Exploitation of adult *Anopheles arabiensis* behaviour and ecology for the dissemination of pyriproxyfen, a novel technique for malaria vector control in Tanzania

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ABSTRACT

Effective larviciding to manage mosquito aquatic habitats offers an additional strategy for malaria vector control by complementing benefits already achieved by long lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS). Sustainable implementation of larviciding requires comprehensive understanding of the ecology of disease vectors and robust monitoring of factors governing local disease transmission. Treatment of aquatic habitats with the juvenile hormone analogue Pyriproxyfen (PPF), inhibits adult mosquito emergence at extremely low concentrations that are potentially deliverable by PPF-contaminated gravid adult females, a phenomenon termed 'autodissemination'.

The primary aim of this thesis was to investigate a range of adult mosquito behaviours that might be exploited to disseminate PPF. The effectiveness of PPF to sterilize adult mosquitoes for malaria vector control was also assessed in a controlled system. Vector dynamics, malaria transmission intensity and risk factors were evaluated at the field site where the PPF autodissemination strategy would be evaluated in field trials and potentially implemented.

Field monitoring of indoor malaria transmission risk factors revealed that even in the communities with high coverage of bednets, LLINs did not reduce the indoor densities of *An. gambiae* s.l (RR= 0.74 (0.50 - 1.11, $p > 0.05$) but reduced *An. funestus* indoor densities by 56% (RR= 0.44 (0.23 - 0.87, $p < 0.05$)). Houses with eave gaps had 3.3 and 5.5 times more *An. gambiae* s.l. (RR= 3.3 (2.39 - 4.56, $p < 0.05$)) and *An. funestus* ((RR = 5.55 (3.25 - 9.46, $p < 0.05$)) respectively. Intact screening over windows reduced up to 66% (RR = 0.34 (0.17 - 0.69)) and 83% (RR = 0.17 (0.08 - 0.39)) indoor entry of *An. gambiae* s.l. and *An. funestus* respectively.

Furthermore, surveillance of wild malaria vectors populations and susceptibility to insecticide resistance demonstrated significant increase in *An. funestus* densities in 2012 (RR=1.56 (1.33-1.69)) compared to *An.gambiae* s.l. ($p < 0.0001$). In 2014, the proportion of *An. gambiae* s.l. catches (67%; 4373/6373) was higher than *An. funestus* (33%; 2100/6373). PCR results revealed change in relative proportion between the two sibling species of *An.gambaie* s.l. with a significant decrease in *An. gambiae* s.s. from approximately 14% (414/2,924) in 2008 to 0% (0/435) in 2014. Insecticide susceptibility tests indicated high resistance in *An. funestus* against deltamethrin (mortality rate in discriminating dose assay = 87%), lambda cyhalothrin (74%), permethrin (65%), bendiocarb (65%), and DDT (66%). Similarly, *An. arabiensis* showed insecticide resistance to permethrin (77%), deltamethrin (64%) and lambda cyhalothrin (42%) in 2014.

In large screened cages it was demonstrated that adult *An. arabiensis* can disseminate PPF from clay pots treated with PPF to the aquatic habitats, resulting in 76.5% reduction in adult emergence, with higher mean proportion of adult emerging from untreated chamber, 0.95 (0.56 - 1.34) compared to the treated chamber, 0.21 (0.09 - 0.51, $p < 0.0001$). Treatment of a single clay pot resulted in 58% reduction in adult emergence in six habitats, with mean proportion of 0.34 (0.21 – 0.45) compared to the controls, 0.98 (0.96 – 1.00, $p < 0.0001$), showing a high level of habitats coverage amplification of the autodissemination event. After treating the walls and ceilings of cattle shelters with PPF, mosquito sterilization resulted in > 95% (89.3 - 102.9%) reduction in adult *An. arabiensis* production.

This research provides evidence on the need of better housing and larviciding to complement LLINs in controlling the remaining malaria transmission transmitted by *An. funestus* and *An. arabiensis*. It also demonstrated for the first time that the PPF autodissemination strategy and sterilization of adult females present a promising malaria vector control option for field trial. PPF-autodissemination can be integrated into a vector management toolbox to control outdoor malaria transmission and also target multiple disease-carrying mosquitoes that share aquatic habitats with malaria vectors. These findings highlight the potential of PPF for controlling outdoor and indoor malaria vectors and call for further testing in the field.

DECLARATION

I declare that this thesis is my own original work and that none of the material in this thesis has been presented or submitted to any other University for a similar degree award. Chapters 2, 3, 4 and 5 have already been published as reports in peer-reviewed journals, as Lwetoijera *et al.* and are presented in this thesis with minimal modification. Since those publications had multiple authors, the individual contributions of the co-authors are described here:-

Chapter 2: *A need for better housing to further reduce indoor malaria transmission in areas with high bed net coverage.* Dickson W. Lwetoijera (DWL) proposed the study, supervised field data collection, analysed the data and drafted the manuscript under the supervision of Silas Majambere (SM), Samson K. Kiware (SSK) and Brian Faragher (BF) assisted in data analysis. The additional authors, Zawadi D. Mageni (ZDM), Caroline Harris (CH), Stefan Dongus (SD) and Gregor J. Devine (GJD), contributed to and approved the final manuscript.

Chapter 3: *Increasing role of Anopheles funestus and Anopheles arabiensis in malaria transmission in the Kilombero Valley, Tanzania*. DWL conceived the study hypothesis, designed and supervised data collection, performed data analysis and drafted the manuscript under supervision of SM and Philip J. McCall (PJM). CH, SSK, SD and GJD contributed to the study design, and contributed to and approved the final manuscript.

Chapter 4: *Effective autodissemination of pyriproxyfen to breeding sites by the exophilic malaria vector Anopheles arabiensis in semi-field settings in Tanzania.* DWL, SM and GD conceived the study hypothesis. DWL developed the study protocols and conducted the experiments; DWL, SSK and CH performed statistical analysis and interpreted the results. SM and PJM supervised the experimental progress and

supervised drafting of the manuscript. All authors contributed to and approved the final manuscript.

Chapter 5: *Comprehensive sterilization of resilient malaria vectors: a step closer to malaria elimination.* DWL and SM conceived the study hypothesis, summarized and analysed the data under guidance of SSK and CH, and wrote the first manuscript draft. DWL prepared the study protocols, and performed and supervised the experiments under supervision of SM and PJM. All authors contributed to and approved the final manuscript.

All remaining parts of the thesis were written by DWL, under the guidance of PJM and SM.

Signed :.....(Candidate)

Date:.....

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LIST OF ABBREVIATIONS

AI	Active ingredient
CDC	Centre for Disease Control
CI	Confidence interval
DALYS	Daily Adjusted Life Years
DDT	Dichlorodiphenyltrichloroethane
EIR	Entomological inoculation rate
ELISA	Enzyme linked-immuno-sorbent assay
GDP	Gross domestic product
G	Granular
GLM	Generalized linear model
IHI	Ifakara Health Institute
IRS	Indoor residual spraying
ITN	Insecticide treated net
IVM	Integrated vector management
JH	Juvenile hormone
JHA	Juvenile hormone analogue
LLINs	Long lasting insecticide treated nets
LSM	Larval source management

ME	Mode estimate
OBS	Odour baited station
PCR	Polymerase chain reaction
PPF	Pyriproxyfen
RR	Relative rate
SEC	Solubilized emulsifiable concentrate
SD	Standard deviation
SFS	Semi-field system
SPSS	Statistical package for social sciences (Program for statistical analyses)
WHO	World Health Organization
WHOPES	World Health Organization pesticides scheme
WP	Wettable powder

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Malaria

Throughout the world, vector borne diseases cause significant morbidity and mortality (Becker *et al.*, 2006). Malaria is one of the most important vector borne diseases and continues to put 3.2 billion people at risk, with more than one malaria case occurring per 1000 people (WHO, 2014). In 2013, malaria was estimated to have caused 198 million clinical cases and claimed approximately 584,000 lives worldwide, of which 80% of total cases and 90% of deaths occurred in Africa (WHO, 2014). Another analysis of malaria deaths from all endemic countries (Murray *et al.*, 2012), estimated that malaria killed 1.24 million people globally in 2010. Although mainly impacting children under 5 years of age and pregnant women (Adam *et al.*, 2005; Lawn *et al.*, 2005), it also significantly affects adults across all age groups (Murray *et al.*, 2012).

The disease is disproportionately abundant in tropical and subtropical regions of Africa where it continues to destabilize social and economic development (Fig. 1.1.1). A decade ago, malaria was estimated to account for 10% of the African disease burden in terms of disability adjusted life years or DALYs (Goodman *et al.*, 2001). Moreover, the disease was estimated to account for an annual loss of 1.3% Gross Domestic Product (GDP) in Africa and approximately 40% of public health expenditure in sub-Saharan Africa (RMB, 2008).

