intervention might be targeted in time for 4-5 months over the main transmission season only, which would further reduce demand on personnel and costs.

The potential of adding larval control interventions to ongoing indoor vector control interventions has been recently explored by a range of mathematical models that all come to the conclusion that targeting different life stages of the mosquito including larvae (and reproduction) can have a huge added benefit for reducing malaria transmission and contribute to malaria eradication. The impact of targeted interventions has also been highlighted (Killeen *et al.* 2000; Gu and Novak 2005; Yakob and Yan 2009; White *et al.* 2011b; Smith *et al.* 2013).

The commercially available slow-release granular formulation of PPF, Sumilarv®0.5G, was easily applied by hand therefore not requiring expensive application equipment. It is therefore concluded that Sumilarv®0.5G presents a promising new tool for larval control integration in malaria vector control programmes.

8.1.2 Exposure to PPF 24 hours before to 24 hours after a blood meal sterilizes *An. gambiae s.s.* and *Cx. quinquefasciatus* females

In addition to being effective for immature control, PPF has also shown to have a pronounced impact on female vectors when exposed to PPF as adult. PPF sterilized *An. gambiae s.s. and Cx. quinquefasciatus* when adults were in contact with the insecticide between 24 hours before and 24 hours after blood meal. Sterilization included reduced number of females laying eggs, reduced mean number of eggs laid per female that laid and reduced hatching rates in eggs laid leading overall to a reduction of over 90% of the viable offspring from exposed *An. gambiae s.s.* females as compared to unexposed females. Similar results have been found by Harris *et al.* (2013) for *An. arabiensis* exposed to PPF 24 hours after blood meal. These findings provide an exciting new opportunity for vector control targeting the reproduction of vectors rather than their survival, using a completely different mode of action than current public health insecticides therefore providing prospects to manage insecticide resistant vectors. Sumitomo Chemical has just recently developed a new mosquito net (Olyset Duo) that incorporates permethrin (a pyrethroid) and PPF and its effectiveness will be tested in an upcoming trial (Sagnon *et al.* 2015; Tiono *et al.* 2015).

Exposure of females to PPF 48 hours after a blood meal or later when the female is already gravid and close to egg-laying did not affect either mosquito species' reproduction.

8.1.3 Improved mechanisms to optimise release of attractive odorants are required for successful development of the 'attract and kill' strategy

One objective of this thesis was to evaluate strategies for attracting gravid females to a potential oviposition site to kill her offspring. One possible way to apply such an 'attract and kill' strategy would be to combine an attractant with a potent residual larvicide, like

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AMF and PPF tested here. However, the oviposition behaviour of *An. gambiae s.l.* has only recently received increased attention (Sumba *et al.* 2004; Sumba *et al.* 2008; Okal *et al.* 2013; Herrera-Varela *et al.* 2014; Lindh *et al.* 2015) and to date only one oviposition attractant, the sesquiterpene alcohol cedrol, has been reported (Lindh *et al.* 2015). However, when added to a small artificial pond cedrol did not increase the attractiveness of the pond for gravid *An. gambiae s.s.* Consequently, the anticipated 'attract and kill' strategy tested here by combining cedrol with AMF or PPF could not be confirmed. It is likely that the lack of attraction was due to the poor release mechanism of cedrol from the habitat since here a passive release was tested as compared to the published work (Lindh *et al.* 2015) where cedrol was dispensed in water in traps that produce an air current and therefore likely a far more pronounced odour plume. Interestingly, in the experiment where a baiting station for PPF was developed (see below) cedrol did attract females compared to ponds that did not include it, possibly because females could approach the pond more closely in the baiting station experiment where the water was only covered by a sticky screen as compared to the experiment where a square of E-nets was surrounding the ponds at a larger distance to the water. This clearly indicates that there is value to explore the 'attract and kill' strategy combining larvicides with attractants further (see Future Work below).

8.1.4 Auto-dissemination is not a feasible strategy for control of Afrotropical malaria vectors

Auto-dissemination is a technique in which the obligate behaviours of adult mosquitoes are exploited for transfer of lethal doses of insecticide in a far more targeted approach to the preferred larval habitats of mosquitoes during egg-laying (Devine *et al.* 2009; Devine and Killeen 2010). This strategy has been tested a few times for container breeding *Aedes* (Itoh *et al.* 1994; Dell Chism and Apperson 2003; Devine *et al.* 2009) and was suggested for *Anopheles* (Devine and Killeen 2010) control in sub-Saharan Africa as a means to treat difficult to access habitats with the insecticide. However, for this approach to work there are a number of crucial considerations that were investigated in this thesis: When is the best time to contaminate the adult female given that PPF sterilizes females?; How much PPF is transferred by a single female and therefore how many females would be

required to effectively suppress emergence of vectors from an aquatic habitat? How far does an individual female distribute PPF from the place of contamination?

Laboratory assays implemented in small cages showed that the best time to contaminate female *An. gambiae s.s.* and *Cx. quinquefasciatus* for auto-dissemination is while already gravid. Female *An. gambiae s.s.* exposed to PPF while gravid and close to egg-laying transferred PPF to the oviposition substrate leading to approximately 50% emergence inhibition of larvae introduced into the oviposition cup. Contrary, females exposed to PPF around blood meal delivered little or no PPF to the oviposition substrate indicating that a female without viable eggs has little urge to visit the aquatic habitats and therefore is unlikely to transfer PPF. Furthermore, it is likely that most of the PPF contacted at the time of blood feeding has been adsorbed by the body or lost by flight activity between exposure around blood feeding and egg-laying 3-5 days later (Medina *et al.* 2002).

Chromatographic analyses showed that whilst a single gravid *An. gambiae s.s.* could pick up on average 112 µg PPF (active ingredient) from contaminated surfaces, the amount transferred by the female to 100 ml of water was on average only 0.023 µg, therefore around 4,800 times less than that picked up from a contaminated netting screen. The amount required to lead to complete emergence inhibition was identified in dose-response tests as EI_{99} 0.02860 mg/l or 28.6 µg/l suggesting that thousands of females would be required to deliver a sufficient amount of PFF to a puddle of 1 m^2 and a depth of 10 cm (100 l volume). This could be highly improved if the female would in fact deliver to the habitat the total amount she had picked up. If the female would transfer the 112 µg it would be sufficient to completely inhibit adult vector emergence from water bodies of a capacity of 4 L. Based on this, a minimum of 25 females would be required to deliver PPF to a puddle (100 l) to completely inhibit adult vector emergence in the field. However, based on published data on early instar larval densities in natural habitats per 1m^2 (Ndenga *et al.* 2011) and assuming that the median number of eggs laid by a female is 50 (Herrera-Varela *et al.* 2014), even 25 females per m^2 seems unrealistic.

In this study the females were contaminated by landing on a netting screen that was powdered with a Sumilarv® formulation that contained 10% PPF. Likely, the females lost most of the powder when flying and resting before laying eggs. To minimize loss of PPF from the mosquito body and maximize the amount of active ingredient transferred to water requires the exploration of improved contamination mechanisms such as the use of

electrostatically charged PPF particles (Huang *et al.* 2010) that adhere to the mosquito's body more strongly but are delivered from the body when the female gets in contact with water. Additionally, the use of formulation that has a higher content of the active ingredient such as technical powders containing 100% PPF would increase the impact on adult vector emergence inhibition. However such formulations would be highly costly (personal communication, Sumitomo Chemical), and given that only a small proportion of what would be required in a baiting station would actually be picked up and transferred to aquatic habitats, this appears little cost-effective in resource-deprived Africa.

This is corroborated by the findings from the semi-field tests where it was shown that even if gravid females are strongly lured to an attractive baiting station to pick up PPF, lethal doses of PPF will eventually only be transferred to larval habitats closest to the dissemination station. This suggests that numerous dissemination stations would be needed for vector control under field conditions where habitats are numerous and where the most inaccessible habitats might be further away than others therefore the method would fail to reach those habitats for which it was developed.

Based on the here presented findings it is concluded that for an effective auto-dissemination approach for malaria vector control heavy investments would be required for the development of highly attractive baiting stations, improved mechanisms to ensure that gravid females pick up PPF, improved formulations that contain a higher content of the active ingredient and a large number of stations in areas with high numbers of aquatic habitats. Such an approach might be working in areas with low habitat numbers or during dry seasons but seems otherwise highly impractical and very costly. Thus the monthly application of PPF to aquatic habitats already shown in this study to be effective in suppressing adult vector production from treated aquatic habitats is a more attainable approach for control of immature stages of these malaria vectors.

8.2   Limitations of the study

As with all studies it needs to be considered that the findings apply to the local eco-epidemiological settings in which they have been tested and possibly to the local vector species and strains used in the laboratory. This has to be kept in mind when generalizing results.

The field work investigating the monthly impact of PPF application has been implemented in the western Kenya highlands where habitats are focal, defined, and accessible and where the climate does not lead to a very fast larval development. It can be expected that similar results will be found under similar conditions; however, optimal re-treatment intervals have to be investigated in other eco-epidemiological settings. The field work could have been strengthened by having comparable entomological baseline data for a 'controlled before after study design' however, since three study clusters were investigated in the non-intervention and intervention arm and findings did not vary strongly between clusters in each arm, the results are considered reliable.

The methodology used for routine surveys to sample mosquito larvae and pupae in the field was not standardized. The presence of mosquito larvae and pupae was assessed by taking only 10 dips from habitats irrespective of their size. Moreover the abundance of mosquito larvae and pupae was assessed by sampling only 20 m length of the water surface area when the habitat exceeded this size. Yet the sampling efficiency of mosquito larvae and pupae in aquatic habitats varies with the size of the water body sampled (Service 1971). Thus the results presented here on the colonisation and abundance of mosquito immatures in aquatic habitats need to be interpreted with some caution. However, since this same sampling scheme was used throughout the study, the same bias has likely been introduced everywhere and whilst the data might not be very precise in quantitative terms it is considered reliable qualitatively.

The field work could have been extended to shed some light on abiotic factors on the performance of PPF. Under standardized field conditions it was found that turbidity and pH affect the activity of PPF in controlling mosquitoes, however, the impact of these factors were not further explored in studies carried out under natural field conditions. Other factors that are known to influence the activity of larvicides such as water salinity and temperature were not monitored (Rydzanicz *et al.* 2010). These limitations were given by the extent of the number of habitats monitored in the field.

The extended development periods of immature stages of odonata and other non-target aquatic insects found in the aquatic habitats that takes months and at time years (Stoks and Cordoba-Aguilar 2012) was a hindrance in the assessment of the impact of PPF on emergence of adult insects from these non-target immature stages. Thus collection of the immature stages of the non-target aquatic insects from the habitats for observing adult

emergence in the laboratory was considered impractical. The impact of PPF on non-target insects was therefore assessed by monitoring the fluctuations in the abundance of these non-target organisms which is flawed since PPF's mode of action is the prevention in emergence of adult insects from immature stages while having minimal impacts on the immature stages (Invest and Lucas 2008).

The standardized field tests to evaluate the residual activity of PPF were conducted during the dry season when mosquito densities are generally low in Mbita where these tests were conducted (Fillinger *et al.* 2004). Thus tests to assess the impact of PPF were conducted with insectary-reared late instar *An. gambiae s.s.* and *An. arabiensis* larvae introduced into treated ponds. Whilst PPF is a pupicide that has little impact on mosquito larvae the impact in reducing adult emergence is higher in larvae that have prolonged exposure to the insecticide (Invest and Lucas 2008). Thus it is hypothesized that higher emergence inhibition rates would be observed if the effect on emergence inhibition was assessed on mosquito larvae exposed to the insecticide as from early instars.

Although the research regarding the sterilizing effects of PPF on *An. gambiae s.s.* and *Cx. quinquefasciatus* forms a very valuable insight in the potential of this strategy for mosquito control, it would have been desirable to monitor sterilized females over successive gonotrophic cycles and to investigate the survival of exposed females since it has been suggested that a single point of exposure has long-ranging impact, however the evidence for this is very limited (Ohashi *et al.* 2012; Harris *et al.* 2013).

Studies to evaluate the 'attract and kill' strategies that involve the use of semiochemicals to lure gravid females to baiting stations did not explore alternative robust dispensing mechanisms of attractive odorants from a source such as the use of low density polyethylene (LDPE) or nylon strips (Mukabana *et al.* 2012). Moreover, the interaction between cedrol and PPF or AMF was also not explored, even though these might have important consequences when both attractant and larvicide are applied into the same aquatic habitat.

8.3   Future work

Several new research questions resulted from the findings of this thesis that are worth perusing in further studies.

Although the self-spreading surface film AMF was found to be highly effective for mosquito control in laboratory and standardized field tests, the effectiveness of this surface film for mosquito control under natural field conditions was not evaluated. Such a surface film would be especially beneficial in the control of mosquito larvae in extensive and vegetated aquatic habitats. There is still very limited published data on AMF for field use. Only Bukhari *et al.* (2011) has evaluated AMF for control of *An. gambiae s.l.* in the field. They found AMF to suppress production of adult anophelines by 88% and adult culicines by 82% in rice paddies treated with AMF at 2 ml/m^2. More studies are however needed to evaluate the potential of the surface film for mosquito control in extensive habitats that are highly inaccessible for larvicide application personnel (Majambere *et al.* 2010) as well as in smaller confined habitats where this film might provide a long-lasting solution. The ease of handling makes this control agent especially interesting for future evaluations. However, based on the mode of action, it will also be important to test non-target effects.

Based on the promising findings with operational PPF applications in the western Kenya highlands, it would be desirable to evaluate this intervention in different eco-epidemiological settings and also to evaluate its impact on disease outcome.

The failure of cedrol to lure gravid females to treated ponds from a distance in some of the experiments was hypothesised to be due to the lack of good release mechanisms of the odorant when applied directly in water. The non-repellent effect of both AMF and PPF suggest they can be effectively combined with chemicals that are attractive to gravid females to lure females to lays eggs in ponds treated with these larvicides. Thus the development of baits that attract gravid *An. gambiae s.s.* to aquatic habitats needs to be prioritized. Of importance is the exploration of additional substances that are attractive to gravid *An. gambiae s.s* that can be used in synergy with cedrol. In addition, the exploration of improved mechanisms to release attractive odorants from a source without directly introducing the chemical in water, such as the use of low density polyethylene (LDPE) sachets and nylon strips (Mukabana *et al.* 2012) is considered necessary.

The success of auto-dissemination for malaria vector control is partly dependent on the number of PPF-contaminated gravid females that visit a larval habitat to lay eggs (Devine and Killeen 2010). Thus studies to determine the number of gravid females that visit an aquatic habitat to lay eggs are considered vital. This can be done by use of molecular

tools to investigate the genetic relatedness of mosquito larvae contained in an aquatic habitat (Chen *et al.* 2006; Chen *et al.* 2008b).

8.4   General conclusions

The findings of this thesis provide considerable evidence of the potential of two persistent insecticides with novel modes of action, the silicone-based surface film Aquatain Mosquito Formulation (AMF) and the insect growth regulator pyriproxyfen (PPF) for the control of malaria vectors in Africa. At the same time this thesis emphasises the need to explore mechanisms to improve novel methods of larvicide application by combining those with oviposition attractants.

These conclusions are derived from research findings that were carried out stepwise. First, laboratory and field tests demonstrated the effective control of mosquito immatures with AMF and PPF. *Anopheles gambiae s.s.* and *An. arabiensis* were highly susceptible to both insecticides when applied at low doses. The field study in the western Kenya highlands confirmed that 15 rounds of PPF application per year effectively suppress more than 80% of the vector emergence from treated aquatic habitats making larviciding with PPF a promising tool for integrated vector management.