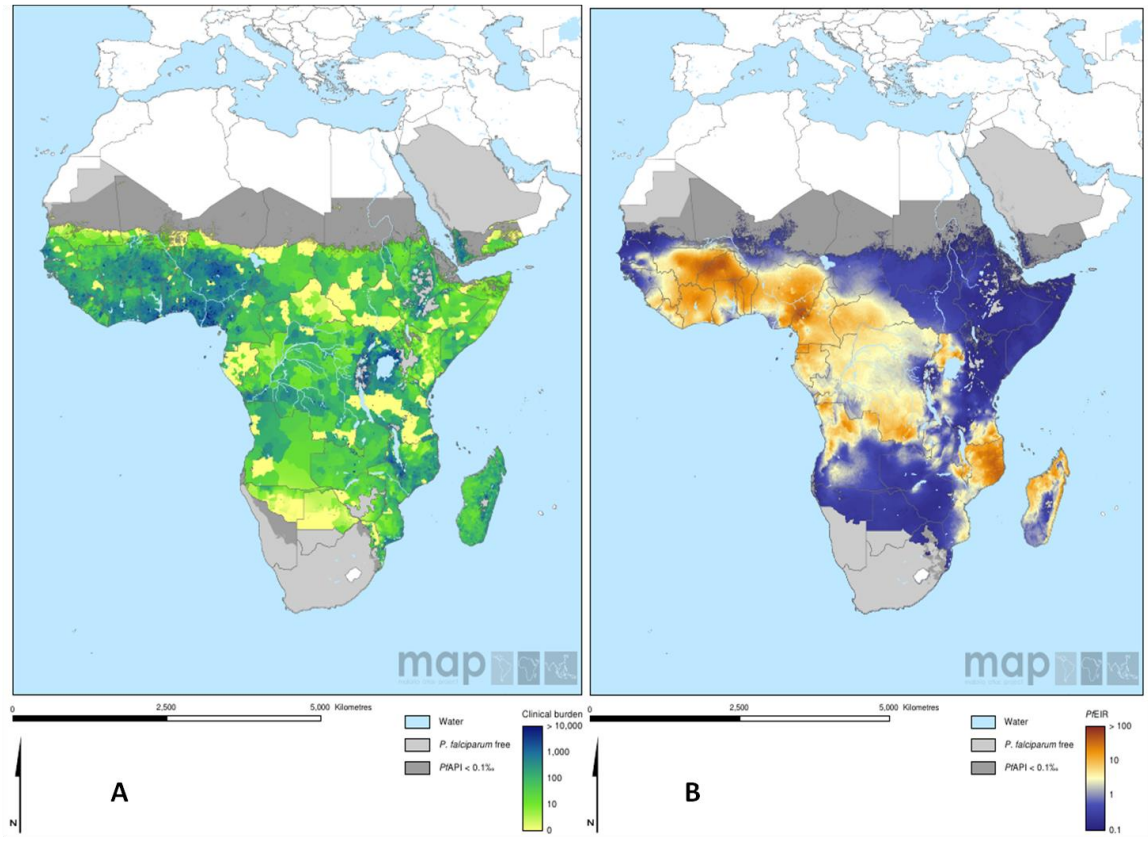

Figure 1.1.1: Maps showing A) clinical burden of *Plasmodium falciparum* in all age groups, in 2007, B) spatial distribution of *Plasmodium falciparum* entomological inoculation rate in 2010 in Africa (from http://www.map.ox.ac.uk/browse-resources/).

1.1.1 Malaria parasites and transmission

Malaria is caused by single-celled protozoan parasites within the genus *Plasmodium*. To date, there are five species that are known to infect humans naturally: *Plasmodium falciparum, P.vivax, P.ovale, P.malariae* (Beier, 1998; Weller, 2003) and *P. knowlesi* (Singh *et al.*, 2004). However, *P.malariae* also infects non-human primates (Garnham, 1966). Transmission occurs from human to human via the vector mosquito in most cases. *P. falciparum* is by far the most common and deadliest form, accounting for the

majority of human malaria cases worldwide (Mueller *et al.*, 2007), with most severe disease occurring in people with low protective immunity (Warrell *et al.*, 1990; Baird, 1998). The severity of *P. falciparum* is associated with the parasite blood stages' binding ability to the endothelial blood capillaries as well as the sequestration ability in the liver, brain and kidney, which eventually results in large parasite loads and harmful toxins produced by the parasites in the bloodstream (Buffet *et al.*, 2011). These pathological processes lead to initial nonspecific disease symptoms which include headache, fatigue, fever, chill, joint aches, abdominal discomfort, vomiting and body malaise (Reyburn, 2010; WHO, 2010). Delayed or ineffective treatment of the disease usually results in clinical complications of malaria such as liver failure, encephalopathy, coma as well as anaemia (Mackintosh *et al.*, 2004). Several studies have reported that the severity of the *P.falciparum* can either be reduced (Maitland *et al.*, 1997) or amplified (Rogerson & Carter, 2008) during co-infection with *P.vivax*.

Malaria parasites are transmitted only by female mosquitoes within the genus *Anopheles* (Diptera: Culicidae) during feeding on human blood (Bruce-Chwatt *et al.*, 1966). The parasite transmission pathway is complex and cyclical between human and mosquito vectors, with a sporogonic or sexual stage in mosquitoes (Beier, 1998), and a schizogony or asexual stage in humans, in both non-blood (exo-erythrocyte) and blood (erythrocyte) stages (Cox, 2010) (Fig 1.1.2).

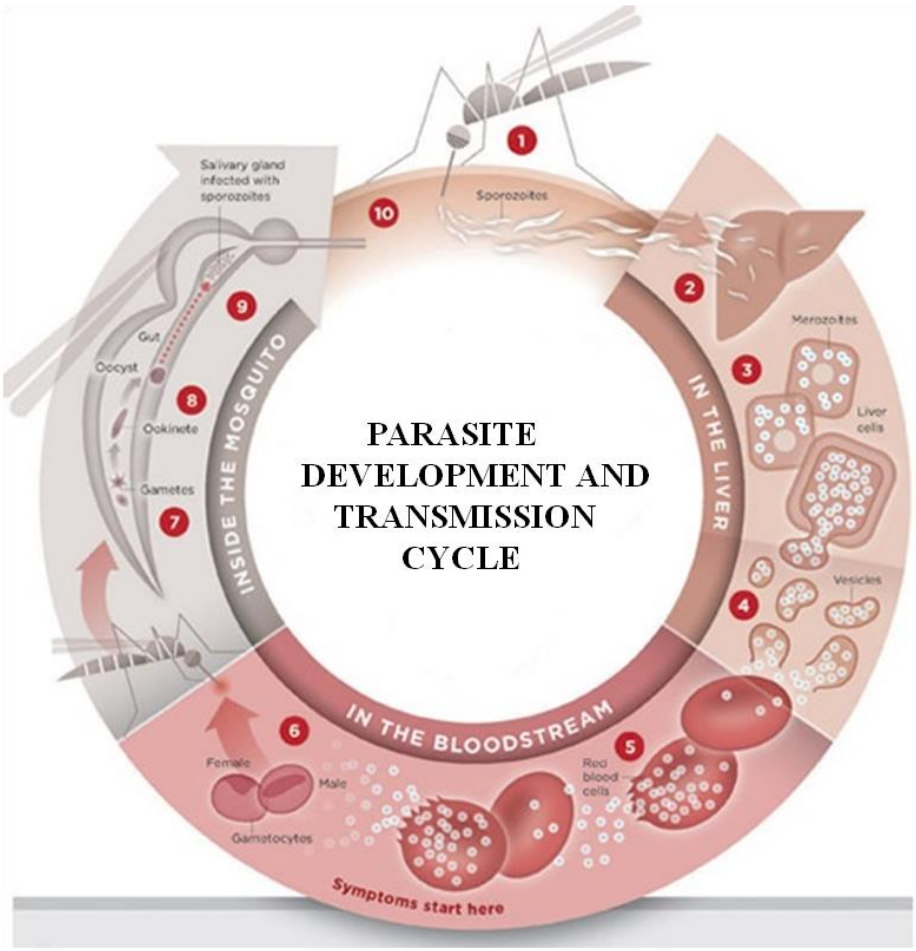

Figure 1.1.2: A schematic illustration of the generalized malaria parasite life cycle. Infected mosquito bite and person and inject asexual form of parasites (1) which migrate to the liver, multiply and released (2, 3, 4) into bloodstream where the parasite invade red blood cells, multiply and develop to sexual form of parasites (5, 6) which are ingested when mosquito bites an infected person and develop inside mosquito to an infective asexual parasites (7, 8, 9) transmitted via mosquito bite (10), (from www.malariavaccine.org/malvac-lifecycle.php).

1.1.2 Malaria vectors

There are more than 400 species of *Anopheles* mosquitoes, and 70 that are known to transmit malaria worldwide (Service, 1993; Sinka *et al.*, 2012). Of these, *Anopheles gambiae* Giles, and *An. funestus* Giles are the most widespread and efficient vectors in Africa (Coetzee *et al.*, 2000; Fontenille & Simard, 2004; Okara *et al.*, 2010) (Fig. 1.1.3). Both of these are species complexes (Section 1.1.3), and include species that feed preferentially on humans and that are highly efficient at transmitting malaria (Bruce-Chwatt *et al.*, 1966). Adult mosquitoes develop through four developmental stages, eggs, larvae, pupae and adults, of which the first three are aquatic.

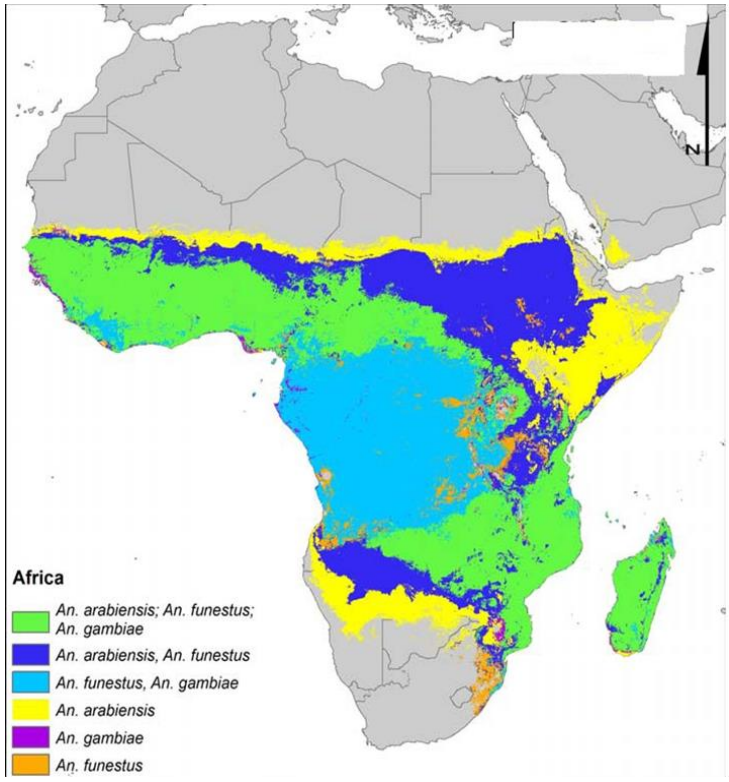

Figure 1.1.3: Map showing distribution of the dominant malaria vectors in Africa (Sinka *et al.*, 2012).

1.1.3 Biology of the main African anopheline vectors

The *An. gambiae* complex (*Anopheles gambiae sensu lato,* or s.l.) comprises seven sibling species, of which *Anopheles arabiensis* and *Anopheles gambiae sensu stricto* (hereafter termed *An. gambiae* s.s.) are primary vectors of malaria (White, 1974). *An. arabiensis* and *An. gambiae* s.s. typically share the same freshwater habitats (Gilles *et al*., 1961; Takken & Lindsay, 2004), ranging from small temporary water bodies to margins of permanent streams or ponds or large irrigated expanses (Fillinger & Lindsay, 2006).

Anopheles funestus group or *An. funestus* s.l., is also an important malaria vector, comprising nine species. *An. funestus* s.s. is the most efficient malaria vector due to its anthropophagic and endophilic behaviour, while the rest are mainly zoophilic. *An. funestus* prefers to breed in large and permanent or semi-permanent fresh and turbid shaded water bodies with emergent vegetation (Gilles *et al.*, 1961; Gillies, 1962; Gillies & de Meillon, 1968; Coetzee *et al.*, 2000). Members of both complexes are widely distributed all over Africa and often co-exist (Sinka *et al*., 2012) (Fig. 1.1.3).

During the rainy season, *An. gambiae* s.s. numbers typically increase, possibly due to this species' ability to outcompete *An. arabiensis* population in the larval habitat under the environmental conditions in that season (Schneider *et al.*, 2000; Koenraadt & Takken, 2003). Conversely, during the dry season, *An. arabiensis* survives better than *An. gambiae* s.s. and typically becomes dominant (Lindsay *et al.*, 1998). *An. funestus* mosquitoes which breed typically in permanent and semi-permanent larger, clear and turbid shaded water bodies (Gilles *et al.*, 1961; Mosha & Subra, 1983), also increases in

numbers in the dry season and often account for much of the dry season transmission (Charlwood *et al.*, 2000).

Characterization of malaria vector resting behaviour post-blood feeding has been well established across different mosquito species, as a crucial facet of effective mosquito control and successful evaluation of the interventions involved (Gillies & Smith, 1960). Although mosquito resting behaviour between species is generally categorized either as endophilic or exophilic, it has been also documented that the same mosquito species can display both endophilic and exophilic resting behaviours (Lines *et al.*, 1986; Githeko *et al.*, 1996).

Endophilic behaviour is the tendency for mosquitoes to prefer resting inside human houses; typically this is during the period after feeding and before the onset of the search for an oviposition site. Exophilic behaviour is when mosquitoes prefer to rest outside human dwellings (Pates & Curtis, 2005). Although efficient vectors of malaria, notably *An. gambiae* s.s. and *An. funestus* s.s. are mostly recognised as endophilic mosquitoes (Gillies, 1954; Mnzava *et al.*, 1995; Githeko *et al.*, 1996), exophilic behaviour has been also observed (Fontenille *et al.*, 1990; Bockarie *et al.*, 1994). *An. arabiensis*, is a mainly exophilic mosquito (Mnzava *et al.*, 1995; Tirados *et al.*, 2006), also found to rest indoors (Faye *et al.*, 1997; Animut *et al.*, 2013). Development of new vector control tools to target different species of malaria vector must appreciate these resting behaviour preferences to maximize the efficacy of targeted interventions.

1.2 Control of malaria vectors and challenges to existing approaches

Available vector control strategies can target immature or adult stages of mosquitoes. The current, most widely used and most efficient approaches are those that target the adult mosquito stages. These control strategies primarily involve two tools: insecticide-treated nets (ITNs; currently long-lasting insecticidal nets are most used, termed LLINs) and indoor residual spraying (IRS). LLINs target mosquitoes that feed on humans indoors at night by reducing human-vector contacts through physical barrier of a net and impregnated insecticide which increase LLINs protective efficacy, either through excito-repellency, reducing survival or killing mosquitoes that come into contact with the LLINs (Takken, 2002; Lengeler, 2004). IRS targets mosquitoes that rest on the interior surfaces (walls, eaves and ceiling) of the houses or domestic animal shelters that have been sprayed with insecticides by killing/repelling and reducing adult mosquito longevity. LLINs target host-seeking mosquitoes, while IRS is more effective in targeting blood fed resting mosquitoes compared to non-blood fed host seeking due to its extended residual effect which can kill mosquitoes resting on sprayed surfaces inside houses and/or animal shelters during blood digestion (WHO, 2006, 2013a). Both have contributed to control programmes and helped to significantly reduce malaria transmission in many African settings (WHO, 1995; Mabaso *et al.*, 2004; Griffin *et al.*, 2010; Russell *et al.*, 2010; WHO, 2014). However, these methods are designed to address indoor malaria transmission and even where they have been successful and in use over many years, transmission has not been interrupted completely and there are still high estimates of human exposure to malaria (Seyoum *et al.*, 2012; Huho *et al.*, 2013; Bayoh *et al.*, 2014). This is termed 'residual transmission' and most experts agree that it cannot be addressed by those two main control measures alone (Griffin *et al.*, 2010; Killeen, 2014).

Outdoor malaria transmission undoubtedly contributes a high proportion of residual malaria transmission (Durnez & Coosemans, 2013; Killeen, 2014). Several studies suggest that continuous use of indoor' based interventions (LLINs and IRS), while reducing the density of indoor' biting mosquitoes in targeted communities, may have resulted in an increased proportion of outdoor transmission. Accelerated behavioural changes in malaria vector populations leading to higher proportions feeding outdoors earlier times in the morning and evening, to avoid contact with insecticides, has been reported, although evidence to date shows that this has resulted from species shifts rather than evolved changes in a single target species (Pates & Curtis, 2005; Van Bortel *et al.*, 2010; Bugoro *et al.*, 2011a; Russell *et al.*, 2011b; Kitau *et al.*, 2012; Reddy *et al.*, 2012; Kiware *et al.*, 2012a; Kiware *et al.*, 2012b).

These shifts in species, arose due to LLINs and IRS killing species that were predominantly endophagic, endophilic and anthropophilic, particularly *An. gambiae* s.s., which historically was dominant in malaria transmission (Gillies & de Meillon, 1968; White, 1974). *An. arabiensis*, which feeds outdoors as well as indoors and on cattle as well as humans benefited from that selective targeting and has become increasingly important in sustaining residual malaria transmission (Coluzzi *et al.*, 1979; Bayoh *et al.*, 2010; Russell *et al.*, 2010). It is clear that *An. arabiensis* cannot be controlled adequately with existing LLINs and IRS strategies (Durnez & Coosemans, 2013; Killeen, 2014).

Moreover, the future of both LLINs and IRS are threatened by insecticide resistance. Today, target site and metabolic forms of resistance to all four major classes of insecticides that are widely used in vector control (organochlorines, organophosphates, carbamates and pyrethroids) have been reported widely (Kelly-Hope *et al.*, 2008; Ranson *et al.*, 2009; Ranson *et al.*, 2011; Jones *et al.*, 2013). Of particular and

immediate concern is resistance to the pyrethroids, which constitute over 80% of the global spray utility (the surface area covered by an active ingredient) (van den Berg *et al.*, 2012), and are the only insecticide class approved for use on LLINs, due to their low human toxicity, rapid knockdown of mosquitoes and residual effect (Hougard *et al.*, 2002; Hougard *et al.*, 2003). Following the rapid spread of resistance in recent years (Ranson *et al.*, 2011), there is no country in Africa where the major malaria vectors remain fully susceptible to pyrethroids (WHO, 2012a; Hemingway, 2014). Consequently, WHO strongly recommend a pro-active strategy for resistance management as an essential component of vector control programmes i.e. early monitoring for the possibility of resistance development in mosquitoes rather than waiting and react when a vector control product/intervention fails (WHO, 2011a, 2012a).

Hence, despite the substantial reductions in mosquito-vector densities that have been achieved with LLINs and IRS, their efficiency and longevity have been compromised to the point where the ability to continue to combat malaria transmission with these tools alone is under threat. Clearly, new tools and strategies are urgently needed.

1.2.1 Integrated vector management

As part of ongoing efforts to address the aforementioned challenges, WHO has recommended an integrated vector management (IVM) approach (WHO, 2004a; Beier *et al.*, 2008), emphasising the importance of resistance management strategies (Nauen, 2007; Penilla *et al.*, 2007; Sharp, *et al.*, 2007; Kleinschmidt *et al.*, 2009) and optimal use of resources to ensure comprehensive future control of malaria vectors. Integrated vector management is defined as "a rational decision-making process for the optimal use

of resources for vector control" with ultimate aim of reducing or interrupting transmission of vector borne diseases (WHO, 2004a).

Integration of non-chemical based approaches with existing vector control tools, robust entomological and epidemiological surveillance systems to guide evidence-based decision making and targeted control are among the five key elements emphasized by the Global Strategic Framework for IVM (WHO, 2004a; Beier *et al.*, 2008).

In recent years there have been concerted efforts for developing tools to tackle the major challenge of outdoor malaria transmission, involving approaches such as repellents (Barnard, 2000), outdoor baited traps augmented with killing agents (Lwetoijera *et al.*, 2010; Okumu *et al.*, 2010c; Matowo *et al.*, 2013) and larval source reduction (Walker & Lynch, 2007; Fillinger & Lindsay, 2011; Tusting *et al.*, 2013). All are intended to complement the use of LLINs and IRS, by protecting people when not under bed nets or while outside their houses.

1.2.2 Sustainable larval control

Various active formulations and compounds have been tested, recommended and approved by WHOPES for controlling mosquito larvae (WHO, 2013b). These larvicides can be categorized into different classes as follows

1) Oil and surface agent such as petroleum distillates which kill larvae via suffocation by preventing them from resting at the surface due to the reduced water surface tension (Rozendaal, 1997).

2) Synthetic organic compounds can comprise of a wide range of active ingredients for larviciding, such as malathion, pirimiphos-methyl, fenthion and temephos; all of these

compounds kill mosquito larvae by blocking the nerve transmission pathways by interference with enzymatic activities (Rozendaal, 1997; WHO, 2013b).

3) Bacterial larvicides, such as *Bacillus thuringiensis* var. *israelensis* (Bti) and *Bacillus sphaericus* (Bs).

4) Insect growth regulators (IGR), such as pyriproxyfen and methoprene, represent an ideal in many ways because they combine high efficaciousness with low mammalian toxicity and minimal impact to non-target organisms (Schaefer *et al*., 1988; López *et al.*, 2005). While bacterial larvicides kill larvae by destroying the gut with endotoxin-proteins (Charles *et al.*, 1996; Bravo *et al.*, 2007), the insect growth regulators prevent the development of mosquito larvae and pupae into adults by disrupting the normal function of insect developmental hormones (Wilson, 2004).

The use of larvicides to target immature mosquito stages is considered by many to be a potentially effective supplementary approach to existing LLINs and IRS based programmes which target adult-disease transmitting mosquitoes, since even with maximum scaling up, LLINSs and/or IRS may eventually reach a threshold that cannot be further reduced by these interventions alone (Townson *et al.*, 2005; Fillinger *et al.*, 2009; Tusting *et al.*, 2013). However, when adult populations are small, the capacity for population recovery increases, therefore, the addition of larval insecticides might be used to maintain strong larval density dependence that will limit mosquito population's potential for recovery by rendering the aquatic habitats which sustain the remain population unproductive (Russell *et al.*, 2011a).