Second, this study showed that the optimum time to contaminate female *An. gambiae s.s.* and *Cx. quinquefasciatus* with PPF for sterilizing effects is close to blood meal time. Strong sterilizing effects were observed when females of both species were topically exposed to PPF between 24 hours before and 24 hours after blood meal

The potential of 'attract and kill' strategies that combines treatment of water bodies with larvicides and oviposition attractants to lure gravid *An. gambiae s.s.* to lay eggs in larvicide-treated water was not confirmed in this study. Additional research is needed to develop improved mechanisms for slow, consistent and long-lasting release of attractive odours from water sources.

Based on the combined results from laboratory and semi-field experiments, this study found auto-dissemination not to be a feasible strategy for the control of Afrotropical malaria vectors.

9   References

Abad-Franch, F., E. Zamora-Perea, G. Ferraz, S. D. Padilla-Torres and S. L. B. Luz (2015). "Mosquito-disseminated pyriproxyfen yields high breeding-site coverage and boosts juvenile mosquito mortality at the neighborhood scale." PLoS Negl Trop Dis **9**(4): e0003702.

Abbott, W. S. (1987). "A method of computing the effectiveness of an insecticide." J Am Mosq Control Assoc **3**(2): 302-303.

Abdalla, S. I., E. M. Malik and K. M. Ali (2007). "The burden of malaria in Sudan: incidence, mortality and disability - adjusted life - years." Malar J **6**: 97.

Abramides, G. C., D. Roiz, R. Guitart, S. Quintana, I. Guerrero and N. Gimenez (2011). "Effectiveness of a multiple intervention strategy for the control of the tiger mosquito (*Aedes albopictus*) in Spain." Trans R Soc Trop Med Hyg **105**(5): 281-288.

Afrane, Y. A., T. J. Little, B. W. Lawson, A. K. Githeko and G. Yan (2008). "Deforestation and vectorial capacity of *Anopheles gambiae* Giles mosquitoes in malaria transmission, Kenya." Emerg Infect Dis **14**(10): 1533-1538.

Afrane, Y. A., G. Zhou, A. K. Githeko and G. Yan (2014). "Clinical malaria case definition and malaria attributable fraction in the highlands of western Kenya." Malar J **13**: 405.

Aiku, A. O., A. Yates and M. Rowland (2006). "Laboratory evaluation of pyriproxifen treated bednets on mosquito fertility and fecundity. A preliminary study." West Afri J Med **25**(1): 22-26.

Al-Sarar, A. S., D. Al-Shahrani, A. E. Bayoumi, Y. Abobakr and H. I. Hussein (2011). "Laboratory and field evaluation of some chemical and biological larvicides against *Culex* spp. (Diptera: Culicidae) immature stages." Int J Agr Biol **13**: 115-119.

Ali, A., M. A. Chowdhury, M. I. Hossain, A. Mahmud Ul, D. B. Habiba and A. F. Aslam (1999). "Laboratory evaluation of selected larvicides and insect growth regulators against field-collected *Culex quinquefasciatus* larvae from urban Dhaka, Bangladesh." J Am Mosq Control Assoc **15**(1): 43-47.

Ali, A., J. K. Nayar and R. D. Xue (1995). "Comparative toxicity of selected larvicides and insect growth regulators to a Florida laboratory population of *Aedes albopictus*." J Am Mosq Control Assoc **11**(1): 72-76.

Alonso, P. L., S. W. Lindsay, J. R. Armstrong, M. Conteh, A. G. Hill, P. H. David, G. Fegan, A. de Francisco, A. J. Hall, F. C. Shenton and et al. (1991). "The effect of insecticide-treated bed nets on mortality of Gambian children." Lancet **337**(8756): 1499-1502.

Ameneshewa, B. and M. W. Service (1996). "The relationship between female body size and survival rate of the malaria vector *Anopheles arabiensis* in Ethiopia." Med Vet Entomol **10**: 170-172.

Andrighetti, M. T. M., F. Cerone, M. Rigueti, K. C. Galvani and M. L. G. Macoris (2008). "Effect of pyriproxyfen in *Aedes aegypti* populations with different levels of susceptibility to the organophosphate temephos." Dengue Bulletin **32**: 186-198.

Ansari, M. A., V. P. Sharma, P. K. Mittal and R. K. Razdan (1991). "Evaluation of juvenile hormone analogue JHM/S-31183 against immature stages of mosquitoes in natural habitats." Indian J Malariol **28**: 39-43.

Antonio, G. E., D. Sanchez, T. Williams and C. F. Marina (2009). "Paradoxical effects of sublethal exposure to the naturally derived insecticide spinosad in the dengue vector mosquito, *Aedes aegypti*." Pest Manag Sci **65**: 323–326.

Arab, A., M. C. Jackson and C. Kongoli (2014). "Modelling the effects of weather and climate on malaria distributions in West Africa." Malar J **13**: 126.

Araujo, M. S., L. H. S. Gil and A. A. e-Silva (2012). "Larval food quantity affects development time, survival and adult biological traits that influence the vectorial capacity of *Anopheles darlingi* under laboratory conditions." Malar J **11**: 261.

Atieli, H. E., G. Zhou, M. C. Lee, E. J. Kweka, Y. Afrane, I. Mwanzo, A. K. Githeko and G. Yan (2011). "Topography as a modifier of breeding habitats and concurrent vulnerability to malaria risk in the western Kenya highlands." Parasit Vectors **4**: 241.

Awolola, T. S., A. O. Oduola, J. B. Obansa, N. J. Chukwurar and J. P. Unyimadu (2007). "*Anopheles gambiae s.s.* breeding in polluted water bodies in urban Lagos, southwestern Nigeria." J Vector Borne Dis **44**(4): 241-244.

Ayele, D. G., T. T. Zewotir and H. G. Mwambi (2013). "Spatial distribution of malaria problem in three regions of Ethiopia." Malar J **12**: 207.

Bai, H., D. B. Gelman and S. R. Palli (2011). "Mode of action of methoprene in affecting female reproduction in the African malaria mosquito, *Anopheles gambiae*." *Pest Manag Sci* **66**(9): 936-943.

Bang, Y. H., N. GRatz and C. P. Pant (1972). "Suppression of a field population of *Aedes aegypti* by malathion thermal fogs and Abate larvicide " Bull World Health Organ **46**: 554-558.

Barbazan, P., T. Baldet, F. Darriet, H. Escaffre, D. H. Djoda and J. M. Hougard (1998). "Impact of treatments with *Bacillus sphaericus* on *Anopheles* populations and the transmission of malaria in Maroua, a large city in a savannah region of Cameroon." J Am Mosq Control Assoc **14**: 33–39.

Barrera, R., A. J. Mackay and M. Amador (2013). "A novel autocidal ovitrap for the surveillance and control of *Aedes aegypti*." J Am Mosq Control Assoc **29**(3): 293-296.

Bartlett-Healy, K., I. Unlu, P. Obenauer, T. Hughes, S. Healy, T. Crepeau, A. Farajollahi, B. Kesavaraju, D. Fonseca, G. Schoeler, R. Gaugler and D. Strickman (2012). "Larval mosquito habitat utilization and community dynamics of *Aedes albopictus* and *Aedes japonicus* (Diptera: Culicidae)." J Med Entomol **49**(4): 813-824.

Batra, C. P., P. K. Mittal, T. Adak and S. K. Subbarao (2006). "Efficacy of Agnique MMF monomolecular surface film against *Anopheles stephensi* breeding in urban habitats in India." J Am Mosq Control Assoc **22**(3): 426-432.

Bayoh, M. N. and S. W. Lindsay (2003). "Effect of temperature on the development of the aquatic stages of *Anopheles gambiae sensu stricto* (Diptera: Culicidae)." Bull Entomol Res **93**(5): 375-381.

Bayoh, M. N., D. K. Mathias, M. R. Odiere, F. M. Mutuku, L. Kamau, J. E. Gimnig, J. M. Vulule, W. A. Hawley, M. J. Hamel and E. D. Walker (2010). "*Anopheles gambiae*: historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya." Malar J **9**: 62.

Bayoh, M. N., E. D. Walker, J. Kosgei, M. Ombok, G. B. Olang, A. K. Githeko, G. F. Killeen, P. Otieno, M. Desai, N. F. Lobo, J. M. Vulule, M. J. Hamel, S. Kariuki and J. E. Gimnig (2014). "Persistently high estimates of late night, indoor exposure to malaria vectors despite high coverage of insecticide treated nets." Parasit Vectors **7**: 380.

Beck-Johnson, L. M., W. A. Nelson, K. P. Paaijmans, A. F. Read, M. B. Thomas and O. N. Bjørnstad (2013). "The effect of temperature on *Anopheles* mosquito population dynamics and the potential for malaria transmission." PLoS ONE **8**(11): e79276.

Becker, N. and F. Rettich (1994). "Protocol for the introduction of new *Bacillus thuringiensis israelensis* products into the routine mosquito control program in Germany." J Am Mosq Control Assoc **10**(4): 527-533.

Begum, M. N., T. U. Ahmed and M. E-Khoda (1985). "Gonotrophic cycle of *Culex quinquefasciatus* Say (Diptera: Culicidae) in Dhaka." Bangladesh J Zool **14**(2): 111-115.

Beier, J. C., J. Keating, J. I. Githure, M. B. Macdonald, D. E. Impoinvil and R. J. Novak (2008). "Integrated vector management for malaria control." Malar J **7 Suppl 1**: S4.

Beier, J. C., G. F. Killeen and J. I. Githure (1999). "Short report: entomologic inoculation rates and *Plasmodium falciparum* malaria prevalence in Africa." Am J Trop Med Hyg **61**(1): 109-113.

Bentley, M. D. and J. F. Day (1989). "Chemical ecology and behavioral aspects of mosquito oviposition." Annu Rev Entomol **34**: 401-421.

Berlin, J. A. and D. W. Micks (1973). "Cellular and subcellular effects of petroleum hydrocarbons on mosquito larvae." Ann Entomol Soc Am **66**(4): 775-780.

Binka, F. N., A. Kubaje, M. Adjuik, L. A. Williams, C. Lengeler, G. H. Maude, G. E. Armah, B. Kajihara, J. H. Adiamah and P. G. Smith (1996). "Impact of permethrin impregnated bednets on child mortality in Kassena-Nankana district, Ghana: a randomized controlled trial." Trop Med Int Health **1**(2): 147-154.

Bogh, C., S. E. Clarke, M. Jawara, C. J. Thomas and S. W. Lindsay (2003). "Localized breeding of the *Anopheles gambiae* complex (Diptera: Culicidae) along the River Gambia, West Africa." Bull Entomol Res **93**(4): 279-287.

Boreman, P. F. L. and G. R. Port (1982). "The distribution and movement of engorged females of *Anopheles gambiae* Giles (Diptera: Culicidae) in a Gambian village." Bull Entomol Res **72**: 489-495.

Boudjelida, H., A. Bouaziz, T. Soin, G. Smagghe and N. Soltani (2005). "Effects of ecdysone agonist halofenozide against *Culex pipiens*." Pest Biochem Physiol **83**: 115-123.

Bousema, T., J. T. Griffin, R. W. Sauerwein, D. L. Smith, T. S. Churcher, W. Takken, A. Ghani, C. Drakeley and R. Gosling (2012). "Hitting hotspots: spatial targeting of malaria for control and elimination." PLoS Med **9**(1): e1001165.

Briegel, H. (1990). "Fecundity, metabolism, and body size in *Anopheles* (Diptera:Culicidae), vectors of malaria." Malar J **27**: 839-850.

Brown, T. M., D. H. Devries and A. W. A. Brown (1978). "Induction of resistance to insect growth regulators." J Econ Entomol **71**(2): 223-229.

Brown, T. M. and G. H. S. Hooper (1979). "Metabolic detoxication as a mechanism of methoprene resistance in *Culex pipiens pipiens*." Pestic Biochem Physiol **12**: 79-86.

Bruce-Chwatt, L. J. (1966). "The epidemiology of malaria." Bull Soc Pathol Exot Filiales **59**: 459-466.

Buczkowski, G., C. W. Scherer and G. W. Bennett (2008). "Horizontal transfer of bait in the German cockroach: indoxacarb causes secondary and tertiary mortality." J Econ Entomol **101**(3): 894-901.

Bukhari, T. and B. G. Knols (2009). "Efficacy of Aquatain, a monomolecular surface film, against the malaria vectors *Anopheles stephensi* and *An. gambiae* s.s. in the laboratory." Am J Trop Med Hyg **80**(5): 758-763.

Bukhari, T., W. Takken, A. K. Githeko and C. J. Koenraadt (2011). "Efficacy of aquatain, a monomolecular film, for the control of malaria vectors in rice paddies." PLoS One **6**(6): e21713.

Burkot, T. R., T. Handzel, M. A. Schmaedick, J. Tufa, J. M. Roberts and P. M. Graves (2007). "Productivity of natural and artificial containers for *Aedes polynesiensis* and *Aedes aegypti* in four American Samoan villages." Med Vet Entomol **21**(1): 22-29.

Caputo, B., A. Ienco, D. Cianci, M. Pombi, V. Petrarca, A. Baseggio, G. J. Devine and A. della Torre (2012). "The ''auto-dissemination'' approach: A novel concept to fight *Aedes albopictus* in urban areas." PLoS ONE **6**(8): e1793.

Carlson, D. B. (2006). "Source reduction in Florida's salt marshes: Management to reduce pesticide use and enhance the resource." J Am Mosq Control Assoc **22**(3): 534-537.

Carlson, J. C., B. D. Byrd and F. X. Omlin (2004). "Field assessments in western Kenya link malaria vectors to environmentally disturbed habitats during the dry season." BMC Public Health **4**: 33.

Carrieri, M., A. Masetti, A. Albieri, B. Maccagnani and R. Bellini (2009). "Larvicidal activity and influence of *Bacillus thuringiensis* var *israelensis* on *Aedes albopictus* oviposition in ovitraps during a two-week check-interval protocol." J Am Mosq Control Assoc **25**(2): 149-155.

Carroll, M. K. (1979). "Methoprene briquetes as an attractant for gravid *Aedes aegypti* (L.)." Mosq News **39**(3): 680-681.

Carter, R., K. N. Mendis and D. Roberts (2000). "Spatial targeting of interventions against malaria." Bull World Health Organ **78**(12): 1401-1411.

Chadee, D. D. (2010). "The diel oviposition periodicity of *Aedes aegypti* (L.) (Diptera: Culicidae) in Trinidad, West Indies: effects of forced egg retention." Bull Entomol Res **100**: 599-603.

Chadee, D. D. and S. A. Ritchie (2010). "Efficacy of sticky and standard ovitraps for *Aedes aegypti* in Trinidad, West Indies." J Vector Ecol **35**(2): 395-400.

Chaki, P. P., N. J. Govella, B. Shoo, A. Hemed, M. Tanner, U. Fillinger and G. F. Killeen (2009). "Achieving high coverage of larval-stage mosquito surveillance: challenges for a community-based mosquito control programme in urban Dar es Salaam, Tanzania." Malar J **8**: 311.

Chanda, E., F. Masaninga, M. Coleman, C. Sikaala, C. Katebe, M. Macdonald, K. S. Baboo, J. Govere and L. Manga (2008). "Integrated vector management: The Zambian experience." Malar J **7**: 164.

Chavasse, D. C., J. D. Lines, K. Ichimori, A. R. Majala, J. N. Minjas and J. Marijani (1995a). "Mosquito control in Dar es Salaam. II. Impact of expanded polystyrene beads and pyriproxyfen treatment of breeding sites on *Culex quinquefasciatus* densities." Med. Vet. Entomol. **9**(2): 147-154.

Chavasse, D. C., J. D. Lines, K. Ichimori and J. Marijani (1995b). "Mosquito control in Dar es Salaam. I. Assessment of *Culex quinquefasciatus* breeding sites prior to intervention." Med Vet Entomol **9**(2): 141-146.

Chaves, L. F., M. Hashizume, A. Satake and N. Minakawa (2012). "Regime shifts and heterogeneous trends in malaria time series from Western Kenya Highlands." Parasitology **139**(1): 14-25.

Cheikh, H. B. (1993). "Resistance to temephos, an organophosphorus insceticide in *Culex pipiens* form Tunisia, North Africa." J Am Mosq Control Assoc **9**: 335-337.

Chen, H., U. Fillinger and G. Yan (2006). "Oviposition behavior of female *Anopheles gambiae* in western Kenya inferred from microsatellite markers." Am J Trop Med Hyg **75**(2): 246-250.

Chen, H., A. K. Githeko, J. I. Githure, J. Mutunga, G. Zhou and G. Yan (2008a). "Monooxygenase levels and knockdown resistance (kdr) allele frequencies in *Anopheles gambiae* and *Anopheles arabiensis* in Kenya." J Med Entomol **45**(2): 242-250.

Chen, H., N. Minakawa, L. Cui and G. Yan (2008b). "Conspecific sharing of breeding sites by anopheline female mosquitoes (Diptera: Culicidae) inferred from microsatellite markers." J insect behav **21**: 24–33

Choe, D. H. and M. K. Rust (2008). "Horizontal transfer of insecticides in laboratory colonies of the Argentine ant (Hymenoptera: Formicidae)." J Econ Entomol **101**(4): 1397-1405.

Chouaibou, M., J. Etang, T. Brevault, P. Nwane, C. K. Hinzoumbe, R. Mimpfoundi and F. Simard (2008). "Dynamics of insecticide resistance in the malaria vector *Anopheles gambiae s.l.* from an area of extensive cotton cultivation in Northern Cameroon." Trop Med Int Health **13**(4): 476-486.

Clements, A. N. (2000). "The biology of mosquitoes. Development nutrition and reproduction." CABI Publishers.

Cleveland, C. B., M. A. Mayes and S. A. Cryer (2001). "An ecological risk assessment for spinosad use on cotton." Pest Manag Sci **58**: 70-84.

Clive, S. (2002). "Integrated approach to malaria control." Clin Microbiol Rev **15**: 278–293.

Colton, Y. M., D. D. Chadee and D. W. Severson (2003). "Natural skip oviposition of the mosquito *Aedes aegypti* indicated by codominant genetic markers." Med Vet Entomol **17**(2): 195-204.

Coluzzi, M. (1999). "The clay feet of the malaria giant and its African roots: hypotheses and inferences about ongin, spread and control of *Plasmodium falciparum*." <u>Parassitologia</u> **41**: 277-283.

Coluzzi, M., A. Sabatini, V. Petrarca and M. A. Di Deco (1979). "Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex." <u>Trans R Soc Trop Med Hyg</u> **73**(5): 483-497.

Constantini, C., N. F. Sagnon, A. della Torre and M. Coluzzi (1999). "Mosquito behavioural aspects of vector-human interactions in the *Anopheles gambiae* complex." <u>Parassitologia</u> **41**: 209-217.

Coosemans, M., M. Wery, J. Mouchet and P. Carnevale (1992). "Transmission factors in malaria epidemiology and control in Africa." <u>Mem. Inst. Oswaldo Cruz</u> **87**: 385-391.

Corbet, S. A., C. Tiley, T. Moorhouse, C. Giam, S. Pursglove, J. Raby and M. Rich (2000). "Surface films as mosquito larvicides: partitioning the mode of action." <u>Entomology</u> **94** 295–307.

Crawley, J. (2004). "Reducing the burden of anemia in infants and young children in malaria-endemic countries of Africa: from evidence to action." <u>Am J Trop Med Hyg</u> **71**(2 Suppl): 25-34.

Crowder, D. W., C. Ellers-Kirk, C. M. Yafuso, T. J. Dennehy, B. A. Degain, V. S. Harpold, B. E. Tabashnik and Y. Carriere (2008). "Inheritance of resistance to pyriproxyfen in *Bemisia tabaci* (Hemiptera: Aleyrodidae) males and females (B Biotype)." <u>J. Econ. Entomol.</u> **101**(3): 927-932.

Cuker, B. E. (1987). "Field experiment on the influences of suspended clay and P on the plankton of a small lake." <u>Limnol Oceanogr</u> **32**: 840–847.

Dame, D. A., G. J. Wichterman and J. A. Hornby (1998). "Mosquito (*Aedes taeniorhynchus*) resistance to methoprene in an isolated habitat." <u>J Am Mosq Control Assoc</u> **14**(2): 200-203.

Darwazeh, H. A., R. C. Fox and D. J. Ramke (1972). "Efficacy of fortified petroleum oils as mosquito larvicides in Irrigated pastures." <u>Proc. Calif. Mosq. Cont. Assoc.</u>: 46-48.

Das, S., L. Garver and G. Dimopoulos (2007). "Protocol for mosquito rearing (*A. gambiae*)." <u>J Vis Exp</u>(5): 221.

David, M. R., G. S. Ribeiro and R. M. Freitas (2012). "Bionomics of *Culex quinquefasciatus* within urban areas of Rio de Janeiro, Southeastern Brazil." <u>Rev Saude Publica</u> **46**(5): 858-865.

de Jong, R. and B. G. Knols (1995). "Olfactory responses of host-seeking *Anopheles gambiae s.s.* Giles (Diptera: Culicidae)." <u>Acta Trop</u> **59**(4): 333-335.

Dean, S. R., R. W. Meola, S. M. Meola, H. Sittertz-Bhatkar and R. Schenker (1998). "Mode of action of lufenuron on larval cat fleas (Siphonaptera: Pulicidae)." <u>J Med Entomol</u> **35**(5): 720-724.

Dejenie, T., M. Yohannes and T. Assmelash (2011). "Characterization of mosquito breeding sites in and in the vicinity of tigray microdams." <u>Ethiop J Health Sci</u> **21**(1): 57-66.

Dell Chism, B. and C. S. Apperson (2003). "Horizontal transfer of the insect growth regulator pyriproxyfen to larval microcosms by gravid *Aedes albopictus* and *Ochlerotatus triseriatus* mosquitoes in the laboratory." <u>Med Vet Entomol</u> **17**(2): 211-220.

Deul, D. H., B. J. de Jong and J. A. M. Kortenbach (1976). "Inhibition of chitin synthesis by two 1-(2,6-disubstituted benzoyl)-3-phenylurea insecticides." <u>Pest Biochem Physiol</u> **8**: 98-105.

Devine, G. J. and G. F. Killeen (2010). "The potential of a new larviciding method for the control of malaria vectors." <u>Malar J</u> **9**: 142.

Devine, G. J., E. Z. Perea, G. F. Killeen, J. D. Stancil, S. J. Clark and A. C. Morrison (2009). "Using adult mosquitoes to transfer insecticides to *Aedes aegypti* larval habitats." <u>PNAS Early Edition</u>: 0901369106.

Dinan, L., P. Whiting, J. Girault, R. Lafont, S. Dhadialla, D. E. Cress, B. Mugat, C. Antoniewski and J. Lepesant (1997). "Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor." <u>Biochem J</u> **327**: 643-650.

Dossou-yovo, J., J. Doannio, F. Riviere and G. Chauvancy (1995). "Urbanization and establishment of *Culex quinquefasciatus* in a west African rural area." <u>Acta Trop</u> **59**(3): 251-253.

Dugassa, S., J. M. Lindh, S. J. Torr, F. Oyieke, S. W. Lindsay and U. Fillinger (2012). "Electric nets and sticky materials for analysing oviposition behaviour of gravid malaria vectors." <u>Malar J</u> **11**: 374.

Dukeen, M. Y. H. (1986). "Ecology of the malaria vector *Anopheles arabiensis* Patton (Diptera:Culicidae) by the Nile in northern Sudan." <u>Bull Ent Res</u> **76**: 451-467.

Durnez, L. and M. Coosemans (2014). "Residual transmission of malaria: an old issue for new approaches." In Manguin, S. (Ed.). *Anopheles Mosquitoes – New Insights into Malaria Vectors*. ISBN: 978-953-951-1188-1187, InTech.

Eiras, A. E., T. S. Buhagiar and S. A. Ritchie (2014). "Development of the gravid *Aedes* trap for the capture of adult female container-exploiting mosquitoes (Diptera: Culicidae)." <u>J Med Entomol</u> **51**(1): 200-209.

Eisele, T. P., D. Larsen and R. W. Steketee (2010). "Protective efficacy of interventions for preventing malaria mortality in children in *Plasmodium falciparum* endemic areas." <u>Int J Epidemiol</u> **39**: 88-101.

El-Shazly, M. M. and B. M. Refaie (2002). "Larvicidal effect of the juvenile hormone mimic pyriproxyfen on *Culex pipiens*." <u>J Am Mosq Control Assoc</u> **18**(4): 321-328.

Ernst, K. C., S. O. Adoka, D. O. Kowuor, M. L. Wilson and C. C. John (2006). "Malaria hotspot areas in a highland Kenya site are consistent in epidemic and non-epidemic years and are associated with ecological factors." <u>Malar J</u> **5**: 78.

Fales, J. H., P. J. Spangler, O. F. Bodenstein, G. D. Mills Jr and C. G. Durbin (1968). "Laboratory and field evaluation of Abate against a backswimmer (*Notonecta undulata* Say) (Hemiptera: *Notonectidae*)." <u>Mosq News</u> **28**: 77-81.

Farnesi, L. C., J. M. Brito, J. G. Linss, M. Pelajo-Machado, D. Valle and G. L. Rezende (2012). "Physiological and morphological aspects of *Aedes aegypti* developing larvae: effects of the chitin synthesis inhibitor novaluron." <u>PLoS ONE</u> **7**(1): e30363.

Faye, O., L. Konate, J. Mouchet, D. Fontenille, S. Y. Ngayo, G. Hebrard and J. P. Herve (1997). "Indoor resting by outdoor biting females of *Anopheles gambiae* complex (Diptera: Culicidae) in the Sahel of Northern Senegal." <u>J Med Entomol</u> **34**(3): 285-289.

Ferguson, H. M., A. Dornhaus, A. Beeche, C. Borgemeister, M. Gottlieb, M. S. Mulla, J. E. Gimnig, D. Fish and G. F. Killeen (2010). "Ecology: A prerequisite for malaria elimination and eradication." <u>PLoS Med</u> **7**(8): e1000303.

Fillinger, U., K. Kannady, G. William, M. J. Vanek, S. Dongus, D. Nyika, Y. Geissbuhler, P. P. Chaki, N. J. Govella, E. M. Mathenge, B. H. Singer, H. Mshinda, S. W. Lindsay, M. Tanner, D. Mtasiwa, M. C. de Castro and G. F. Killeen (2008). "A tool box for operational mosquito larval control: preliminary results and early lessons from the Urban Malaria Control Programme in Dar es Salaam, Tanzania." <u>Malar J</u> **7**: 20.

Fillinger, U., B. G. Knols and N. Becker (2003). "Efficacy and efficiency of new *Bacillus thuringiensis* var *israelensis* and *Bacillus sphaericus* formulations against Afrotropical anophelines in Western Kenya." <u>Trop Med Int Health</u> **8**(1): 37-47.

Fillinger, U. and S. W. Lindsay (2006). "Suppression of exposure to malaria vectors by an order of magnitude using microbial larvicides in rural Kenya." <u>Trop Med Int Health</u> **11**(11): 1629-1642.

Fillinger, U. and S. W. Lindsay (2011). "Larval source management for malaria control in Africa: myths and reality." <u>Malar J</u> **10**: 353.

Fillinger, U., B. Ndenga, A. Githeko and S. W. Lindsay (2009a). "Integrated malaria vector control with microbial larvicides and insecticide-treated nets in western Kenya: a controlled trial." <u>Bull World Health Organ</u> **87**(9): 655-665.

Fillinger, U., H. Sombroek, S. Majambere, E. van Loon, W. Takken and S. W. Lindsay (2009b). "Identifying the most productive breeding sites for malaria mosquitoes in The Gambia." Malar J **8**: 62.

Fillinger, U., G. Sonye, G. F. Killeen, B. G. Knols and N. Becker (2004). "The practical importance of permanent and semipermanent habitats for controlling aquatic stages of *Anopheles gambiae sensu lato* mosquitoes: operational observations from a rural town in western Kenya." Trop Med Int Health **9**(12): 1274-1289.

Floore, T. G. (2006). "Mosquito larval control practices: past and present." J Am Mosq Control Assoc **22**(3): 527-533.

Fortin, C., A. Maire and R. Leclair (1987). "The residual effect of temephos (Abate 4-E) on nontarget communities." J Am Mosq Control Assoc **3**: 282-288.

Fournet, F., C. Sannier and N. Monteny (1993). "Effects of the insect growth regulators OMS 2017 and diflubenzuron on the reproductive potential of *Aedes aegypti*." J Am Mosq Control Assoc **9**(4): 426-430.

Freeborn, S. B. and R. F. Atsatt (1918). "The effects of petroleum oils on mosquito larva." J Econ Entomol **2**: 299-307.

Gallup, J. L. and J. D. Sachs (2001). "The economic burden of malaria." Am J Trop Med Hyg **64**(1-2 Suppl): 85-96.

Garcia-Rejon, J. E., J. A. Farfan-Ale, A. Ulloa, L. F. Flores-Flores, E. Rosado-Paredes, C. Baak-Baak, M. A. Lorono-Pino, I. Fernandez-Salas and B. J. Beaty (2008). "Gonotrophic cycle estimate for *Culex quinquefasciatus* in Merida, Yucatan, Mexico." J Am Mosq Control Assoc **24**(3): 344-348.

Garrett-Jones, C. and G. R. Shidrawi (1969). "Malaria vectorial capacity of a population of *Anopheles gambiae*: an exercise in epidemiological entomology." Bull World Health Organ **40**: 531-545.

Garrett, W. D. and S. A. White (1977). "Mosquito control with monomolecular organic surface films: I - Selection of optimum film-forming agents." Mosq News **37**(3): 344-348.

Gaugler, R., D. Suman and Y. Wang (2012). "An autodissemination station for the transfer of an insect growth regulator to mosquito oviposition sites " Med. Vet. Entomol. **26**: 37–45.

Gautam, B. K., G. Henderson and R. W. Davis (2012). "Toxicity and horizontal transfer of 0.5% fipronil dust against Formosan subterranean termites." J Econ Entomol **105**(5): 1766-1772.

Geissbühler, Y., K. Kannady, P. P. Chaki, B. Emidi, N. J. Govella, V. Mayagaya, M. Kiama, D. Mtasiwa, H. Mshinda, S. W. Lindsay, M. Tanner, U. Fillinger, M. C. Castro and G. F. Killeen (2009). "Microbial larvicide application by a large-scale, community-based program reduces malaria infection prevalence in urban Dar es Salaam, Tanzania." PLoS ONE **4**(3): e5107.