The benefits of integrating larval control with existing malaria adult control interventions have been documented in a number of studies, many of which have achieved significant reductions in adult vector densities and associated malaria transmission (Utzinger *et al.*, 2001; Gu *et al.*, 2006; Killeen *et al.*, 2006; Fillinger *et al.*, 2008; Gu *et al.*, 2008; Tusting *et al.*, 2013).

Sustainable larval control implementation needs an in-depth understanding of local disease transmission driven by the ecology of disease vectors (Fillinger & Lindsay, 2006; Baragatti *et al.*, 2009). In urban areas, where larval habitats are more identifiable and accessible, it is relatively easy to target the habitats and complement the widely used LLINs and IRS (Fillinger *et al.*, 2008). However, in rural areas, especially during the rainy season, larval habitats can be myriad, ranging from small and ephemeral pools to large and permanent swamps (Gimnig *et al.*, 2001; Minakawa *et al.*, 2004). The nature of these habitats presents difficulties in their identification as well as treatment, processes that are expensive, time-consuming and labour intensive (Majambere *et al.*, 2007; Gu *et al.*, 2008).

In rural Gambia, both standardized and non-standardized field trials achieved 95% reductions in *Anopheles gambiae* s.l. densities when either *Bacillus thuringiensis* var. *israelensis* (Bti) or *Bacillus sphaericus* (Bs) were applied in the surveyed breeding habitats at weekly intervals (Majambere *et al.*, 2007). Although the reduction in abundance and larval mortality might sometimes serve as proxy indicators for microbial efficacy, it is imperative to show an impact on adult density reduction and especially on malaria transmission within the targeted community, before effectiveness in disease control can be inferred (WHO, 2012b). Fillinger and others (Fillinger *et al.*, 2008) reported 96% and 31% density reductions in anophelines and malaria transmission respectively within one year of starting larval intervention in urban Dar es Salaam. These findings were not only associated with the efficaciousness of larvicides used but also because breeding habitats in urban settings are relatively easier to find and treat compared to rural settings (WHO, 2013b). Although larviciding with *Bti* and *Bs* are operationally cost effective in comparison to LLINs and IRS (Worrall & Fillinger, 2011), safe for the environment and highly efficacious against malaria vectors, issues such as selective action and low persistence in the environment at high temperatures

(Majambere *et al.*, 2008; WHO, 1999; Fillinger *et al.*, 2003; Fillinger & Lindsay, 2006), remain among the limitations for the strategy (Fillinger & Lindsay, 2006).

In efforts to address the aforementioned challenges to existing chemical and biological larvicides, it is worth exploring the other larvicides with relative high persistence under field settings that are also potentially efficient and cost-effective tools for field implementation. This necessitates the development of novel, ecologically sound, cost-effective and sustainable approaches. One such approach that offers considerable potential is the autodissemination of larvicides (Schlein & Pener, 1990) by adult mosquitoes.

1.2.3 Larval control by autodissemination of pyriproxyfen

Pyriproxyfen (PPF) is a synthetic hormone analogue derived from insects' naturally occurring juvenile hormones (JH) responsible for growth and reproduction (Wigglesworth, 1934; Wilson, 2004). The autodissemination technique for larviciding is an approach whereby insects are co-opted to perform transfer of insecticides to other insects via different behavioural activities including oviposition, mating, aggregation, resting and host seeking (Devine *et al.*, 2009; Gaugler *et al.*, 2011; Geden & Devine, 2012). The auto-transfer of insecticides can either be horizontally (*i.e.* the transfer of Pyriproxyfen (PPF) from contaminated adults to larvae or pupae in the breeding habitats) (Chism & Apperson, 2003) or vertically (*i.e.* failure in production of viable eggs within PPF contaminated females or sperm formation in male mosquitoes, without interfering with adult fitness) (Itoh *et al.*, 1994; Sihuincha *et al.*, 2005). Ohashi and others also demonstrated the occurrence of adult mortality and reduced survival when mosquitoes are contaminated with high doses of PPF (Ohashi *et al.*, 2012).

The horizontal transfer of PPF is mediated through 1) picking up the insecticides by insects (contamination), 2) retention of the picked-up particles / dosage and 3) successful delivery of the dosage either to the breeding habitats or an individual mosquito (dissemination). The autodissemination approach with PPF is environmentally benign (accurate targeting of potential habitats) and economically sound (labour and product cost saving) than conventional larviciding approaches which directly target the breeding environment and are expensive and less accurate in application (Killeen *et al.*, 2006; Vanek *et al.*, 2006; Chaki *et al.*, 2009; Gaugler *et al.*, 2011). Furthermore, it is has been demonstrated mathematically that larviciding with non-repellent larvicide, which does not prevent mosquitoes to utilized the treated/contaminated aquatic habitats, can result to significant reduction of mosquito density up to 96% with 80% treatment / removal of the productive habitats (Smith *et al.*, 2013). Similarly, the deployment of autodisssemination approach, which solely relies on adult mosquito' egg laying behaviour to disseminate non-repellent larvicides such as PPF to their aquatic habitats, offers high possibilities of covering most productive aquatic habitats especially when the habitats are limited.

1.3 Insect Development and Reproduction Hormones

Development and reproduction are regulated by juvenile and ecdysone hormones in all insects, where ecdysone control insect moulting and transformation (metamorphosis), and juvenile hormone (JH) regulates larval growth and inhibit metamorphosis (Wyatt & Davey, 1996; Dhadialla *et al.*, 1998; Riddiford, 2008). The coordination of insect development is maintained under a balanced ratio of these hormones. Juvenile hormone has been found to be most common in several insect species in a form of JHIII (Cusson & Palli, 2000) and is responsible for controlling metamorphosis, reproduction and insect behaviour (Wyatt & Davey, 1996; Hiruma, 2003; Riddiford *et al.*, 2003). Juvenile

hormone is produced by the *corpus allatum* region of the insect's brain where its production and removal from an insect is under neurohormonal (Wigglesworth, 1934., 1936) and enzymatic control respectively (Feng *et al.*, 1999). In the adult insect, JH is also responsible for regulating reproductive processes (Wyatt & Davey, 1996).

1.3.1 Insect Growth Regulators

Understanding of hormones mode of action on development and reproduction at the molecular level has led to successful development of synthetic compounds or analogues, termed insect's growth regulators (IGRs), which operate as mimics to deregulate the normal functioning of the insect's natural developmental hormones (Dhadialla *et al.*, 1998). This has led to the exploitation of IGRs to control many insect pests of medical and agricultural importance (Wilson, 2004).

In the past, IGRs were labelled as bio-rational and third-generation insecticides due to their extreme minimal human risk, selective action, low likelihood for resistance development and environmental safety compared to existing conventional adult insecticides such as DDT and organophosphates (Mulla, 1991). However, recent findings indicate the likelihood for resistance development in the targeted insects (Schaefer & Mulligan III, 1991; Crowder *et al.*, 2008; Shah *et al.*, 2015). While the term IGR encompasses all hormone mimics capable of disrupting normal functioning of an insect's endocrine hormones, this review limits itself to only those IGRs that interfere with juvenile hormone (JH) activity.

The intrinsic role of JH and its potential to inhibit insect development was first described in the blood sucking bug, *Rhodnius prolixus* (Wigglesworth, 1934). In light of this finding and after several decades of concerted efforts in synthesis, the first juvenile hormone analogues (JHA) were suggested as safe agents for controlling insect pests and disease vectors (Williams, 1967). Synthetic JHAs can either be classified as *terpenoids* such as kinoprene and methoprene (Slama *et al.*, 1974) or *phenoxy* e.g. pyriproxyfen and fenoxycarb (Dhadialla *et al.*, 2005).

Based on mode of action, IGRs can be classified as: (Harris & Waindle, 1980).

1) Hormonal inhibitors (e.g. Pyriproxyfen, methoprene and fenoxycarb), encompassing JHAs and ecdysone inhibitors that interfere with normal functions of JH and ecdysone hormone respectively.

2) Enzymatic / Chitin inhibitors (e.g. Diflubenzuron) - inhibit enzymatic activities during cuticle formation in insect larvae as well as disrupting chitin synthesis which is required for exoskeleton formation in pharate adults.

3) Anti-Juvenile hormones which work by blocking JH production, and in turn result in malfunctioning adult insects and death from premature moulting

1.3.2 Mechanism of action of Juvenile Hormone Analogues

While the mode of action of JHAs is not fully understood (Charles *et al.*, 2011), it is believed that JHAs are involved in regulating the activation and expression of genes responsible for the release of the insect's developmental hormones, most notably juvenile and ecdysone hormones. Physiologically, larval growth requires the presence of juvenile hormone, after when the endogenous JH titre must drop below a threshold level

for larval-pupal transformation to occur, an event that is controlled by ecdysone hormone (Wilson, 2004). Hence, by treating the immature mosquito stage with JH exactly when low levels are critical, the ecdysone-mediated metamorphic transformation from pupa to adult is severely disrupted resulting in emergence inhibition and eventual mortality of pupae / pharate adults (Wilson, 2004).

At the molecular level, by selectively binding to the insect DNA, JHAs interact with many different proteins required for signal transduction and transcription regulation (Wheeler & Nijhout, 2003; Zhang *et al.*, 2011). Following absorption by contaminated insects, JHAs binds to putative transcriptional receptor proteins such as *methoprene-tolerant* (*MET*) (Ashok *et al.*, 1998) and *Ultraspiracle* (USP) (Riddiford *et al.*, 2001; Iwema *et al.*, 2007) and form a JHA–transcription protein receptor complex that either modifies normal gene expression or partially interferes with primary ecdysone-regulated gene products such as *broad complex (BR-C)* (Wilson, 2004) Thus, JHAs prevent pupal-adult transition (Kiss *et al.*, 1988; Konopova & Jindra, 2008) by preventing normal developmental physiology (Restifo & Wilson, 1998; Wilson *et al.*, 2006) via interference with certain essential genes that regulate normal developmental processes (Dunne *et al.*, 2002; Sempere *et al.*, 2003; Wilson, 2004). Ultimately, treated pharate adults fail to emerge and die.