Gillies, M. T. and M. Coetzee (1987). "A supplement to the Anophelinae of Africa South of the Sahara (Afrotropical region)." South African Medical Research Institute, Johannesburg.

Gillies, M. T. and T. J. Wilkes (1965). "A study of the age-composition of populations of *Anopheles gambiae* Giles and *A. funestus* Giles in North-Eastern Tanzania." Bull Entomol Res **56**(2): 237-262.

Gimnig, J. E., M. Ombok, L. Kamau and W. A. Hawley (2001). "Characteristics of larval anopheline (Diptera: Culicidae) habitats in western Kenya." J. Med. Entomol **38**(2): 282-288.

Gimnig, J. E., M. Ombok, S. Otieno, M. G. Kaufman, J. M. Vulule and E. D. Walker (2002). "Density-dependent development of *Anopheles gambiae* (Diptera: Culicidae) larvae in artificial habitats." J Med Entomol **39**: 162-172.

Githeko, A. K., J. M. Ayisi, P. K. Odada, F. K. Atieli, B. A. Ndenga, J. I. Githure and G. Yan (2006). "Topography and malaria transmission heterogeneity in western Kenya highlands: prospects for focal vector control." Malar J **5**: 107.

Gouagna, L. C., M. Rakotondranary, S. Boyer, G. Lemperiere, J. S. Dehecq and D. Fontenille (2012). "Abiotic and biotic factors associated with the presence of *Anopheles arabiensis*

immatures and their abundance in naturally occurring and man-made aquatic habitats." <u>Parasit Vectors</u> **5**: 96.

Govella, N. J., P. P. Chaki and G. F. Killeen (2013). "Entomological surveillance of behavioural resilience and resistance in residual malaria vector populations." <u>Malar J</u> **12**: 124.

Govoetchan, R., V. Gnanguenon, E. Ogouwale, F. Oke-Agbo, R. Azondekon, A. Sovi, R. Attolou, K. Badirou, R. A. Youssouf, R. Osse and M. Akogbeto (2014). "Dry season refugia for anopheline larvae and mapping of the seasonal distribution in mosquito larval habitats in Kandi, northeastern Benin." <u>Parasit Vectors</u> **7**: 137.

Graf, J. F. (1993). "Role of insect growth regulators in arthropod control." <u>Parasitol Today</u> **9**(12): 471-474.

Griffin, J. T., T. D. Hollingsworth, L. C. Okell, T. S. Churcher, M. White, W. Hinsley, T. Bousema, C. J. Drakeley, N. M. Ferguson and M. G. Basáñez (2010). "Reducing *Plasmodium falciparum* malaria transmission in Africa: A model-based evaluation of intervention strategies." <u>PLos Med</u> **7**: e1000324.

Gu, W. and R. J. Novak (2005). "Habitat-based modeling of impacts of mosquito larval interventions on entomological inoculation rates, incidence, and prevalence of malaria." <u>Am J Trop Med Hyg</u> **73**(3): 546-552.

Gu, W., J. Utzinger and R. J. Novak (2008). "Habitat-based larval interventions: A new perspective for malaria control." <u>Am J Trop Med Hyg</u> **78**(1): 2–6.

Guerra, C. A., P. W. Gikandi, A. J. Tatem, A. M. Noor, D. L. Smith, S. I. Hay and R. W. Snow (2008). "The limits and intensity of *Plasmodium falciparum* transmission: Implications for malaria control and elimination worldwide." <u>PLoS Med</u> **5**(2): e38.

Guerra, C. A., R. E. Howes, A. P. Patil, P. W. Gething, T. P. Van Boeckel, W. H. Temperley, C. W. Kabaria, A. J. Tatem, B. H. Manh, I. R. F. Elyazar, J. K. Baird, R. W. Snow and S. I. Hay (2010). "The international limits and population at risk of *Plasmodium vivax* transmission in 2009." <u>PLoS Negl Trop Dis</u> **4**(8): e774.

Hagstrum, D. W. and M. S. Mulla (1968). "Petroleum oils as mosquito larvicides and pupicides. I. Correlation of physicochemical properties with biological activity." <u>J Econ Entomol</u> **61**(1): 220-225.

Hale, K. A. and D. E. Portwood (1996). "The aerobic soil degradation of spinosad – a novel natural insect control agent." <u>J Environ Sci Health B</u> **31**: 477-484.

Harris, C., D. W. Lweitoijera, S. Dongus, N. S. Matowo, L. M. Lorenz, G. J. Devine and S. Majambere (2013). "Sterilising effects of pyriproxyfen on *Anopheles arabiensis* and its potential use in malaria control." <u>Parasit Vectors</u> **6**: 144.

Hartfelder, K. (2000). "Insect juvenile hormone: from "status quo" to high society." <u>Braz J Med Biol Res</u> **33**(2): 157-177.

Hatakoshi, M., H. Kawada, S. Nishida, H. Kisida and I. Nakayama (1987). "Laboratory evaluation of 2-[1-methyl-2-(4-phenoxyphenoxy)-ethoxy] pyridine against larvae of mosquitoes and housefly." <u>Jpn. J. Sanit. Zool.</u> **38**(4): 271-274.

Hawley, W. A., P. A. Phillips-Howard, F. O. ter Kuile, D. J. Terlouw, J. M. Vulule, M. Ombok, B. L. Nahlen, J. E. Gimnig, S. K. Kariuki, M. S. Kolczak and A. W. Hightower (2003). "Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in western Kenya." <u>Am J Trop Med Hyg</u> **68**(4 Suppl): 121-127.

Hayward, R. E., B. Tiwari, K. P. Piper, D. I. Baruch and K. P. Day (1999). "Virulence and transmission success of the malarial parasite *Plasmodium falciparum*." <u>Proc Natl Acad Sci U S A</u> **96**(8): 4563-4568.

Hemingway, J. (2014). "The role of vector control in stopping the transmission of malaria: threats and opportunities." <u>Philos Trans R Soc Lond B Biol Sci</u> **369**(1645): 20130431.

Hemingway, J., B. C. Boning, K. G. I. Jayawardena, I. S. Weerasinghe, P. R. J. Herath and H. Oouchi (1988). "Possible selective advantage of *Anopheles* spp. (Diptera: Culicidae) with the oxidase- and acelylcholincsierase-based insecticide resistance genes after exposure to

organophosphates or an insect growth regulator in Sri Lankan rice fields." Bull Ent Res **78**: 471-478.

Herrera-Varela, M., J. Lindh, S. W. Lindsay and U. Fillinger (2014). "Habitat discrimination by gravid *Anopheles gambiae sensu lato* - a push-pull system." Malar J **13**: 133.

Hertlein, M. B., C. Mavrotas, C. Jousseaume, M. Lysandrou, G. D. Thompson, W. Jany and S. A. Ritchie (2010). "A review of spinosad as a natural product for larval mosquito control." J Am Mosq Control Assoc **26**(1): 67-87.

Hester, P. G., M. A. Olson and J. C. Dukes (1991). "Effects of Arosurf MSF on a variety of aquatic nontarget organisms in the laboratory." J Am Mosq Control Assoc **7**(1): 48-51.

Hill, J., J. Lines and M. Rowland (2006). "Insecticide-treated nets." Adv Parasitol **61**: 77-128.

Himeidan, Y. E., G. Zhou, L. Yakob, Y. Afrane, S. Munga, H. Atieli, A. El-Rayah el, A. K. Githeko and G. Yan (2009). "Habitat stability and occurrences of malaria vector larvae in western Kenya highlands." Malar J **8**: 234.

Huang, J., L. L. Stelinski and L. J. Gut (2010). "Mating behaviors of *Cydia pomonella* (Lepidoptera: Tortricidae) as influenced by sex pheromone in electrostatic powder." J Econ Entomol **103**(6): 2100-2106.

Huang, J., E. D. Walker, P. Y. Giroux, J. Vulule and J. R. Miller (2005). "Ovipositional site selection by *Anopheles gambiae*: influences of substrate moisture and texture." Med Vet Entomol **19**(4): 442-450.

Huestis, D. L., A. S. Yaro, A. I. Traore, A. Adamou, Y. Kassogue, M. Diallo, S. Timbine, A. Dao and T. Lehmann (2011). "Variation in metabolic rate of *Anopheles gambiae* and *A. arabiensis* in a Sahelian village." J Exp Biol **214**(Pt 14): 2345-2353.

Impoinvil, D. E., J. Keating, C. M. Mbogo, M. D. Potts, R. R. Chowdhury and J. C. Beier (2008). "Abundance of immature *Anopheles* and culicines (Diptera: Culicidae) in different water body types in the urban environment of Malindi, Kenya." J Vector Ecol **33**(1): 107-116.

Invest, J. F. and J. R. Lucas (2008). "Pyriproxyfen as a mosquito larvicide." Proceedings of the Sixth International Conference on Urban Pests: 239-245.

Itoh, K. (1993). "Control of DF/DHF vector, *Aedes* mosquito, with insecticides." Trop. Med. **35**(4): 259-267.

Itoh, T., H. Kawada, A. Abe, Y. Eshita, Y. Rongsriyam and A. Igarashi (1994). "Utilization of bloodfed females of *Aedes aegypti* as a vehicle for the transfer of the insect growth regulator pyriproxyfen to larval habitats." J Am Mosq Control Assoc **10**(3): 344-347.

Iwashita, H., G. O. Dida, G. O. Sonye, T. Sunahara, K. Futami, S. M. Njenga, L. F. Chaves and N. Minakawa (2014). "Push by a net, pull by a cow: can zooprophylaxis enhance the impact of insecticide treated bed nets on malaria control?" Parasit Vectors **7**: 52.

Jacobs, D. E., M. J. Hutchinson, K. J. Krieger and D. Bardt (1996). "A novel approach to flea control on cats, using pyriproxyfen." Vet Rec **139**: 559-561.

Jawara, M., M. Pinder, C. J. Drakeley, D. C. Nwakanma, E. Jallow, C. Bogh, S. W. Lindsay and D. J. Conway (2008). "Dry season ecology of *Anopheles gambiae* complex mosquitoes in The Gambia." Malar J **7**: 156.

Jones, C. O. and H. A. Williams (2004). "The social burden of malaria: what are we measuring?" Am J Trop Med Hyg **71**(2 Suppl): 156-161.

Jones, G. and B. D. Hammock (1985). "Critical roles for juvenile hormone and its esterase+ in the prepupa of *Trichoplusia ni*." Arch Insect Biochem Physiol **2**: 397-404.

Judson, C. L. and H. Z. de Lumen (1976). "Some effects of juvenile hormone and analogues on the ovarian follicles of the mosquito *Aedes aegypti* (Diptera: Culicidae)." J Med Entomol **13**(2): 197-201.

Kabbale, F. G., A. M. Akol, J. B. Kaddu and A. W. Onapa (2013). "Biting patterns and seasonality of *Anopheles gambiae sensu lato* and *Anopheles funestus* mosquitoes in Kamuli District, Uganda." Parasit Vectors **6**: 340.

Kamal, H. A. and E. I. M. Khater (2010). "The biological effects of the insect growth regulators; pyriproxyfen and diflubenzuron on the mosquito *Aedes aegypti*." J Egypt Soc Parasitol **40**(3): 565 - 574.

Kamimura, K. and A. Arakawa (1991). "Field evaluation of an insect growth regulator, pyriproxyfen, against *Culex pipiens* Pallens and *Culex tritaeniorhynchus*." Jap. J. Sanit. Zool. **42**(3): 249-252.

Karanja, D. M. S., A. K. Githeko and J. M. Vulule (1994). "Small-scale field evaluation of the monomolecular surface film Arosurf MSF' against *Anopheles arabiensis* Patton." Acta Trop **56**: 365-369.

Karatolos, N. K., M. S. Williamson, I. Denholm, K. Gorman, H. f.-C. Richard and C. Bass (2012). "Over-expression of a cytochrome P450 is associated with resistance to pyriproxyfen in the greenhouse whitefly *Trialeurodes vaporariorum*." PLoS ONE **7** (2): e31077.

Karch, S., N. Asidi, Z. M. Manzambi and J. J. Salaun (1992). "Efficacy of *Bacillus sphaericus* against the malaria vectors *Anopheles gambiae* and other mosquitoes in swamps and rice fields in Zaire." J Am Mosq Control Assoc **8**: 376-380.

Karch, S., Z. A. Manzambi and J. J. Salaun (1991). "Field trials with Vectolex (*Bacillus sphaericus*) and Vectobac (*Bacillus thuringiensis* (H-14)) against *Anopheles gambiae* and *Culex quinquefasciatus* breeding in Zaire." J Am Mosq Control Assoc **7**: 176-179.

Karch, S., N. Monteny, J. L. Jullien, G. Sinegre and J. Coz (1990). "Control of *Culex pipiens* by *Bacillus sphaericus* and role of nontarget arthropods in its recycling." J Am Mosq Control Assoc **6**: 47-54.

Katz, M. H. (2006). "Multivariable analysis: A practical guide for clinicians." Cambridge University Press **2nd edition**: 11-13.

Kaufman, M. G., E. Wanja, S. Maknojia, N. M. Bayoh, J. M. Vulule and E. D. Walker (2006). "Importance of algal biomass to growth and development of *Anopheles gambiae* larvae." J Med Entomol **43**: 669-676.

Kawada, H., G. O. Dida, K. Ohashi, E. Kawashima, G. Sonye, S. M. Njenga, C. Mwandawiro and N. Minakawa (2014). "A small-scale field trial of pyriproxyfen-impregnated bed nets against pyrethroid-resistant *Anopheles gambiae s.s.* in Western Kenya." PLoS ONE **9**(10): e111195.

Kawada, H., K. Dohara and G. Shinjo (1988). "Laboratory and field evaluation of an insect growth regulator, 4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether, as a mosquito larvicide." Jap. J. Sanit. Zool. **39**(4): 339-346.

Kawada, H., K. Futami, O. Komagata, S. Kasai, T. Tomita, G. Sonye, C. Mwatele, S. M. Njenga, C. Mwandawiro, N. Minakawa and M. Takagi (2011a). "Distribution of a knockdown resistance mutation (L1014S) in *Anopheles gambiae s.s.* and *Anopheles arabiensis* in Western and Southern Kenya." PLoS ONE **6**(9): e24323.

Kawada, H. D., G. O. Dida, K. Ohashi, O. Komagata, S. Kasai, T. Tomita, G. Sonye, Y. Maekawa, C. Mwatele, S. M. Njenga, C. Mwandawiro, N. Minakawa and M. Takagi (2011b). "Multimodial pyrethroid resistance in malaria vectors, *Anopheles gambiae s.s.*, *Anopheles arabiensis*, and *Anopheles funestus s.s.* in Western Kenya." PLoS ONE **6**(8): e22574.

Kawada, Y., Y. Shono, T. Ito and Y. Abe (1993). "Laboratory evaluation of insect growth regulators against several species of anopheline mosquitoes." Jap. J. Sanit. Zool. **44**(4): 349-353.

Kazembe, L. N. (2007). "Spatial modelling and risk factors of malaria incidence in northern Malawi." Acta Trop **102**: 126-137.