1.4 Pyriproxyfen for control of mosquito vectors and other pests

Pyriproxyfen has been used against various insect pests of agricultural and medical importance including whiteflies (Hemiptera: Aleyrodidae), fruit flies (Diptera: Drosophilidae), tsetse flies (Diptera: Glossinidae), fleas (Pulicidae: Siphonaptera), midges (Diptera: Chironomidae), houseflies (Diptera: Muscidae), cockroaches

(Blattodea: Blattidae) and other insects (Meola *et al.*, 2001; Saltzmann *et al.*, 2006; Tassou & Schulz, 2009; Riddiford *et al.*, 2010; Geden & Devine, 2012; Moshitzky & Morin, 2014).

In agriculture and horticulture, PPF has been the most widely used insect growth regulator for controlling Lepidoptera and Hemiptera following the development of resistance to widely used organophosphate, carbamate and pyrethroids pesticides (Grafton-Cardwell *et al.*, 2005; Sullivan & Goh, 2008). PPF has been applied extensively in controlling pests such as California red scale *(Aonidiella aurantii)* on citrus crops, apple leafminers *(Phyllonorycter* species*)*, silverleaf whitefly *(Bemisia argentifolii)* on cotton, pear psylla *(Cacopsylla pyricola)* and pests such as the San Jose scale *(Diaspidotus perniciosus)* on Stone and pome fruits and nuts, mainly in North America (Grafton-Cardwell *et al.*, 2005; Sullivan & Goh, 2008). However, in routine practice, PPF must be managed like any other insecticide in order to reduce its impact on non-target insects or natural enemies of pests (Ellsworth & Martinez-Carrillo, 2001) and to minimize the risks of resistance developing in treated populations, as already reported in whiteflies on cotton (Crowder et al 2008).

In public health, the development of PPF for mosquito control dates back over three decades, when laboratory experiments demonstrated full susceptibility of *Culex*, *Anopheles* and *Aedes* mosquitoes to PPF (Hatakoshi *et al.*, 1987). Afterwards, evaluation of PPF efficacy in the field against immature stages of *Anopheles* (Okazawa *et al.*, 1991; Kawada *et al.*, 1993;Yapabandara & Curtis, 2004), *Aedes* (Lee, 2001; Sihuincha *et al.*, 2005; Vythilingam *et al.*, 2005), and *Culex* mosquitoes (Kamimura and Arakawa., 1991; Chavasse *et al.*, 1995; Lee, 2002) demonstrated significant adult emergence inhibition when PPF was applied directly to the breeding habitats. PPF persistence in a granular

form has been demonstrated to be very high in water with residual activities of up to 6 months recorded under field conditions (Kawada *et al.*, 1988; Nayar *et al.*, 2002;Yapabandara & Curtis, 2002; Sulaiman *et al.*, 2004; Seng *et al.*, 2006), reducing the need for frequent reapplication and making field interventions with this product cost-effective, especially during dry-season when new habitats cannot be created and existing ones cannot be diluted. In addition, PPF-based interventions are environmental friendly owing to the lower doses of PPF (<1 ppb) required to deliver lethal effect against immature mosquitoes in the breeding habitats (Sihuincha *et al.*, 2005).

To overcome environmental degradation and short persistence of PPF when deployed in the field (Mian & Mulla, 1982), various formulations classified into solubilized emulsifiable concentrate (SEC), wettable powder (WP) and granular (G) have been proposed and tested for their effectiveness against mosquitoes. Granular formulations have been more effective under laboratory and field conditions than WP and SEC formulations (Kawada *et al.*, 1988). To date, PPF has been made available through various registered commercial products such as Sumilarv®, Admiral® and Knack® (Dhadialla *et al.*, 2005).

PPF is a very potent compound with high levels of activity and specificity, compared to other classes of chemical insecticides recommended for mosquito control (Mulla *et al.*, 1989). It is a safe compound with minimal level toxicity to mammals and a high margin of safety to non-target organisms in PPF-contaminated habitats (Schaefer *et al.*, 1988; Mulla *et al.*, 1989). WHO has approved PPF for public health uses, including mosquito control, at a dose of 50 - 100 ppb, below the recommended limit of 300 ppb in human drinking water (WHO, 2004b; Sihuincha *et al.*, 2005).

1.4.1 Effect of pyriproxyfen on non-target organisms

Mosquito aquatic habitats are also occupied by a wide range of organisms including nematodes, other insects (*e.g.* dragonflies, damselflies, beetles, copepods, cladocerans), fish and countless other micro-organisms all of which contribute to the balance of this aquatic ecosystem, including roles as mosquito prey or predator (Schaefer *et al*., 1988). Clearly, the impact of PPF on non-target organisms depends on the concentration and accumulation of PPF applied as well as the species sensitivity to PPF itself (Schaefer *et al.*, 1988; Wang *et al*., 2005). Laboratory and field experiments conducted using an effective PPF dosage of 0.01 ppm for mosquito control reported no adverse effect against a wide range of non-target mosquito predators (Schaefer *et al*., 1988).

However, some minimal impact has been documented in crustaceans, particularly cladocerans and copepods, where PPF mimics the crustacean juvenile hormone methyl farnesoate (Reddy *et al.*, 2004; Nagaraju & Borst, 2008). However, the impact is usually short-lived and does not compromise the crustacean's survival (Schaefer & Miura, 1990) although this might also be due to the low persistence of PPF under sunlight and its high adsorption to organic material (Sullivan & Goh, 2008).

A separate laboratory study conducted by (Wang *et al.*, 2005) assessing the impact of PPF at high concentration of 0.1 ppm on two species of crustaceans copepods (*Mesocyclops pehpeiensis* and *Megacyclops viridis*), showed significant mortality and reduced reproductive success on *Megacyclops viridis* but not on the *Mesocyclops pehpeiensis.* Although, the adverse effects were observed on the immature stages of *M. viridis,* it was proposed that the improved reproductive fitness, development and longevity, and predation ability in the surviving individuals was likely to sustain the population (Schaefer & Miura, 1990). In addition, it has been also demonstrated that rapid accumulation of PPF via repeated application at short intervals can cause

significant mortalities in non-target organisms in the treated mosquito aquatic habitats (Wang *et al*., 2005). Regarding PPF that can be naturally transferred by contaminated mosquitoes, it seems likely that the extremely low quantities transferred would be unlikely to raise significantly the concentration of PPF in the aquatic habitats to levels approaching those that might impact on non-target organisms.

1.4.2 Potential for pyriproxyfen to impact on malaria transmission

The impact of PPF on malaria transmission would be achieved through reduction of mosquito abundance, a key determinant of vectorial capacity and malaria transmission (Macdonald, 1957). In the contaminated breeding habitats, PPF acts on 4th larva and especially on non-feeding pupal stages by preventing emergence into adults until pupae die without developing further. Although PPF neither kills nor prevents oviposition by adult mosquitoes transmitting malaria it might impact on female mosquito reproductive and feeding success at higher doses (Itoh *et al.*, 1994; Sihuincha *et al.*, 2005). Adult feeding inhibition has been observed in *An. balabacensis* (Iwanaga & Kanda, 1988), and sterility induction has been documented in *Ae. aegypti* (Sihuincha *et al.*, 2005), *An. gambiae* s.s. (Ohashi *et al.*, 2012) and *An. arabiensis* (Harris *et al.*, 2013) following their exposure to PPF.

Most of the existing empirical evidence on mosquito control using PPF has largely come from laboratory and small-scale field trials. The potential for using adult mosquitoes for PPF dissemination has been established in controlled environments (Itoh *et al.*, 1994; Chism & Apperson, 2003; Sihuincha *et al.*, 2005; Gaugler *et al.*, 2011). Unlike the traditional chemical insecticides which quickly kill the adult after exposure, PPF does

not kill adult mosquito (Kawada *et al.*, 1993; Devine *et al.*, 2009), but it slightly interfere with adult's longevity (Ohashi *et al.*, 2012).

Field trials in Peru demonstrated that the primary vector of dengue, *Ae. aegypti*, can be used to disseminate PPF into their own breeding habitats (Devine *et al.*, 2009). In the study, a 4% coverage of the mosquito resting sites with PPF resulted in 98% larval mortality and 42-98% adult emergence reduction. For this process to be successful wild mosquitoes had to pick up the larvicide from contamination surfaces and retain it until reaching a breeding habitat, where during the oviposition process, they contaminated the water. In addition, the biology and ecology of the mosquito involved contributed to the success of these trials. *Ae. aegypti* has pulvilli on the feet and a relatively hairy body, exhibits skip oviposition (*i.e.* small batch of eggs are distributed in multiples water bodies) and oviposits in small, multiple and mostly man-made containers, partly explaining the success of autodissemination approach with this species (refs).

Most African anophelines have less hairy body, lack pulvilli and are not efficient skip ovipositor when compared to *Aedes sp.* mosquitoes (Chadee & Corbet, 1991; Chen *et al.*, 2006; Herrera-Varela *et al.*, 2014; Okal *et al.*, 2015), factors which might limit successful auto-transfer of PPF to the targeted destinations. It can be hypothesized that purpose-built or contamination surfaces treated with PPF, which are highly preferred for visiting and resting by host-seeking and resting mosquitoes, could be developed by baiting the surfaces with mosquito attractants/cues such as sugar, or honey so that mosquitoes will visit the surfaces and stay longer while feeding (Gary & Foster, 2004 ; Muller & Schlein, 2006). Furthermore, hosts such as cattle could be used as the PPF contamination or sterilization strategy, exploiting their innate attractiveness to zoophagic and exophilic anopheline mosquitoes. In addition, creating suitable resting sites such as

clay pots made dark inside could retain mosquitoes over a longer period of time and amplify PPF uptake. Most importantly, designing suitable PPF formulations which can easily be picked up by mosquitoes, retained and successfully released at the aquatic habitats is fundamental for successful field implementation of autodissemination approach.

Malaria vectors such as *An. gambiae* s.s., *An. arabiensis* and *An. funestus* utilize diverse and cryptic aquatic habitats within rural settings. In these settings, where numerous aquatic habitats of different sizes are scattered, the PPF-autodissemination strategy might be beneficial and an optimal solution in identifying and targeting mosquito breeding habitats, which cannot be effectively located with human efforts. However, acquisition of blood meal is a prerequisite prior to the mosquito's visit and transfer of PPF to the breeding habitats.