Keiser, J., M. C. de Castro, M. F. Maltese, R. Bos, M. Tanner, B. H. Singer and J. Utzinger (2005a). "Effect of irrigation and large dams on the burden of malaria on a global and regional scale " Am J Trop Med Hyg **72**(4): 392-406.

Keiser, J., B. H. Singer and J. Utzinger (2005b). "Reducing the burden of malaria in different eco-epidemiological settings with environmental management: a systematic review." Lancet Infect Dis **5**(11): 695-708.

Killeen, G. F. (2013). "A second chance to tackle African malaria vector mosquitoes that avoid houses and don't take drugs." Am J Trop Med Hyg **88**(5): 809-816.

Killeen, G. F. (2014). "Characterizing, controlling and eliminating residual malaria transmission." Malar J **13**: 330.

Killeen, G. F. and N. Chitnis (2014). "Potential causes and consequences of behavioural resilience and resistance in malaria vector populations: a mathematical modelling analysis." Malar J **13**: 97.

Killeen, G. F., U. Fillinger, I. Kiche, L. C. Gouagna and B. G. Knols (2002a). "Eradication of *Anopheles gambiae* from Brazil: lessons for malaria control in Africa?" Lancet Infect Dis **2**(10): 618-627.

Killeen, G. F., U. Fillinger and B. G. J. Knols (2002b). "Advantages of larval control for African malaria vectors: Low mobility and behavioural responsiveness of immature mosquito stages allow high effective coverage." Malar J **1**: 8.

Killeen, G. F., F. E. McKenzie, B. D. Foy, C. Schieffelin, P. F. Billingsley and J. C. Beier (2000). "The potential impact of integrated malaria transmission control on entomologic inoculation rate in highly endemic areas." Am J Trop Med Hyg **62**(5): 545-551.

Killeen, G. F., T. A. Smith, H. M. Ferguson, H. Mshinda, S. Abdulla, C. Lengeler and S. P. Kachur (2007). "Preventing childhood malaria in Africa by protecting adults from mosquitoes with insecticide-treated nets." PLoS Med **4**(7): e229.

Killeen, G. F., M. Tanner, W. R. Mukabana, M. S. Kalongolela, K. Kannady, S. W. Lindsay, U. Fillinger and M. C. de Castro (2006). "Habitat targeting for controlling aquatic stages of malaria vectors in Africa." Am J Trop Med Hyg **74**(4): 517-518.

Kirst, H. A. (2010). "The spinosyn family of insecticides: realizing the potential of natural products research." J Antibiot (Tokyo) **63**: 101-111.

Kitau, J., R. M. Oxborough, P. K. Tungu, J. Matowo, S. M. Magesa, J. Bruce, F. W. Mosha and M. W. Rowland (2012). "Species shifts in the *Anopheles gambiae* complex: Do LLINs successfully control *Anopheles arabiensis*?" PLoS ONE **7**(3): e31481.

Kitron, U. and A. Spielman (1989). "Suppression of transmission of malaria through source reduction: Antianopheline measures applied in Israel, the United States, and Italy." Rev Infect Dis **11**(3): 391-406.

Klinkenberg, E., K. A. Onwona-Agyeman, P. J. McCall, M. D. Wilson, I. Bates, F. H. Verhoeff, G. Barnish and M. J. Donnelly (2010). "Cohort trial reveals community impact of insecticide-treated nets on malariometric indices in urban Ghana." Trans R Soc Trop Med Hyg **104**(7): 496-503.

Knols, B. G., L. E. Mboera and W. Takken (1998). "Electric nets for studying odour-mediated host-seeking behaviour of mosquitoes." Med Vet Entomol **12**(1): 116-120.

Koenraadt, C. J., M. Kormaksson and L. C. Harrington (2010). "Effects of inbreeding and genetic modification on *Aedes aegypti* larval competition and adult energy reserves." Parasit Vectors **3**: 92.

Koenraadt, C. J. M., A. K. Githeko and W. Takken (2004). "The effects of rainfall and evapotranspiration on the temporal dynamics of *Anopheles gambiae s.s.* and *Anopheles arabiensis* in Kenyan highland village." Acta Trop **90**: 141-153.

Kweka, E. J., G. Zhou, L. B. Beilhe, A. Dixit, Y. Afrane, T. M. Gilbreath, 3rd, S. Munga, M. Nyindo, A. K. Githeko and G. Yan (2012). "Effects of co-habitation between *Anopheles gambiae s.s.* and *Culex quinquefasciatus* aquatic stages on life history traits." Parasit Vectors **5**: 33.

Kweka, E. J., G. Zhou, M. C. Lee, T. M. Gilbreath III, F. Mosha, S. Munga, A. K. Githeko and G. Yan (2011). "Evaluation of two methods of estimating larval habitat productivity in western Kenya highlands." Parasit Vectors **4**: 110.

Lefevre, T., L. C. Gouagna, K. R. Dabire, E. Elguero, D. Fontenille, F. Renaud, C. Costantini and F. Thomas (2009). "Beyond nature and nurture: phenotypic plasticity in blood-feeding behavior of *Anopheles gambiae s.s.* when humans are not readily accessible." Am J Trop Med Hyg **81**(6): 1023-1029.

Lehmann, T., R. Dalton, E. H. Kim, E. Dahl, A. Diabate, R. Dabire and J. P. Dujardin (2006). "Genetic contribution to variation in larval development time, adult size, and longevity of starved adults of *Anopheles gambiae*." Infect Genet Evol **6**: 410-416.

Lengeler, C. (2004). "Insecticide-treated bed nets and curtains for preventing malaria (Review)." Cochrane Database Syst Rev(2): CD000363.

Levy, R., J. J. Chizzonite, W. D. Garrett and T. W. Miller Jr. (1981). "Ground and aerial application of a monomolecular organic surface film to control salt-marsh mosquitoes in natural habitats of Southwestern Florida." Mosq News **41**(2): 219-301.

Levy, R., J. J. Chizzonite, W. D. Garrett and T. W. Miller Jr. (1982). "Efficacy of the organic surface film isostearyl alcohol containing two oxyethylene groups for control of *Culex* and *Psorophora* mosquitoes: laboratory and field studies " Mosq News **42**(1): 1-11.

Lindh, J. M., M. N. Okal, M. Herrera-Varela, A. Borg-Karlson, B. Torto, S. W. Lindsay and U. Fillinger (2015). "Discovery of an oviposition attractant for gravid malaria vectors of the *Anopheles gambiae* species complex." Malar J **14**: 119.

Lindsay, S. W., J. H. Adiamah, J. E. Miller and J. R. Armstrong (1991). "Pyrethroid-treated bednet effects on mosquitoes of the *Anopheles gambiae* complex in The Gambia." Med Vet Entomol **5**(4): 477-483.

Lindsay, S. W. and W. J. M. Martens (1998). "Malaria in the African highlands: past, present and the future." Bull World Health Organ **76**(1): 33-45.

Lindsay, S. W., L. Parson and C. J. Thomas (1998). "Mapping the ranges and relative abundance of the two principal African malaria vectors, *Anopheles gambiae sensu stricto* and *An. arabiensis*, using climate data." Proc R Soc Lond B **265**: 847-854.

Lindsay, S. W., R. W. Snow, G. L. Broomfield, M. Semega Janneh, R. A. Wirtz and B. M. Greenwood (1989). "Impact of permethrin-treated bednets on malaria transmission by the *Anopheles gambiae* complex in The Gambia." Med Vet Entomol **3**: 263-271.

Liu, N. (2015). "Insecticide resistance in mosquitoes: impact, mechanisms, and research directions." Annu Rev Entomol **60**: 537-559.

Loh, P. Y. and H. H. Yap (1989). "Laboratory studies on the efficacy and sublethal effects of an insect growth regulator, pyriproxyfen (S-31183) against *Aedes aegypti* (Linnaeus)." Trop Biomed **6**: 7-12.

Lopes, A., S. M. da Rosa-Osman, M. Teresa and M. T. F. Piedade (2009). "Effects of crude oil on survival, morphology, and anatomy of two aquatic macrophytes from the Amazon floodplains." Hydrobiologia **636**: 295-305.

Lounibos, L. P., D. C. Lima, R. Lourenco-de-Oliveira, R. L. Escher and N. Nishimura (1998). "Egg maturation in neotropical malaria vectors: one blood meal is usually enough." J Vector Ecol **23**(2): 195-201.

Lowe, R. E., J. E. Fowler, C. S. Lofgren, D. A. Dame, K. E. Savage and D. L. Bailey (1980). "Field and laboratory assessment of temephos for larval control of *Anopheles albimanus* in El Salvador and evidence for resistance." Mosq News **4**(3): 418-423.

Lwetoijera, D., C. Harris, S. Kiware, S. Dongus, G. J. Devine, P. J. McCall and S. Majambere (2014). "Effective autodissemination of pyriproxyfen to breeding sites by the exophilic malaria vector *Anopheles arabiensis* in semi-field settings in Tanzania." Malar J **13**: 161.

Lyimo, E. O., W. Takken and J. C. Koella (1992). "Effect of rearing temperature and larval density on larval survival, age at pupation and adult size of *Anopheles gambiae*." Entomol Exp Appl **63**: 265-271.

Lyons, C. L., M. Coetzee and S. L. Chown (2013). "Stable and fluctuating temperature effects on the development rate and survival of two malaria vectors, *Anopheles arabiensis* and *Anopheles funestus*." Parasit Vectors **6**: 104.

Macdonald, G. (1956). "Epidemiological basis of malaria control." <u>Bull World Health Organ</u> **15**(3-5): 613-626.

Mackay, A. J., M. Amador and R. Barrera (2013). "An improved autocidal gravid ovitrap for the control and surveillance of *Aedes aegypti*." <u>Parasit Vectors</u> **6**(1): 225.

Magesa, S. M., T. J. Wilkes, A. E. Mnzava, K. J. Njumwa, J. Myamba, M. D. Kivuyo, N. Hill, J. D. Lines and C. F. Curtis (1991). "Trial of pyrethroid impregnated bednets in an area of Tanzania holoendemic for malaria. Part 2. Effects on the malaria vector population." <u>Acta Tropica</u> **49**(2): 97-108.

Maheu-Giroux, M. and M. C. Castro (2013). "Impact of community-based larviciding on the prevalence of malaria infection in Dar es Salaam, Tanzania." <u>PLoS One</u> **8**(8): e71638.

Majambere, S., U. Fillinger, D. R. Sayer, C. Green and S. W. Lindsay (2008). "Spatial distribution of mosquito larvae and the potential for targeted larval control in The Gambia." <u>Am J Trop Med Hyg</u> **79**(1): 19-27.

Majambere, S., S. W. Lindsay, C. Green, B. Kandeh and U. Fillinger (2007). "Microbial larvicides for malaria control in The Gambia." <u>Malar J</u> **6**: 76.

Majambere, S., M. Pinder, U. Fillinger, D. Ameh, D. J. Conway, C. Green, D. Jeffries, M. Jawara, P. J. Milligan, R. Hutchinson and S. W. Lindsay (2010). "Is mosquito larval source management appropriate for reducing malaria in areas of extensive flooding in The Gambia? A cross-over intervention trial." <u>Am J Trop Med Hyg</u> **82**: 176-184.

Majori, G., A. Ali and G. sabatinelli (1987). "Laboratory and field efficacy of *Bacillus thuringiensis* var *israelensis* and *Bacillus sphaericus* against *Anopheles gambiae s.l.* and *Culex quinquefasciatus* in Ouagadougou, Burkina Faso." <u>J Am Mosq Control Assoc</u> **3**: 20-25.

Mala, A. O. and L. W. Irungu (2011). "Factors influencing differential larval habitat productivity of *Anopheles gambiae* complex mosquitoes in a western Kenyan village." <u>J Vector Borne Dis</u> **48**: 52-57.

Mala, A. O., L. W. Irungu, J. I. Shililu, E. J. Muturi, C. C. Mbogo, J. K. Njagi and J. I. Githure (2011). "Dry season ecology of *Anopheles gambiae* complex mosquitoes at larval habitats in two traditionally semi-arid villages in Baringo, Kenya." <u>Parasit Vectors</u> **4**: 25.

Marchand, R. P. (1985). "A new cage for observing mating behaviour of wild *Anopheles gambiae* in the laboratory." <u>J Am Mosq Control Assoc</u> **1**(2): 234-236.

Marina, C. F., J. G. Bond, M. Casas, J. Munoz, A. Orozco, J. Valle and T. Williams (2011). "Spinosad as an effective larvicide for control of *Aedes albopictus* and *Aedes aegypti*, vectors of dengue in southern Mexico." <u>Pest Manag Sci</u> **67**(1): 114-121.

Matowo, N. S., J. Moore, S. Mapua, E. P. Madumla, I. R. Moshi, E. W. Kaindoa, S. P. Mwangungulu, D. R. Kavishe, R. D. Sumaye, D. W. Lweitoijera and F. O. Okumu (2013). "Using a new odour-baited device to explore options for luring and killing outdoor-biting malaria vectors: a report on design and field evaluation of the Mosquito Landing Box." <u>Parasit Vectors</u> **6**: 137.

Mayagaya, V. S., G. Nkwengulila, I. N. Lyimo, J. Kihonda, H. Mtambala, H. Ngonyani, T. L. Russell and H. M. Ferguson (2015). "The impact of livestock on the abundance, resting behaviour and sporozoite rate of malaria vectors in southern Tanzania." <u>Malar J</u> **14**(1): 17.

Mbare, O., S. W. Lindsay and U. Fillinger (2013). "Dose–response tests and semi-field evaluation of lethal and sub-lethal effects of slow release pyriproxyfen granules (Sumilarv®0.5G) for the control of the malaria vectors *Anopheles gambiae sensu lato*." <u>Malar J</u> **12**: 94.

Mbare, O., S. W. Lindsay and U. Fillinger (2014a). "Aquatain(R) Mosquito Formulation (AMF) for the control of immature *Anopheles gambiae sensu stricto* and *Anopheles arabiensis*: dose-responses, persistence and sub-lethal effects." <u>Parasit Vectors</u> **7**: 438.

Mbare, O., S. W. Lindsay and U. Fillinger (2014b). "Pyriproxyfen for mosquito control: female sterilization or horizontal transfer to oviposition substrates by *Anopheles gambiae sensu stricto* and *Culex quinquefasciatus*." <u>Parasit Vectors</u> **7**(1): 280.

McCrae, A. W. R. (1984). "Oviposition by African malaria vector mosquitoes II. Effects of site tone, water type and conspecific immatures on target selection by freshwater *Anopheles gambiae* Giles, *sensu lato*." J Trop Med **73**(3): 307-318.

McHugh, C. P. (1990). "Survivorship and gonotrophic cycle length of *Culex tarsalis* (Diptera: Culicidae) near Sheridan, Placer County, California." J Med Entomol **27**(6): 1027-1030.

Medina, P., G. Smagghe, F. Budia, P. Del Estal, L. Tirry and E. Vinuela (2002). "Significance of penetration, excretion, and transovarial uptake to toxicity of three insect growth regulators in predatory lacewing adults." Arch Insect Biochem Physiol **51**(2): 91-101.

Mereta, S. T., D. Yewhalaw, P. Boets, A. Ahmed, L. Duchateau, N. Speybroeck, S. O. Vanwambeke, W. Legesse, L. De Meester and P. L. Goethals (2013). "Physico-chemical and biological characterization of anopheline mosquito larval habitats (Diptera: Culicidae): implications for malaria control." Parasit Vectors **6**(1): 320.