The success of the PPF autodissemination strategy is most likely to be influenced by malaria mosquito host-seeking and resting behaviours which have been well documented elsewhere (Gillies & de Meillon, 1968; Boreham & Port, 1982). While malaria vectors can come into contact with PPF-treated surfaces either before or after blood feeding (Aiku *et al.*, 2006; Harris *et al.*, 2013), targeting them while resting after blood meal would not only ensure longer PPF-mosquito contact times during blood digestion but also eventual dissemination of picked-up PPF to the habitats during egg-laying events. Importantly, suitable contamination sites that will be visited by a large proportion of the adult female mosquito population must be identified. Previous studies have suggested that bed nets, curtains, and interior house walls (Lengeler, 2004; Pluess *et al.*, 2010), cattle (Rowland *et al.*, 2001), odour baited stations (OBS) (Okumu *et al.*, 2010c), and clay pots (Odiere *et al.*, 2007; Farenhorst *et al.*, 2008) could be used to

expose mosquitoes to insecticides and potentially could be used to transfer PPF to mosquitoes.

The deployment of a PPF-autodissemination strategy is envisaged to interrupt malaria transmission through reducing adult density, and therefore biting, by rendering the breeding habitats unproductive (*i.e.* larval source reduction). The integration of the PPF-autodissemination technique in combination with other malaria prevention measures such as LLINs and IRS could simultaneously provide community-wide benefits and personal protection through adult mortality and reduced survival of contaminated mosquito populations (Ohashi *et al.*, 2012).

Furthermore, at low adult mosquito densities, where the capacity for population recovery is high and the existing LLINs and IRS interventions are less effective in regulating it (Russell *et al.*, 2011a), the deployment of PPF-autodissemination offers the possibility to limit the vector population's potential for recovery. This could be achieved through robust density-dependent regulation of larval/pupal stages by rendering larval habitats unproductive (Killeen *et al.*, 2002). Finally, at high levels of LLINs coverage (exceeding 80%) the use of the PPF- autodissemination strategy could be expected to be more beneficial and cost-effective than extensive IRS because of its potential to target mosquito juveniles at aquatic habitats (Okumu & Moore, 2011; White *et al.*, 2012).

1.4.3 Evolution of resistance to PPF

As with all insecticides and control measures there has been concern over the possible evolution of resistance to pyriproxyfen in mosquitoes. While the impetus of JHA

development was motivated by the assumption that they would be unable to develop resistance (Williams, 1967), studies have documented resistance to methoprene in insects such as beetles, houseflies (Cerf & Georghiou, 1972; Dyte, 1972) and in *Ae. nigromaculis* mosquitoes (Cornel *et al.*, 2002). To date there is no evidence on PPF resistance development in mosquitoes (Invest & Lucas, 2008). Moreover, many studies have demonstrated lack of cross resistance of PPF with other classes of conventional insecticides, but also mosquitoes resistant to other chemical insecticides show susceptibility to PPF (Hemingway & Bonning, 1988; Schaefer & Mulligan III, 1991; Kawada *et al.*, 1993).

Following recent evidence on high level of PPF resistance demonstrated by houseflies, *Musca domestica* (Diptera: Muscidae) (Shah *et al.*, 2015) and whiteflies, *Bemisia tabaci* (Hemiptera: Aleyrodidae) (Crowder *et al.*, 2008), the possibility of targeted malaria mosquitoes to develop resistance against PPF should be considered as a potential future threat to this intervention. Therefore, despite the potential of integrating PPF with the existing tools for malaria vectors control (Nauen, 2007), PPF must be used as part of integrated resistance and pest management strategies (Schaefer & Mulligan III, 1991; Geden & Devine, 2012).

1.4.4 Dry season implementation of autodissemination strategy

The dry season, which is characterized by few, semi-permanent and permanent mosquito breeding habitats compared to rainy season (Charlwood *et al.*, 2000; Killeen *et al.*, 2002; Fillinger *et al.*, 2004), provides an optimal timing for future field implementation of the autodissemination strategy in interrupting malaria transmission. Although this season would be associated with low mosquito abundance, it is envisaged that the effective

transfer of lethal PPF dosage to the breeding habitats would be derived from a PPF accumulation effect derived from multiple visits over the course of a mosquito's consecutive and overlapping gonotrophic cycles (Devine & Killeen, 2010).

Deployment of the autodissemination strategy in the rainy season is likely to be impractical due to the dilution of PPF concentration in the breeding habitats by continuous water flooding and flushing (Devine & Killeen, 2010). Moreover, during the rainy season, breeding habitats are abundant and expansive, and this might pose challenges in attaining optimum concentration required to prevent adult emergence and cause lethal effect against immature mosquitoes regardless of high mosquito densities (Fillinger *et al.*, 2004; Koenraadt *et al.*, 2004).

1.5 Rationale of the study

Larval source management (LSM) with conventional larviciding can be an additional strategy to support adult vector control in Africa (WHO, 2013b; Tusting *et al.*, 2013). The transfer of PPF by mosquitoes (autodissemination) as previously demonstrated with container breeding *Aedes sp.* mosquitoes (Itoh *et al.*, 1994; Devine *et al.*, 2009; Caputo *et al.*, 2012; Suman *et al.*, 2014) offers a possible method to overcome these limitations.

For the first time, exploiting malaria mosquitoes to kill their own offspring presents a unique opportunity for sustainable malaria vector control. An additional benefit of this strategy is the potential to also impact mosquito vectors of other diseases and nuisance mosquitoes coexisting in the same habitats.

This study aimed to investigate and identify effective mechanisms for wild mosquitoes to pick up PPF and successfully transfer sufficient levels to contaminate targeted breeding habitats, thus preventing further adult mosquito emergence from those sites.

1.5.1 Project goal and specific objectives

The overall goal of the study was to evaluate the potential for exploiting adult *An. arabiensis* behaviour and ecology to disseminate PPF into mosquito breeding habitats for population control, and to assess its effectiveness in sterilizing adult malaria vectors, *An. arabiensis* under controlled semi-field settings.

This goal was approached under the following specific objectives:

1. Assess malaria vector dynamics, transmission intensity and associated risk factors under field settings
2. Design and evaluate potential tools for delivering PPF to *An. arabiensis* mosquitoes under semi-field settings
3. Evaluate the potential of pyriproxyfen-treated cattle shelters for sterilizing *An. arabiensis* under semi-field settings

1.5.2 Thesis outline

Chapter 2 provides a description of how entry behaviour and indoor densities of *An. arabiensis* and *An. funestus* are associated with different house characteristics in villages

in Kilombero Valley, the location where the proposed PPF-based vector control strategies eventually would be deployed and evaluated.

Chapter 3 documents the recent shift in malaria vectors distribution in Kilombero Valley, with significant increases seen in the abundance and vectorial role of *An. arabiensis* and *An. funestus* and coincident reduction of *An. gambiae* s.s. to negligible levels.

Chapter 4 aims to provide a proof of principle for autodissemination of PPF to breeding habitats by malaria vectors, in a semi-field system.

Chapter 5 examines the sterilization rates in malaria vectors achieved by applying PPF on walls and ceiling of cattle shelters.

Chapter 6 discusses and synthesises the research findings in terms of their implications for future PPF-based vector control strategies aimed at interrupting malaria transmission in the context of IVM, insecticide resistance and outdoor transmission.

CHAPTER 2

A NEED FOR BETTER HOUSING TO FURTHER REDUCE INDOOR MALARIA TRANSMISSION IN AREAS WITH HIGH BED NET COVERAGE

The results reported in this chapter have been published in a slightly different form as Lwetoijera *et al.* *Parasites & Vectors 2013, Volume 6, Issue 57*

2.1 Abstract

Introduction: The suppression of indoor malaria transmission requires additional interventions that complement the use of insecticide treated nets (LLINs) and indoor residual spraying (IRS). Previous studies have examined the impact of house structure on malaria transmission in areas of low transmission. This study was conducted in a high transmission setting and presents further evidence about the association between specific house characteristics and the abundance of endophilic malaria vectors.

Methodology: Mosquitoes were sampled using CDC light traps from 72 randomly selected houses in two villages, at monthly intervals from 2008 to 2011 in rural southern Tanzania. Negative binomial regression with robust error estimates and with adjustment for clustering effects within houses was used to analyse the association of house characteristics, number of occupants and ITN usage with mean catches of malaria vectors (*An.gambiae* s.l. and *An. funestus*). Furthermore, analysis of linear trend between indoor mosquito densities and covariates was performed using correlation analysis.

Results: A total of 36,490 female *An. gambiae* s.l. were collected in Namwawala village and 21,266 in Idete village. When both villages were combined, less number of mosquitoes were collected in large houses (RR = 0.66 (0.45 - 0.97)), with many (>4) rooms (RR = 0.50 (0.27 - 0.95)), many (>5) windows (RR= 0.44 (0.28 - 0.68)), intact net over windows (RR = 0.34 (0.17 - 0.69)), plastered walls (RR = 0.62 (0.43 - 0.89), metal roofing (RR = 0.48 (0.34 - 0.68)) relative to their reference category for mean catches for *An. gambiae* s.l., ($p < 0.05$). However, more mosquitoes were collected inside the house with eave gaps (RR= 3.3 (2.39 - 4.56, $p < 0.05$)) and many (>3) occupants (RR= 1.91 (1.35 – 2.69, $p < 0.05$)). The presence of treated bednets had no impact in reducing number of mosquitoes indoor (RR= 0.74 (0.50 - 1.11, $p > 0.05$).

Furthermore, a total, 2,268 *An. funestus* females were collected in Namwawala and 3,398 in Idete villages. Similarly, when both villages were combined, less number of

mosquitoes were collected in houses with many rooms (RR = 0.65 (0.54 - 0.78)), doors (RR= 0.61 (0.45 - 0.82)), windows (RR= 0.84 (0.78 - 0.91)), intact net over windows (RR = 0.17 (0.08 - 0.39)), plastered walls (RR = 0.29 (0.17 - 0.51), metal roofing (RR = 0.24 (0.13 - 0.43)) relative to their reference category for mean catches for *An. funestus*, ($p < 0.05$). Houses with eave gaps had more mosquitoes (RR = 5.55 (3.25 - 9.46, $p < 0.05$)), and many occupants had no impact on *An. funestus* mean catches indoors (RR = 0.64 (0.29 - 1.42, $p > 0.05$)). The presence of treated bednets reduced the number of mosquitoes indoor (RR= 0.44 (0.23 – 0.87, $p < 0.05$).

Conclusion: Despite significant reductions in vector density and malaria transmission caused by high coverage of LLINs, high numbers of host-seeking malaria vectors are still found indoors partly due to house designs that favour mosquito entry. In addition to LLINs and IRS, significant efforts should focus on improving house design by building modern house structures with screened eaves, windows and doors to prevent mosquito entry and eliminate indoor malaria transmission.