Merzendorfer, H. (2013). "Chitin synthesis inhibitors: old molecules and new developments." Insect Sci **20**(2): 121-138.

Mian, L. S. and M. S. Mulla (1982). "Biological and environmental dynamics of insect growth regulators (IGRs) as used against diptera of public health importance." Residue Rev **84**: 28-35.

Micks, D. W., G. V. Chambers, J. Jennings and A. Rehmet (1967). "Mosquito control agents derived from petroleum hydrocarbons. I. Laboratory effectiveness." J Econ Entomol **60**: 427-429.

Miller, J. E. (1993). "Can pyriproxifen (an insect growth regulator) be used to prevent selection of permethrin resistance by impregnated bednets?" Trans R Soc Trop Med Hyg **18**: 281.

Minakawa, N., S. Munga, F. Atieli, E. Mushinzimana, G. Zhou, A. K. Githeko and G. Yan (2005). "Spatial distribution of anopheline larval habitats in Western Kenyan highlands: effects of land cover types and topography." Am J Trop Med Hyg **73**(1): 157-165.

Minakawa, N., C. M. Mutero, J. I. Githure, J. C. Beier and G. Yan (1999). "Spatial distribution and habitat characterization of anopheline mosquito larvae in Western Kenya." Am J Trop Med Hyg **61**(6): 1010-1016.

Minakawa, N., G. Sonye, M. Mogi and G. Yan (2004). "Habitat characteristics of *Anopheles gambiae s.s.* larvae in a Kenyan highland." Med Vet Entomol **18**: 301-305.

Mireji, P. O., J. Keating, A. Hassanali, C. M. Mbogo, H. Nyambaka, S. Kahindi and J. C. Beier (2008). "Heavy metals in mosquito larval habitats in urban Kisumu and Malindi, Kenya, and their impact." Ecotoxicol Environ Saf **70**(1): 147-153.

MoH (2009). "National malaria strategy 2009–2017." Division of Malaria Control, Ministry of Public Health and Sanitation, Nairobi.

Mohsen, Z. H. and H. H. Zayia (1995). "Long-term sublethal effects of fenoxycarb against *Culex* mosquitoes (Diptera: Culicidae)." Jpn. J. Sanit. Zool. **46**(2): 151-154.

Mosqueira, B., S. Duchon, F. Chandre, J. M. Hougard, P. Carnevale and S. Mas-Coma (2010). "Efficacy of an insecticide paint against insecticide-susceptible and resistant mosquitoes - part 1: laboratory evaluation." Malar J **9**: 340.

Mozley, S. C. and M. G. Butler (1978). "Effects of crude oil on aquatic insects of tundra ponds." Arctic **31**(3): 229-241.

Mukabana, W. R., C. K. Mweresa, B. Otieno, P. Omusula, B. Orindi, R. C. Smallegange, J. J. A. Van Loon and W. Takken (2012). "Evaluation of low density polyethylene and nylon for delivery of synthetic mosquito attractants." Parasit Vectors **50**(2): 123-133.

Mukabana, W. R., W. Takken, R. Coe and B. G. Knols (2002). "Host-specific cues cause differential attractiveness of Kenyan men to the African malaria vector *Anopheles gambiae*." Malar J **1**: 17.

Mulla, M. S. and H. A. Darwazeh (1981). "Efficacy of petroleum larvicidal oils and their impact on some aquatic nontarget organisms." Proc. Calif. Mosq. Cont. Assoc.: 84-87.

Mulla, M. S., H. A. Darwazeh, B. Kennedy and D. M. Dawson (1986). "Evaluation of new insect growth regulators against mosquitoes with notes on nontarget organisms." J Am Mosq Control Assoc **2**(3): 314-320.

Mulla, M. S., H. A. Darwazeh and L. L. Luna (1983). "Monolayer films as mosquito control agents and their effects on nontarget organisms." Mosq News **43**(4): 489-495.

Mulla, M. S., H. A. Darwazeh and R. L. Norland (1974). "Insect growth regulators: evaluation procedures and activity against mosquitoes." J Econ Entomol **67**(3): 329-332.

Mulligan III, F. S. and C. H. Schaefer (1990). "Efficacy of a juvenile hormone mimic, pyriproxyfen (S-31183) for mosquito control in dairy waste water lagoons." J Am Mosq Control Assoc **6**(1): 89-92.

Munga, S., N. Minakawa, G. Zhou, E. Mushinzimana, O. O. J. Barrack, A. K. Githeko and G. Yan (2006). "Association between land cover and habitat productivity of malaria vectors in Western Kenya highlands." Am J Trop Med Hyg **74**(1): 69-75.

Munyekenye, O. G., A. K. Githeko, G. Zhou, E. Mushinzimana, N. Minakawa and G. Yan (2005). "*Plasmodium falciparum* spatial analysis, western Kenya highlands." Emerg Infect Dis **11**(10): 1571-1577.

Murray, D. R. P. (1940). "Problems concerning the efficiency of oils as mosquito larvicides. II. The spreading power of oils and methods of increasing it." Bull Entomol Res **30**: 211-236.

Mutuku, F. M., N. M. Bayoh, J. E. Gimnig, J. M. Vulule, L. Kamau, E. D. Walker, E. Kabiru and W. A. Hawley (2006). "Pupal habitat productivity of *Anopheles gambiae* complex mosquitoes in a rural village in Western Kenya." Am J Trop Med Hyg **74**(1): 54-61.

Muturi, E. J., J. Mwangangi, J. Shililu, B. G. Jacob, C. Mbogo, J. Githure and R. J. Novak (2008). "Environmental factors associated with the distribution of *Anopheles arabiensis* and *Culex quinquefasciatus* in a rice agro-ecosystem in Mwea, Kenya." J Vector Ecol **33**(1): 56-63.

Mwangangi, J. M., C. M. Mbogo, E. J. Muturi, J. G. Nzovu, J. I. Githure, G. Yan, N. Minakawa, R. Novak and J. C. Beier (2007). "Spatial distribution and habitat characterisation of *Anopheles* larvae along the Kenyan coast." J Vector Borne Dis **44**(1): 44-51.

Mweresa, C. K., W. R. Mukabana, P. Omusula, B. Otieno, T. Gheysens, W. Takken and J. J. van Loon (2014). "Evaluation of textile substrates for dispensing synthetic attractants for malaria mosquitoes." Parasit Vectors **7**: 376.

Mweresa, C. K., B. Otieno, P. Omusula, B. T. Weldegergis, N. O. Verhulst, M. Dicke, J. J. van Loon, W. Takken and W. R. Mukabana (2015). "Understanding the long-lasting attraction of malaria mosquitoes to odor baits." PLoS ONE **10**(3): e0121533.

Najera, J. A. (1999). "Malaria control achievements, problems and strategies ": World Health Organization.

Najera, J. A., M. Gonzalez-Silva and P. L. Alonso (2011). "Some lessons for the future from the Global Malaria Eradication Programme (1955-1969)." PLoS Med **8**(1): e1000412.

Nayar, J. K. and A. Ali (2003). "A review of monomolecular surface films as larvicides and pupicides of mosquitoes." J Vector Ecol **28**(2): 190-199.

Nayar, J. K., A. Ali and M. Zaim (2002). "Effectiveness and residual activity comparison of granular formulations of insect growth regulators pyriproxyfen and s-methoprene against Florida mosquitoes in laboratory and outdoor conditions." J Am Mosq Control Assoc **18**(3): 196-201.

Ndenga, B., A. Githeko, E. Omukunda, G. Munyekenye, H. Atieli, P. Wamai, C. Mbogo, N. Minakawa, G. Zhou and G. Yan (2006). "Population dynamics of malaria vectors in western Kenya highlands." J Med Entomol **43**(2): 200-206.

Ndenga, B. A., J. A. Simbauni, J. P. Mbugi and A. K. Githeko (2012). "Physical, chemical and biological characteristics in habitats of high and low presence of anopheline larvae in Western Kenya Highlands." PLoS ONE **7**(10): e47975.

Ndenga, B. A., J. A. Simbauni, J. P. Mbugi, A. K. Githeko and U. Fillinger (2011). "Productivity of malaria vectors from different habitat types in the western Kenya highlands." PLoS **6**(4): e19473.

NEMA (2011). "Vihiga District Environment Action Plan 2009-2013. National Environment Management Authority. Ministry of Environment and Mineral Resources. Republic of Kenya."

Neoh, K. B., J. Hu, B. H. Yeoh and C. Y. Lee (2012). "Toxicity and horizontal transfer of chlorantraniliprole against the Asian subterranean termite *Coptotermes gestroi* (Wasmann): effects of donor:recipient ratio, exposure duration and soil type." Pest Manag Sci **68**(5): 749-756.

Ngufor, C., R. N'Guessan, J. Fagbohoun, A. Odjo, D. Malone, M. Akogbeto and M. Rowland (2014). "Olyset Duo (R) (a pyriproxyfen and permethrin mixture net): an experimental hut trial against pyrethroid resistant *Anopheles gambiae* and *Culex quinquefasciatus* in southern Benin." PLoS ONE **9**: e93603.

Nkuo-Akenji, T., N. N. Ntonifor, M. B. Ndukum, E. L. Abongwa, A. Nkwescheu, D. N. Anong, M. Songmbe, M. G. Boyo, K. N. Ndamukong and V. P. Titanji (2006). "Environmental factors affecting malaria parasite prevalence in rural Bolifamba, South West Cameroon." Afr J Health Sci **13**(1-2): 40-46.

Nwane, P., J. Etang, M. Chouaibou, J. C. Toto, C. Kerah-Hinzoumbe, R. Mimpfoundi, H. P. Awono-Ambene and F. Simard (2009). "Trends in DDT and pyrethroid resistance in *Anopheles gambiae s.s.* populations from urban and agro-industrial settings in southern Cameroon." BMC Infect Dis **9**: 163.

Nwane, P., J. Etang, U. M. Chouasmall yi, J. C. Toto, A. Koffi, R. Mimpfoundi and F. Simard (2013). "Multiple insecticide resistance mechanisms in *Anopheles gambiae s.l.* populations from Cameroon, Central Africa." Parasit Vectors **6**: 41.

Ohashi, K., K. Nakada, J. Miyaguchi, Y. Shono, J. R. Lucas and N. Mito (2012). "Efficacy of pyriproxyfen-treated nets in sterilizing and shortening the longevity of *Anopheles gambiae* (Diptera: Culicidae)." J Med Entomol **49**(5): 1052-1058.

Ohba, S. Y., K. Ohashi, E. Pujiyati, Y. Higa, H. Kawada, N. Mito and M. Takagi (2013). "The effect of pyriproxyfen as a "population growth regulator" against *Aedes albopictus* under semi-field conditions." PLoS One **8**(7): e67045.

Okal, M. N., B. Francis, M. Herrera-Varela, U. Fillinger and S. W. Lindsay (2013). "Water vapour is a pre-oviposition attractant for the malaria vector *Anopheles gambiae sensu stricto*." Malar J **12**: 365.

Okazawa, T., B. Bakote'e, H. Suzuki, H. Kawada and N. Kere (1991). "Field evaluation of an insect growth regulator, pyriproxyfen, against *Anopheles punctulatus* on north Guadalcanal, Solomon Islands." J Am Mosq Control Assoc **7**(4): 604-607.

Okello, P. E., W. Van Bortel, A. M. Byaruhanga, A. Correwyn, P. Roelants, A. Talisuna, U. D'Alessandro and M. Coosemans (2006). "Variation in malaria transmission intensity in seven sites throughout Uganda." Am J Trop Med Hyg **75**(2): 219-225.

Okumu, F. O., E. Mbeyela, G. Lingamba, J. Moore, A. J. Ntamatungiro, D. R. Kavishe, M. G. Kenward, E. Turner, L. M. Lorenz and S. J. Moore (2013). "Comparative field evaluation of combinations of long-lasting insecticide treated nets and indoor residual spraying, relative to either method alone, for malaria prevention in an area where the main vector is *Anopheles arabiensis*." Parasit Vectors **6**: 46.

Okumu, F. O. and S. J. Moore (2011). "Combining indoor residual spraying and insecticide-treated nets for malaria control in Africa: a review of possible outcomes and an outline of suggestions for the future." Malar J **10**: 208.

Oliver, S. V. and B. D. Brooke (2013). "The effect of larval nutritional deprivation on the life history and DDT resistance phenotype in laboratory strains of the malaria vector *Anopheles arabiensis*." Malar J **12**: 44.

Omukunda, E., A. Githeko, M. F. Ndong'a, E. Mushinzimana, H. Atieli and P. Wamae (2013). "Malaria vector population dynamics in highland and lowland regions of western Kenya." J Vector Borne Dis **50**(2): 85-92.

Omukunda, E., A. Githeko, A. M. Ndong, E. Mushinzimana and G. Yan (2012). "Effect of swamp cultivation on distribution of anopheline larval habitats in Western Kenya." J Vector Borne Dis **49**(2): 61-71.

Ong, S. Q. and Z. Jaal (2015). "Investigation of mosquito oviposition pheromone as lethal lure for the control of *Aedes aegypti* (L.) (Diptera: Culicidae)." Parasit Vectors **8**(1): 28.

Ototo, E. N., J. P. Mbugi, C. L. Wanjala, G. Zhou, A. K. Githeko and G. Yan (2015). "Surveillance of malaria vector population density and biting behaviour in western Kenya." Malar J **14**: 244.

Paaijmans, K. P., A. F. Read and M. B. Thomas (2009). "Understanding the link between malaria risk and climate." PNAS **106**: 13844-13849.

Paaijmans, K. P., W. Takken, A. K. Githeko and A. F. Jacobs (2008). "The effect of water turbidity on the near-surface water temperature of larval habitats of the malaria mosquito *Anopheles gambiae*." Int J Biometeorol **52**(8): 747-753.

Paaijmans, K. P., M. O. Wandago, A. K. Githeko and W. Takken (2007). "Unexpected high losses of *Anopheles gambiae* larvae due to rainfall." PLoS ONE **2**(11): e1146.

Padmanabha, H., C. C. Lord and L. P. Lounibos (2011). "Temperature induces trade-offs between development and starvation resistance in *Aedes aegypti* (L.) larvae." Med Vet Entomol **25**: 445-453.

Padmanabha, H., E. Soto, M. Mosquera, C. C. Lord and L. P. Lounibos (2010). "Ecological links between water storage behaviours and *Aedes aegypti* production: implications for dengue vector control in variable climates." Ecohealth **7**: 78-90.

Pates, H. and C. Curtis (2005). "Mosquito behavior and vector control." Annu Rev Entomol **50**: 53-70.

Patterson, R. (1989). "Dr. William Gorgas and his fight with mosquitoes." CMAJ **141**: 596-598.

Perez, C. M., C. F. Marina, J. G. Bond, J. C. Rojas, J. Valle and T. Williams (2007). "Spinosad, a naturally derived insecticide, for control of *Aedes aegypti* (Diptera: Culicidae): efficacy, persistence, and elicited oviposition response." J Med Entomol **44**(4): 631-638.

Pinder, M., M. Jawara, L. B. S. Jarju, B. Kandeh, D. Jeffries, M. F. Lluberas, J. Mueller, D. Parker, K. Bojang, D. J. Conway and S. W. Lindsay (2011). "To assess whether indoor residual spraying can provide additional protection against clinical malaria over current best practice of long-lasting insecticidal mosquito nets in The Gambia: study protocol for a two-armed cluster-randomized trial." Trials **12**: 147.

Pinkney, A. E., P. C. McGowan, D. R. Murphy, P. T. Lowe and D. W. Sparling (1999). "Effects of the mosquito larvicides temephos and methoprene on insect populations in experimental ponds." Environ Toxicol and Chem **19**: 678-684.

Pluess, B., F. C. Tanser, C. Lengeler and B. L. Sharp (2010). "Indoor residual spraying for preventing malaria." Cochrane Database Syst Rev(4): CD006657.

Poopathi, S. and S. Abidha (2010). "Mosquitocidal bacterial toxins (*Bacillus sphaericus* and *Bacillus thuringiensis* serovar *israelensis*): Mode of action, cytopathological effects and mechanism of resistance." J Physiol Pathophysiol **1**(3): 22-28.

Quinones, M. L., J. D. Lines, M. C. Thomson, M. Jawara, J. Morris and B. M. Greenwood (1997). "*Anopheles gambiae* gonotrophic cycle duration, biting and exiting behaviour unaffected by permethrin-impregnated bednets in The Gambia." Med Vet Entomol **11**(1): 71-78.

Ragoonanansingh, R. N., K. J. Njunwa, C. F. Curtis and N. Becker (1992). "A field study of *Bacillus sphaericus* for the control of culicine and anopheline mosquito larvae in Tanzania." Bull Soc Vector Ecol **17**: 45-50.

Ramaiah, K. D., P. K. Das and V. Dhanda (1994). "Estimation of permissible levels of transmission of bancroftian filariasis based on some entomological and parasitological results of a 5-year vector control programme." Acta Trop **56**(1): 89-96.

Ramaiah, K. D., S. P. Pani, N. Balakrishnan, C. Sadanandane, L. K. Das, T. Mariappan, A. R. Rajavel, P. Vanamail and S. Subramanian (1989). "Prevalence of bancroftian filariasis & its control by single course of diethyl carbamazine in a rural area in Tamil Nadu." Indian J Med Res **89**: 184-191.

Ranson, H., H. Abdallah, A. Badolo, W. M. Guelbeogo, C. Kerah-Hinzoumbe, E. Yangalbe-Kalnone, N. Sagnon, F. Simard and M. Coetzee (2009). "Insecticide resistance in *Anopheles gambiae*: data from the first year of a multi-country study highlight the extent of the problem." Malar J **8**: 299.

Ranson, H., B. Jensen, J. M. Vulule, X. Wang, J. Hemingway and F. H. Collins (2000). "Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids." Insect Mol Biol **9**(5): 491-497.

Ranson, H., R. N'Guessan, J. Lines, N. Moiroux, Z. Nkuni and V. Corbel (2011). "Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control?" Trends Parasitol **27**(2): 91-98.

Rapley, L. P., P. H. Johnson, C. R. Williams, R. M. Silcock, M. Larkman, S. A. Long, R. C. Russell and S. A. Ritchie (2009). "A lethal ovitrap-based mass trapping scheme for dengue control in Australia: II. Impact on populations of the mosquito *Aedes aegypti*." Med Vet Entomol **23**(4): 303-316.

Ravoahangimalala, O., I. Thiery and G. Sinegre (1994). "Rice field efficacy of deltamethrin and *Bacillus thuringiensis israelensis* formulations on *Anopheles gambiae s.s.* in the Anjiro Region of Madagascar." Bull Soc Vector Ecol **19**: 169-174.

RBM (2008). "Executive summary. Global Malaria Action Plan." Roll Back Malaria Partnership.

Reddy, M. R., H. J. Overgaard, S. Abaga, V. P. Reddy, A. Caccone, A. E. Kiszewski and M. A. Slotman (2011). "Outdoor host seeking behaviour of *Anopheles gambiae* mosquitoes following initiation of malaria vector control on Bioko Island, Equatorial Guinea." Malar J **10**: 184.

Reiter, P. (1978). "The action of lecithin monolayers on mosquitoes II. Action on the respiratory structures." Ann Trop Med Parasitol **72**(2): 169-176.

Reiter, P. (1979). "The action of lecithin monolayers on mosquitoes III. Studies in irrigated rice-fields in Kenya." J Trop Med **74**(5): 541-557.

Reiter, P. and A. I. McMullen (1978). "The action of lecithin monolayers on mosquitoes I. General observations." *Ann Trop Med Parasitol* **72**(2): 163-168.

Retnakaran, A., P. Krell, Q. Feng and B. Arif (2003). "Ecdysone agonists: mechanism and importance in controlling insect pests of agriculture and forestry." Arch Insect Biochem Physiol **54**(4): 187-199.

Richards, A. G. (1941). "Differentiation between toxic and suffocating effects of petroleum oils on larvae of the house mosquito (*Culex pipiens* L.) (Diptera)." Trans Amer Entomol **67**: 161-193.

Riddiford, L. M. (2012). "How does juvenile hormone control insect metamorphosis and reproduction?" Gen Comp Endocr **179**: 477-484.

Ritchie, S. A. and S. Long (2003). "Does S-methoprene affect oviposition by *Aedes aegypti* in an ovitrap?" J Am Mosq Control Assoc **19**(2): 170-171.

Robert, L. L. and J. K. Olson (1989). "Effects of sublethal dosages of insecticides on *Culex quinquefasciatus*." J Am Mosq Control Assoc **5**(2): 239-246.

Robert, V., H. P. Awono-Ambene and J. Thioulouse (1998). "Ecology of larval mosquitoes, with special reference to *Anopheles arabiensis* (Diptera: Culicidae) in market-garden wells in urban Dakar, Senegal." J Med Entomol **35**(6): 948-955.

Robert, V. and P. Carnevale (1991). "Influence of deltamethrin treatment of bed nets on malaria transmission in the Kou valley, Burkina Faso." Bull World Health Organ **69**(6): 735-740.

Robert, V., G. Le Goff, F. Ariey and J. B. Duchemin (2002). "A possible alternative method for collecting mosquito larvae in rice fields." Malar J **1**: 4.

Romi, R., S. Proietti, M. Di Luca and M. Cristofaro (2006). "Laboratory evaluation of the bioinsecticide Spinosad for mosquito control." J Am Mosq Control Assoc **22**(1): 93-96.

Romoser, W. S. and E. A. Lucas (1999). "Buoyancy and diving behavior in mosquito pupae." J Am Mosq Control Assoc **15**(2): 194-199.

Rozendaal, J. A. (1997). "Vector Control - Methods for use by individuals and communities. Geneva , World Health Organization." **411**.

RTeam (2011). "R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. ISBN 3-900051-07-0. Available: http://www.R-project.org/."

Russell, T. L., N. J. Govella, S. Azizi, C. J. Drakeley, S. P. Kachur and G. F. Killeen (2011). "Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania." Malar J **10**: 80.

Russell, T. L., D. W. Lwetoijera, D. Maliti, B. Chipwaza, J. Kihonda, J. D. Charlwood, T. A. Smith, C. Lengeler, M. A. Mwanyangala, R. Nathan, B. G. J. Knols, W. Takken and G. F. Killeen (2010). "Impact of promoting longer-lasting insecticide treatment of bed nets upon malaria transmission in a rural Tanzanian setting with pre-existing high coverage of untreated nets." Malar J **9**: 187.

Rydzanicz, K., P. DeChant and N. Becker (2010). "Field efficacy of granular formulations of *Bacillus thuringiensis israelensis*--strain AM65-52 against floodwater mosquitoes in Poland and Germany." J Am Mosq Control Assoc **26**(3): 295-301.

Sachs, J. D. (2001). "Macroeconomics and health: Investing in health for economic development." World Health Organziation. Geneva.

Sagnon, N., M. Pinder, E. F. Tchicaya, A. B. Tiono, B. Faragher, H. Ranson and S. W. Lindsay (2015). "To assess whether addition of pyriproxyfen to long-lasting insecticidal mosquito nets increases their durability compared to standard long-lasting insecticidal mosquito nets: study protocol for a randomised controlled trial." Trials **16**(1): 195.

Salgado, V. L. (1998). "Studies on the mode of action of spinosad: insect symptoms and physiological correlates." Pest Biochem Physiol **60**: 91-102.

Sanil, D. and N. J. Shetty (2012). "The effect of sublethal exposure to temephos and propoxur on reproductive fitness and its influence on circadian rhythms of pupation and adult emergence in *Anopheles stephensi* Liston—a malaria vector." Parasitol Res **111**: 423–432

Sattler, M. A., D. Mtasiwa, M. Kiama, Z. Premji, M. Tanner, G. F. Killeen and C. Lengeler (2005). "Habitat characterization and spatial distribution of *Anopheles* sp. mosquito larvae in Dar es Salaam (Tanzania) during an extended dry period." Malar J **4**: 4.

Schaefer, C. H., E. F. Dupras Jr and F. S. Mulligan III (1991). "Studies on the environmental persistence of S-31183 (Pyriproxyfen): Adsorption onto organic matter and potential for leaching through soil." Ecotoxicol. Environ. Saf. **21**: 207-214

Schaefer, C. H. and T. Miura (1990). "Chemical persistence and effects of S-31183, 2-(1-Methy-2(4- phenoxyphenoxy)ethoxy) pyridine on aquatic organisms in field tests." J Econ Entomol **83**(5): 1766-1776.

Schaefer, C. H., T. Miura, E. F. Dupras, Jr., F. S. Mulligan, 3rd. and W. H. Wilder (1988). "Efficacy, nontarget effects, and chemical persistence of S-31183, a promising mosquito (Diptera: Culicidae) control agent." J Econ Entomol **81**: 1648-1655.

Schaefer, C. H. and F. S. Mulligan, 3rd (1991). "Potential for resistance to pyriproxyfen: a promising new mosquito larvicide." J Am Mosq Control Assoc **7**(3): 409-411.

Schantz-Dunn, J. and N. M. Nour (2009). "Malaria and pregnancy: a global health perspective." Rev Obstet Gynecol **2**(3): 186-192.

Schneider, M., G. Smagghe, S. Pineda and E. Vinuela (2008). "The ecological impact of four IGR insecticides in adults of *Hyposoter didymator* (Hym., Ichneumonidae): pharmacokinetics approach." Ecotoxicology **17**(3): 181-188.

Schoof, H. F. (1967). "Mating, resting habits and dispersal of *Aedes aegypti*." Bull World Health Organ **36**(4): 600-601.

Scott, J. A., W. G. Brogdon and F. H. Collins (1993). "Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction." Am J Trop Med Hyg **49**(4): 520-529.

Seavy, N. E., S. Quader, A. J. D. and R. C. J. (2005). "Generalized linear models and point count data: statistical considerations for the design and analysis of monitoring studies. In: Ralph, CJ and Rich TD, Editor Bird conservation implementation and integration in the Americas. USDA Forest Service General Technical Report PSWGTR-191. ." pp 744-753.

Seng, C. M., T. Setha, J. Nealon, D. Socheat and M. B. Nathan (2008). "Six months of *Aedes aegypti* control with a novel controlled-release formulation of pyriproxyfen in domestic water storage containers in Cambodia." SouthEast Asian J Trop Med Public Health **39**(5): 822-826.

Service, M. W. (1971). "Studies on sampling larval population of *Anopheles gambiae* complex." Bull WHO **45**: 169-180.

Seyoum, A. and D. Abate (1997). "Larvicidal efficacy of *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* on *Anopheles arabiensis* in Ethiopia " World J Microb Biot **13**: 21-24.

Shaukat, A. M., J. G. Breman and F. E. McKenzie (2010). "Using the entomological inoculation rate to assess the impact of vector control on malaria parasite transmission and elimination." Malar J **9**: 122.

Shililu, J., T. Ghebremeskel, F. Seulu, S. Mengistu, H. Fekadu, M. Zerom, A. Ghebregziabiher, D. Sintasath, G. Bretas, C. Mbogo, J. Githure, E. Brantly, R. Novak and J. C. Beier (2003a). "Larval habitat diversity and ecology of anopheline larvae in Eritrea." J Med Entomol **40**(6): 921-929.

Shililu, J., C. Mbogo, T. Ghebremeskel, J. Githure and R. Novak (2007). "Mosquito larval habitats in a semiarid ecosystem in Eritrea: Impact of larval habitat management on *Anopheles arabiensis* population." Am J Trop Med Hyg **76** 103-110.

Shililu, J. I., G. M. Tewolde, E. Brantly, J. I. Githure, C. M. Mbogo, J. C. Beier, R. Fusco and R. J. Novak (2003b). "Efficacy of *Bacillus thuringiensis israelensis*, *Bacillus sphaericus* and temephos for managing *Anopheles* larvae in Eritrea." J Am Mosq Control Assoc **19**(3): 251-258.

Siddall, J. B. (1976). "Insect growth regulators and insect control: a critical appraisal." Environ Health Perspect **14**: 119-126.

Sihuincha, M., E. Zamora-Perea, W. Orellana-Rios, J. D. Stancil, V. Lopez-Sifuentes, C. Vidal-Ore and G. J. Devine (2005). "Potential use of pyriproxyfen for control of *Aedes aegypti* (Diptera: Culicidae) in Iquitos, Peru." J Med Entomol **42**(4): 620-630.

Silva-Filha, M. H., L. Regis, C. Nielsen-Leroux and F. Charles (1995). "Low-level resistance to *Bacillus sphaericus* in a field-treated population of *Culex quinquefasciatus* (Diptera: Culicidae)." J Econ Entomol **88**(3): 525-530.

Sinka, M. E., M. J. Bangs, S. Manguin, M. Coetzee, C. M. Mbogo, J. Hemingway, A. P. Patil, W. H. Temperley, P. W. Gething, C. W. Kabaria, R. M. Okara, T. V. Boeckel, H. C. J. Godfray, R. E. Harbach and S. I. Hay (2010). "The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic precis." Parasit Vectors **3**: 117.

Sinka, M. E., M. J. Bangs, S. Manguin, Y. Rubio-Palis, T. Chareonviriyaphap, M. Coetzee, C. M. Mbogo, J. Hemingway, A. P. Patil, W. H. Temperley, P. W. Gething, C. W. Kabaria, T. R. Burkot, R. E. Harbach and S. I. Hay (2012). "A global map of dominant malaria vectors." Parasit Vectors **5**: 69.

Skovmand, O. and S. Bauduin (1996). "Efficacy of a granular formulation of *Bacillus sphaericus* against *Culex quinquefasciatus* and *Anopheles gambiae* in West African countries." J Vector Ecol **22**: 43-51.

Skovmand, O. and E. Sanogo (1999). "Experimental formulations of *Bacillus sphaericus* and *B. thuringiensis israelensis* against *Culex quinquefasciatus* and *Anopheles gambiae* (Diptera: Culicidae) in Burkina Faso." J Med Entomol **36**: 62-67.

Smith, A., W. O. Obudho and S. Esozed (1966). "Resting patterns of *Anopheles gambiae* in experimental huts treated with malathion." Trans R Soc Trop Med Hyg **60**(3): 401-408.

Smith, D. L., K. E. Battle, S. I. Hay, C. M. Barker, T. W. Scott and F. E. McKenzie (2012). "Ross, macdonald, and a theory for the dynamics and control of mosquito-transmitted pathogens." PLoS Pathog **8**(4): e1002588.

Smith, D. L., T. A. Perkins, L. S. Tusting, T. W. Scott and S. W. Lindsay (2013). "Mosquito population regulation and larval source management in heterogeneous environments." PLoS ONE **8**(8): e71247.