2.2 Introduction

The *An. gambiae* and *An. funestus* complexes comprise the major and most efficient malaria vectors in sub-Saharan Africa (Sinka *et al.*, 2012). Their transmission efficiency is mediated by their behavioural adaptation to feed indoors on humans (Gillies & de Meillon, 1968). To date, insecticide treated nets (LLINs) and indoor residual spraying (IRS) are the mainstay for controlling malaria vectors and associated malaria transmission (Pluess *et al.*, 2010; WHO, 2014). Despite the huge success of these interventions, residual malaria transmission cannot be addressed by LLINs and IRS alone, even at very high coverage (Griffin *et al.*, 2010; Kiware *et al.*, 2012b). Moreover,

their sustainability is threatened by a widespread increase in insecticide resistance in the target species (Bayoh *et al.*, 2010; Ranson *et al.*, 2011). In Senegal, the initial successes of an ITN distribution program were partially confounded by an increase in insecticide resistance and a consequent rebound in malaria incidence (Trape *et al.*, 2011) and in northern Tanzania the predominant vector *An. arabiensis* has been reported to display avoidance behaviour against LLINs (Kitau *et al.*, 2012). The integration of existing interventions with environmental management and socio-economic development through house improvement and screening offers a non-insecticidal, complementary approach to increasing protection against mosquito bites (Baragatti *et al.*, 2009; Graves *et al.*, 2009). These additional interventions could enhance the interruption of malaria transmission through the reduction and prevention of human-vector contacts inside human dwellings. It has long been established that the transmission of many vector-borne diseases is facilitated by house designs that favour mosquito entry (Schofield & White, 1984; Webb, 1985; (Lindsay *et al.*, 2002 ; Kumar *et al.*, 2004) and that housing improvements and screening have made substantial contributions to the control and elimination of malaria vectors in many richer countries (Lindsay *et al.*, 2002). Therefore, understanding house risk factors that are associated with reduction of indoor mosquito bites and disease transmission in different settings is crucial for disease vector control and elimination.

Several studies have identified and documented various house characteristics associated with mosquito entry. Presence of eave gaps, lack of a ceiling and lack of screening over windows and doors proved to be the major contributors to mosquito entry (Lindsay & Snow, 1988; Lindsay *et al.*, 2002; Lindsay *et al.*, 2003; Kirby *et al.*, 2008; Kirby *et al.*, 2009). Furthermore, it has been shown in a randomised control trial that blocking all potential house entry points for mosquitoes substantially reduces vector densities and entomological inoculation rates (EIR) (Kirby *et al.*, 2009). Other than protection against

malaria mosquitoes, the use of screened houses offers protection against nuisance bites, other mosquito borne diseases (Kumar *et al.*, 2004; Ogoma *et al.*, 2010) and it associated anaemia reduction in children (Kirby *et al.*, 2009).

While this strategy is deemed efficient in reducing indoor mosquito biting, malaria morbidity and anaemia in children in low malaria transmission settings (Lindsay *et al.*, 2002; Kirby *et al.*, 2009), its impact was yet to be examined in areas experiencing moderate to high malaria transmission and with high LLINs coverage such as the Kilombero valley in south-eastern Tanzania. However, a most recent study conducted in western and south-eastern Uganda in areas with moderate to high malaria transmission has demonstrated lack of an association between house screening/improvement and indoor vector densities where malaria vectors are less endophagic (Wanzirah *et al.*, 2015).

A recent study in northern Tanzania has shown a strong association between houses, individual and behavioural risk factors and malaria transmission that were epidemiologically assessed by testing the presence of malaria parasites in children during household survey (Winskill *et al.*, 2012). However, the authors argued that it was important to complement these findings with entomological data in order to have a fuller understanding of malaria transmission inside human dwellings (Winskill *et al.*, 2012). This study therefore assessed the impact of house characteristics on indoor vector abundance in communities with a high coverage of ITNs.

2.3 Methods and materials

Study site

The study was carried out in Namwawala and Idete villages located in the flood plain of the Kilombero River (8.1° S and 36.6° E) in south-eastern Tanzania (Figure 2.3.1). The epidemiology of malaria transmission and associated disease vector species composition within these villages has been well studied and documented over the past years (Killeen *et al.*, 2007; Russell *et al.*, 2010). Both villages experience an annual rainy season (Dec – May) and the main crops are rice and maize. However, both villages have a relatively similar number of houses (Namwawala = 804 and Idete = 844), Namwawala has a high number of households (3909) compared to Idete (2932). Houses in Idete are built on relatively elevated areas compared to Namwawala. Approximately 92% of community members sleep under a treated net (Russell *et al.*, 2010).

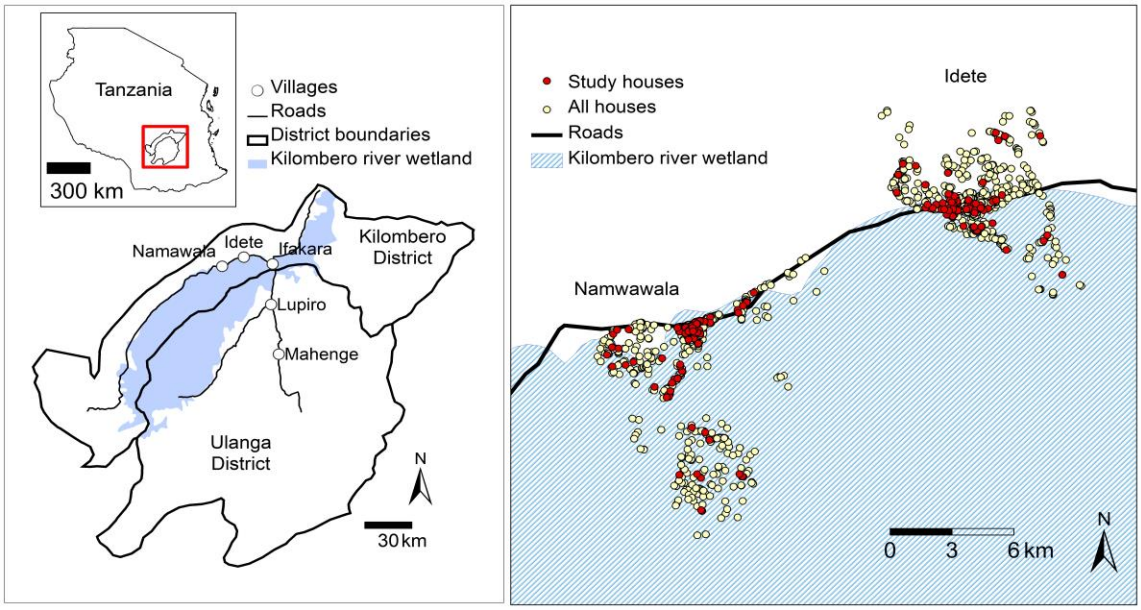

Figure 2.3.1: Kilombero and Ulanga districts (8.1°S and 36.6°E) in Tanzania showing Namwawala and Idete villages (left) and spatial distribution of sentinel houses used for mosquito sampling (right) (Russell *et al.*, 2013).

Study design

This longitudinal study was conducted over four years. A total of 72 houses from each village were randomly selected from Ifakara Health Institute (IHI) Demographic Surveillance System household list (Schellenberg *et al.*, 2001). All selected houses were geo-located using a handheld GPS (eTrex, Vista, Garmin, USA). Each of the 72 houses was sampled monthly (i.e. 6 houses per day, 4 days per week and 3 weeks per month). This longitudinal study was carried out between January 2008 and December 2011, during which mosquitoes were sampled every month during 2008 and 2011, for 6 months of the wet/rain season (January to June) in 2009 and for 6 months of the dry season (July to December) in 2010. This totals 36 months of sampling.

House risk factors

Structured questionnaires were used to record ownership, number and status of bed nets (either treated or untreated) including the one LLINs provided by the research team in this study, and the number of house occupants. The house characteristics which were recorded include house size, number of sleeping rooms, presence and size of eave gaps, number of windows, presence of window screening, number of doors, presence of ceiling, wall and roof types. These factors were correlated with mosquito densities indoors (an indicator of human biting rate) over time in both villages, at house level and were monitored yearly to accommodate any significant changes. Representative house types, which are commonly found in the study area, are shown in Figure 2.3.2

Figure 2.3.2: Representative house types commonly available in Idete and Namwawala villages. A traditional house (A) and a modern house (B)

Mosquito sampling and processing

Mosquitoes were sampled using miniature Centre for Disease Control (CDC) light traps (model 512, USA). One CDC light trap was set per house, placed 1 – 1.5m above the ground close to the foot of a bed with an occupant sleeping under a treated net, and left to run for 12 h (7pm-7am). For every participating house, one LLINs (Olyset, A to Z Textiles Mills, Arusha, Tanzania) was provided to protect the bed occupant where the CDC trap was set. Each morning of a sampling night, mosquitoes were collected and killed using chloroform and were morphologically identified in the field. Furthermore, female mosquitoes were classified as being unfed, partially fed, and fully fed or gravid (M. Gillies & de Meillon, 1968). Sub-samples of five mosquitoes from each trap were individually stored inside a tube containing cotton wad and silica gel beneath. Polymerase chain reaction (PCR) was used for identification of *Anopheles gambiae* (Scott *et al.*, 1993) and *An. funestus* Giles (Koekemoer *et al.*, 2002) complexes, whereas an enzyme-linked immune-sorbent assay (ELISA) was used to determine sporozoite

infection in malaria vectors (Burkot *et al.*, 1984). Unprocessed mosquito samples were stored on silica gel at room temperature.

Data analysis

To assess the impact of individual house factors on the mean catches of *An. gambiae* s.l. and *An. funestus* for both villages, data analyses were performed using negative binomial regression with robust error estimates and with adjustment for clustering effects within houses, in STATA 13 software package (StataCorp 2013. Effect sizes for each house factor are shown as incidence rate ratios (IRRs) with their 95% confidence intervals. Effect sizes significance at the conventional 5% level (or higher) are shown in bold type; effect sizes borderline significant (*i.e.* at the 10% level) are underlined. For each species of a mosquito, the two villages were analysed separately and then combined; for the combined village analyses, effect sizes were covariates adjusted for differences between villages.

We categorized the house factors as follows: Eave gap: present or absent, eave gap size (small: < 9 cm, medium: 9 - 15 cm, large > 15cm), roof type: grass or metal roofs, wall type: mud or cement, number of occupants: up to three or more than three, windows: up to three or more than three, netting over window: intact, present but damaged or absent, doors: one or more than one, rooms: one or more than one, house size: small or large (small house considered to be the one with 1 room and/or 1 door and less than 37.4 m^3), bed nets: treated or untreated. All houses had nets, and they were considered treated if the number of treated nets divided by the total number of nets in the house was greater than 0.5; otherwise they were classed as untreated.