Snetselaar, J., R. Andriessen, R. A. Suer, A. J. Osinga, B. G. Knols and M. Farenhorst (2014). "Development and evaluation of a novel contamination device that targets multiple life-stages of *Aedes aegypti*." Parasit Vectors **7**: 200.

Snow, R. W., C. A. Guerra, A. M. Noor, H. Y. Myint and S. I. Hay (2005). "The global distribution of clinical episodes of *Plasmodium falciparum* malaria." Nature **434**: 214-217.

Soeprono, A. M. and M. K. Rust (2004). "Effect of horizontal transfer of barrier insecticides to control Argentine ants (Hymenoptera: Formicidae)." J Econ Entomol **97**(5): 1675-1681.

Somi, M. F., J. R. Butler, F. Vahid, J. Njau, S. P. Kachur and S. Abdulla (2007). "Is there evidence for dual causation between malaria and socioeconomic status? Findings from rural Tanzania." Am J Trop Med Hyg **77**(6): 1020-1027.

Soper, F. L. (1966). "Paris green in the eradication of *Anopheles gambiae*: Brazil, 1940; Egypt, 1945." Mosquito News **26**(4): 470-476.

Sougoufara, S., S. M. Diédhiou, S. Doucouré, N. Diagne, P. M. Sembène, M. Harry, J. Trape, C. Sokhna and M. O. Ndiath (2014). "Biting by *Anopheles funestus* in broad daylight after use of long-lasting insecticidal nets: a new challenge to malaria elimination." Malar J **13**: 125.

Staal, G. B. (1975). "Insect growth regulators with juvenile hormone activity." Ann Rev Entomol **20**: 417-460.

Steinwascher, K. (1982). "Relationship between pupal mass and adult survivorship and fecundity for *Aedes aegypti*." Environ Entomol **11**: 150-153.

Steketee, R. W. and C. C. Campbell (2010). "Impact of national malaria control scale-up programmes in Africa: magnitude and attribution of effects." Malar J **9**: 299.

Stoks, R. and A. Cordoba-Aguilar (2012). "Evolutionary ecology of Odonata: a complex life cycle perspective." Annu Rev Entomol **57**: 249-265.

Stoops, C. A. (2005). "Influence of *Bacillus thuringiensis* var. *israelensis* on oviposition of *Aedes albopictus* (Skuse)." J Vector Ecol **30**(1): 41-44.

Subra, R. (1981). "Biology and control of *Culex pipiens quinquefasciatus* Say 1823 (Diptera, Culicidae) with special reference to Africa " Insect Sci Application **4**(4): 319-338.

Sufian, S. (2005). "Re-imagining Palestine: scientific knowledge and malaria control in mandatory Palestine." Dynamis **25**: 351-382.

Sumba, L. A., T. O. Guda, A. L. Deng, A. Hassanali, J. C. Beier and B. G. J. Knols (2004). "Mediation of oviposition site selection in the African malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) by semiochemicals of microbial origin." Int. J. Trop. Insect Sci. **24**(3): 260-265.

Sumba, L. A., C. B. Ogbunugafor, A. L. Deng and A. Hassanali (2008). "Regulation of oviposition in *Anopheles gambiae s.s.*: role of inter- and intra-specific signals." J Chem Ecol **34**(11): 1430-1436.

Sutherland, D. D., J. J. McNelly and J. A. Hansen (1989). "Evaluation of granular *Bacillus sphaericus* to control *Culex* in sewage treatments ponds in Cape May." New Jersey Mosqu Contr Assoc **76**: 84-90.

Suzuki, H., T. OKazawa, N. Kere and H. Kawada (1989). "Field evaluation of a new insect growth regulator, pyriproxyfen, against *Anopheles farauti*, the main vector of malaria in the Solomon Islands." Jap. J. Sanit. Zool. **40**(1): 253-257.

Takken, W., R. C. Smallegange, A. J. Vigneau, V. Johnston, M. Brown, A. J. Mordue-Luntz and P. F. Billingsley (2013). "Larval nutrition differentially affects adult fitness and Plasmodium development in the malaria vectors *Anopheles gambiae* and *Anopheles stephensi*." Parasit Vectors **6**(1): 345.

Takken, W., W. B. Snellen, J. P. Verhave and B. G. J. Knols (1990). "Environmental measures for malaria control in Indonesia - an historical review on species sanitation ": Wageningen Agricultural University. Wageningen Agricultural University Papers. pp 60-107.

Teklehaimanot, A. and P. Mejia (2008). "Malaria and poverty." Ann N Y Acad Sci **1136**: 32-37.

Telang, A., L. Frame and M. R. Brown (2007). "Larval feeding duration affects ecdysteroid levels and nutritional reserves regulating pupal commitment in the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae)." J Exp Biol **210**: 854-864.

Tiono, A. B., M. Pinder, S. N'Fale, B. Faragher, T. Smith, M. Silkey, H. Ranson and S. W. Lindsay (2015). "The AvecNet Trial to assess whether addition of pyriproxyfen, an insect juvenile hormone mimic, to long-lasting insecticidal mosquito nets provides additional protection against clinical malaria over current best practice in an area with pyrethroid-resistant vectors in rural Burkina Faso: study protocol for a randomised controlled trial." Trials **16**: 113.

Toms, B. A. (1950). "Mosquito control: an investigation of natural surface films in relation to spreading of larvicidal oils upon water." Bull Entomol Res **40**: 503-510.

Tunaz, H. and N. Uygun (2004). "Insect growth regulators for insect pest control." Turk J Agric For **28**(377-387).

Tusting, L. S., J. Thwing, D. Sinclair, U. Fillinger, J. Gimnig, K. E. Bonner, C. Bottomley and S. W. Lindsay (2013). "Mosquito larval source management for controlling malaria." Cochrane Database Syst Rev **8**: CD008923.

Utzinger, J., Y. Tozan, F. Doumani and B. H. Singer (2002). "The economic payoffs of integrated malaria control in the Zambian copperbelt between 1930 and 1950." Trop Med Int Health **7**(8): 657-677.

Utzinger, J., Y. Tozan and B. H. Singer (2001). "Efficacy and cost-effectiveness of environmental management for malaria control." Trop Med Int Health **6**(9): 677-687.

Vale, G. A. (1974). "New field methods for studying the responses of tsetse flies (Diptera, Glossmidae) to hosts." Bull.ent.Res. **64**: 199-208.

Van den Berg, H. and W. Takken (2007). "A framework for decision-making in integrated vector management to prevent disease." Trop Med Int Health **12**: 1230-1238.

Vanek, M. J., B. Shoo, D. Mtasiwa, M. Kiama, S. W. Lindsay, U. Fillinger, K. Kannady, M. Tanner and G. F. Killeen (2006). "Community-based surveillance of malaria vector larval habitats: a baseline study in urban Dar es Salaam, Tanzania." BMC Public Health **6**: 154.

Vasuki, V. (1992). "The effects of sublethal doses of hexaflumuron on the feeding behaviour of mosquitoes (Diptera: Culicidae)." Bull Entomol Res **82**: 535-538.

Vezzani, D. (2007). "Review: artificial container-breeding mosquitoes and cemeteries: a perfect match." Trop Med Int Health **12**(2): 299-313.

Vythilingam, I., B. M. Luz, R. Hanni, T. S. Beng and T. C. Huat (2005). "Laboratory and field evaluation of the insect growth regulator pyriproxyfen (Sumilarv 0.5G) against dengue vectors." J Am Mosq Control Assoc **21**(3): 296-300.

Walker, K. and M. Lynch (2007). "Contributions of *Anopheles* larval control to malaria suppression in tropical Africa: review of achievements and potential." Med Vet Entomol **21**(1): 2-21.

Wang, L. Y. and Z. Jaal (2005). "Sublethal effects of *Bacillus thuringiensis* H-14 on the survival rate, longevity, fecundity and F1 generation developmental period of *Aedes aegypti*." Dengue Bull **29**: 192-196.

Webb, C. E. and R. C. Russell (2012). "Does the monomolecular film Aquatain Mosquito Formula provide effective control of container-breeding mosquitoes in Australia?" J Am Mosq Control Assoc **28**(1): 53-58.

White, G. B. (1974). "*Anopheles gambiae* complex and disease transmission in Africa." Trans R Soc Trop Med Hyg **68**(4): 278-298.

White, M. T., J. T. Griffin, T. S. Churcher, N. M. Ferguson, M. Basáñez and A. C. Ghani (2011a). "Modelling the impact of vector control interventions on *Anopheles gambiae* population dynamics." Parasit Vectors **4**: 153.

White, M. T., J. T. Griffin, T. S. Churcher, N. M. Ferguson, M. G. Basanez and A. C. Ghani (2011b). "Modelling the impact of vector control interventions on *Anopheles gambiae* population dynamics." Parasit Vectors **4**: 153.

White, N. J., S. Pukrittayakamee, T. T. Hien, M. A. Faiz, O. A. Mokuolu and A. M. Dondorp (2014). "Malaria." Lancet **383**(9918): 723-735.

WHO (1982). "Manual on environmental management for mosquito control with special emphasis on malaria vectors." World Health Organization. Geneva.

WHO (1999a). "International programme on chemical safety (IPCS): Microbial pest control agent *Bacillus thuringiensis*." Environ Health Criteria **217**: 1-105.

WHO (1999b). "The World Health Report." Rolling Back Malaria. World Health Organization.

WHO (2000). "Expert Committee on malaria." WHO Technical Report Series. World Health Organization, Geneva.

WHO (2002). "Scaling-up insecticide treated netting programmes in Africa: A strategic framework for coordinated national action." Roll Back Malaria. World Health Organization.

WHO (2003). "Insecticide-treated mosquito net interventions. A manual for national control programme managers." Roll Back Malaria. World Health Organization.

WHO (2004). "Global strategic framework for integrated vector management." World Health Organization, Geneva, Switzerland.

WHO (2005a). "Guidelines for laboratory and field testing of mosquito larvicides." World Health Organization Communicable Disease Control, Prevention and Eradication. WHO Pesticide Evaluation Scheme.

WHO (2005b). "The Roll Back Malaria strategy for improving access to treatment through home management of malaria." Roll Back Malaria Department.

WHO (2006a). "Malaria vector control and personal protection." WHO Technical Report Series. World Health Organization.

WHO (2006b). "Pesticides and their application for the control of vectors and pests of public health importance ".

WHO (2006c). "Use of indoor residual spraying for scaling up global malaria control and elimination." Global Malaria Programme.

WHO (2007). "Insecticide-treated mosquito nets: a WHO Position Statement." Global Malaria Programme.

WHO (2008a). "Pyriproxyfen in drinking-water: Use for vector control in drinking-water sources and containers. Background document for development of WHO guidelines for drinking-water." World Health Organization.

WHO (2008b). "WHO position statement on integrated vector management." World Health Organization.

WHO (2009). "Malaria case management. Operations manual.": Global Malaria Programme.

WHO (2011). "Integrated vector management to control malaria and lymphatic filariasis: WHO position statement." World Health Organization.

WHO (2012a). "Global plan for insecticide resistance management in malaria vectors." WHO Global Malaria Programme. World Health Organization.

WHO (2012b). "Interim Position Statement: The role of larviciding for malaria control in sub-Saharan Africa." World Health Organization.

WHO (2012c). "Launch of the global plan for insecticide resistance management in malaria vectors. Event report." World Health Organization, Geneva.

WHO (2012d). "World Malaria Report." Geneva, Switzerland.

WHO (2013a). "Indoor Residual Spraying: An operational manual for indoor residual spraying (IRS) for malaria transmission control and elimination." World Health Organization, Geneva.

WHO (2013b). "Larval source management: A supplementary measure for malaria vector control. An operational manual." World Health Organization.

WHO (2013c). "World malaria report." Geneva, Switzerland.

WHO (2014a). "Control of residual malaria parasite transmission. Technical note." Global Malaria Programme. World Health Organization.

WHO (2014b). "A global brief on vector-borne diseases." Geneva, Switzerland.

WHO (2014c). "World Malaria Report." Geneva, Switzerland.

Wigglesworth, V. B. (1934). "The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and 'metamorphosis'." London School of Hygiene and Tropical Medicine. pp 191-222.

Woolhouse, M. E., C. Dye, J. F. Etard, T. Smith, J. D. Charlwood, G. P. Garnett, P. Hagan, J. L. Hii, P. D. Ndhlovu, R. J. Quinnell, C. H. Watts, S. K. Chandiwana and R. M. Anderson (1997). "Heterogeneities in the transmission of infectious agents: implications for the design of control programs." Proc Natl Acad Sci U S A **94**(1): 338-342.

Worrall, E. and U. Fillinger (2011). "Large-scale use of mosquito larval source management for malaria control in Africa: a cost analysis." Malar J **10**: 338.

Wyatt, G. R. (1997). "Juvenile hormone in insect reproduction-a paradox?" Env J Entomol **94**: 323-333.

Yakob, L. and G. Yan (2009). "Modeling the effects of integrating larval habitat source reduction and insecticide treated nets for malaria control." PLoS ONE **4**(9): e6921.

Yapabandara, A. M. and C. F. Curtis (2002). "Laboratory and field comparisons of pyriproxyfen, polystyrene beads and other larvicidal methods against malaria vectors in Sri Lanka." Acta Trop **81**(3): 211-223.

Yapabandara, A. M. and C. F. Curtis (2004). "Control of vectors and incidence of malaria in an irrigated settlement scheme in Sri Lanka by using the insect growth regulator pyriproxyfen." J Am Mosq Control Assoc **20**(4): 395-400.

Yapabandara, A. M., C. F. Curtis, M. B. Wickramasinghe and W. P. Fernando (2001). "Control of malaria vectors with the insect growth regulator pyriproxyfen in a gem-mining area in Sri Lanka." Acta Trop **80**(3): 265-276.

Ye-Ebiyo, Y., R. J. Pollack, A. Kiszewski and A. Spielman (2003). "Enhancement of development of larval *Anopheles arabiensis* by proximity to flowering maize (Zea mays) in turbid water and when crowded." Am. J. Trop. Med. Hyg. **68**(6): 748–752.

Yoshioka, M., J. Couret, F. Kim, J. McMillan, T. R. Burkot, E. M. Dotson, U. Kitron and G. M. Vazquez-Prokopec (2012). "Diet and density dependent competition affect larval performance and oviposition site selection in the mosquito species *Aedes albopictus* (Diptera: Culicidae)." Parasit Vectors **5**: 225.

Yuan, Z. M., Y. M. Zhang and E. Y. Liu (2000). "High-level field resistance to *Bacillus sphaericus* C3-41 in *Culex quinquefasciatus* from Southern China." Biocontrol Sci Technol **10**: 43-51.

Zhou, G., Y. A. Afrane, A. M. Vardo-Zalik, H. Atieli, D. Zhong, P. Wamae, Y. E. Himeidan, N. Minakawa, A. K. Githeko and G. Yan (2011). "Changing patterns of malaria epidemiology between 2002 and 2010 in Western Kenya: the fall and rise of malaria." PLoS ONE **6**(5): e20318.

Zhou, G., A. K. Githeko, N. Minakawa and G. Yan (2010). "Community-wide benefits of targeted indoor residual spray for malaria control in the western Kenya highland." Malar J **9**: 67.

Zhou, G., N. Minakawa, A. K. Githeko and G. Yan (2004). "Association between climate variability and malaria epidemics in the East African highlands." PNAS **101**(8): 2375-2380.