In addition, a correlation analysis (linear trend) was performed for covariates that were either continuous or ordinal and reduced to categories. The generated RR values correlated the proportional change in mosquito numbers for a unit change in the covariate. The percentage relative effect for increasing risk was calculated as (RR – 1) × 100, while for decreasing risk was (1-RR) ×100.

Ethical clearance and protection of human participants

The study approval was granted by the Ifakara Health Institute Institutional Review Board (IHRDC/IRB/No.A-32) and the National Institute of Medical Research (NIMR/HQ/R.8a/Vol. IX/764). The benefits and possible risks associated with the study were explained to all house occupants before commencement. After consenting, the head of the house was asked to sign two copies of the informed consent forms, of which, one remained with the head of the house and the other copy was kept by the study investigator.

2.4 Results

Mosquito collections

A total of 36490 female *An. gambiae s.l.,* were collected in Namwawala village compared to 21266 from Idete village. Of these, approximately 98% were non-blood fed, 1.7% were blood fed and the remaining 0.3% were gravid. Namwawala had fewer female *An. funestus* 2268 than Idete village 3398. Although there were variations in catches, changes in vector abundance patterns between villages were similar over time. A PCR analysis of 6755 mosquitoes of the *Anopheles gambiae* complex yielded 607

(9%) *An. gambiae s.s.* and 6148 (91%) *An. arabiensis.* Furthermore, a sub-sample of 3025 *An. funestus* analysed for species identification comprised 2805 (93%) *An. funestus s.s.*, 120 (4%) *An. rivulorum*, and 100 (3%) *An. leesoni.*

House risk factors associated with* An. gambiae s.l. *indoor abundance

Table 2.4.1 shows effect size with confidence intervals on the mean catches for *An. gambiae* s.l., and the significance level of each house factor when assessed at individual or combined village levels. More mosquitoes were collected during the rainy season compared to the dry season ($p < 0.05$), with up to 99% significant reduction in mosquito density recorded between seasons in both villages, either separately or combined.

All factors, except bed nets, had a significant impact ($p < 0.05$) on the mean catches of indoor mosquitoes when both villages were combined. However, the impact of individual factors on indoor mean catches was between villages and when both villages were combined. All factors had an impact on indoor mosquito entry with exception of number of rooms and wall type in Idete village, whereas bed net status and house size did so in Namwawala village.

Houses with eave openings had 146% increase in *An. gambiae* s.l. mean catches in Idete (RR= 2.46 (1.67 - 3.55)), a 550% increase in Namwawala (RR= 6.50 (4.40 - 9.60)) and a 230% increase when both villages were combined (RR= 3.3 (2.39 - 4.56)) compared to when eaves were closed. Higher mosquito mean catches were correlated with houses with medium eave sizes (9-15cm) as seen by increases of 88% in Idete 148% in Namwawala and 120% when both were combined, ($p < 0.05$). This was not the case with houses where eave sizes were >15cm ($p > 0.05$), when compared to houses with

small eave sizes at both villages, either separately or combined. Houses with more people inside (>3) had high numbers of mosquitoes (increase of 80% in Idete; 97% in Namwawala; 91% when villages are combined) compared to houses with three or fewer people ($p < 0.05$).

Mosquito mean catches significantly reduced with increased number of doors in Namwawala village (RR = 0.58 (0.44 - 0.75), $p < 0.05$)) and both villages were combined (RR = 0.77 (0.64 - 0.91, $p < 0.05$)) but not in Idete village (RR = 0.84 (0.67 - 1.05). While houses with two doors consistently had lower number of mosquitoes at village level and when combined ($p < 0.05$), the effect size within of more than three doors was only observed in Namwawala village (RR = 0.29 (0.18 - 0.45, $p <0.05$)) but not in Idete and when villages were combined ($p > 0.05$).

There was a strong correlation between number of windows and mean mosquito catches at village level; Idete (RR = 0.90 (0.87 - 0.94, $p < 0.05$)), Namwawala (RR = 0.83 (0.71 - 0.98, $p < 0.05$)) and when combined (RR = 0.89 (0.85 - 0.94, $p < 0.05$)). Although, houses with three to four windows had no significant difference in terms of mosquito mean catches, only houses with five or more windows had significantly lower number of mosquitoes mean catches at village levels as well as when combined ($p < 0.05$).

Similarly, houses with many rooms were correlated with low numbers of mosquito collections in Namwawala village and when both villages were combined ($p < 0.05$) but not in Idete village ($p < 0.05$). Compared to a window with no netting, a house with intact netting on the window had 55% lower indoor mean catches of *An. gambiae* s.l. in Idete (RR = 0.45 (0.19 - 1.08), $p > 0.1$)), 81% lower in Namwawala village (RR = 0.19

(0.13 – 0.29)), $p < 0.05$)) and 66% when both villages were combined (RR = (0.34 (0.17 - 0.69)), $p < 0.05$)). However, no decreases in mosquito catches were observed in houses with damaged netting on the windows compared to those without net ($p > 0.05$). Furthermore, houses with either cement plastered walls or metal roofing had 54% lower numbers of mosquitoes (RR = 0.48 (0.30 - 0.76)) in Idete, and 38% lower when both villages were combined (RR = 0.62 (0.43 - 0.89)), when compared to mud walls and grass/thatch roofing ($p < 0.05$). There was no difference in the number of mosquito caught between houses with cement plastered walls with metal roofing and those with mud walls and grass/thatch roofing in Namwawala village ($p > 0.05$).

The presence of bednets was associated with lower mean catches, approximately 38% significant reduction compared to those with treated bed nets in Idete village (RR = 0.62 (0.39 - 0.97), $p < 0.05$)) and 26% non-significant reduction when both villages where combined ($p > 0.05$). However, this was not the case in Namwawala village where houses with treated nets had 32% higher mosquito mean catches (RR = 1.32 (0.69 - 2.50)), than those with untreated nets ($p < 0.05$). The ownership rate of nets in Namwawala village was 89% for treated and 11% for untreated nets, whereas in Idete village it was 50% for treated and 50% for untreated nets.

Table 2.4.1 Factors associated with *Anopheles gambiae* s.l. density in Idete and Namwawala villages

An. gambiae s.l.		Idete (n = 70)			Namwawala (n = 72)			Both villages combined (n = 142)		
Factor		**N**	**Mean (95% CI)**	**RR (95% CI)**	**N**	**Mean (95% CI)**	**RR (95% CI)**	**N**	**Mean (95% CI)**	**RR (95% CI)†**
Season	rainy	70	18.6 (14.0 : 23.2)	---	72	32.8 (24.0 : 41.5)	---	142	25.7 (20.7 : 30.7)	---
	dry	70	0.3 (<0.1 : 0.6)	**0.017 (0.007 : 0.041)**	72	0.3 (0.2 : 0.3)	**0.009 (0.007 : 0.012)**	142	0.3 (0.2 : 0.5)	**0.012 (0.008 : 0.020)**
Number of rooms	1	11	9.3 (5.0 : 13.5)	---	18	16.2 (8.0 : 24.4)	---	29	13.7 (8.1 : 19.3)	---
	2	26	10.9 (6.3 : 15.5)	1.175 (0.637 : 2.167)	39	18.6 (12.3 : 25.0)	1.152 (0.632 : 2.102)	65	15.5 (11.2 : 19.7)	1.150 (0.732 : 1.806)
	3	21	9.7 (5.9 : 13.6)	1.050 (0.580 : 1.901)	8	11.0 (4.4 : 17.6)	0.679 (0.314 : 1.469)	29	10.1 (6.8 : 13.4)	0.869 (0.529 : 1.427)
	4+	12	6.1 (2.1 : 10.2)	0.662 (0.300 : 1.461)	7	5.4 (1.3 : 9.6)	**0.335 (0.135 : 0.828)**	19	5.9 (2.9 : 8.8)	**0.502 (0.266 : 0.948)**
	linear trend	70	---	0.881 (0.748 : 1.038)	72	---	**0.680 (0.532 : 0.869)**	142	---	**0.822 (0.712 : 0.948)**
Number of doors	1	30	11.9 (7.6 : 16.2)	---	51	18.3 (12.7 : 23.9)	---	81	16.0 (12.1 : 19.8)	
	2	30	6.5 (4.3 : 8.7)	**0.545 (0.335 : 0.886)**	18	10.5 (6.4 : 14.7)	**0.575 (0.352 : 0.939)**	48	8.0 (5.9 : 10.2)	**0.551 (0.391 : 0.777)**
	3+	10	10.8 (4.5 : 17.1)	0.906 (0.462 : 1.775)	3	5.3 (3.4 : 7.1)	**0.287 (0.182 : 0.452)**	13	9.4 (4.5 : 14.4)	0.739 (0.397 : 1.374)
	linear trend	70	---	0.840 (0.674 : 1.046)	72	---	**0.579 (0.444 : 0.753)**	142	---	**0.765 (0.643 : 0.909)**
Number of windows	0-2	18	13.6 (7.4 : 19.9)	---	44	17.0 (11.2 : 22.9)	---	62	16.1 (11.5 : 20.6)	---
	3-4	26	9.3 (5.9 : 12.8)	0.684 (0.384 : 1.219)	17	20.4 (12.5 : 28.2)	1.196 (0.719 : 1.990)	43	13.7 (9.7 : 17.7)	0.898 (0.609 : 1.324)
	5+	26	6.7 (4.1 : 9.3)	**0.494 (0.275 : 0.886)**	11	4.6 (2.2 : 7.0)	**0.271 (0.146 : 0.504)**	37	6.1 (4.1 : 8.0)	**0.437 (0.280 : 0.682)**
	linear trend	70	---	**0.904 (0.865 : 0.944)**	72	---	**0.834 (0.712 : 0.977)**	142	---	**0.893 (0.851 : 0.936)**
Netting over window										
	absent	50	10.7 (7.7 : 13.7)	---	60	17.1 (12.6 : 21.7)	---	110	14.2 (11.4 : 17.1)	---
	present but damaged	16	6.5 (3.1 : 9.9)	0.605 (0.337 : 1.084)	9	11.6 (0 : 23.9)	0.679 (0.232 : 1.983)	25	8.4 (3.3 : 13.6)	0.631 (0.357 : 1.157)
	intact	4	4.9 (0.8 : 8.9)	0.452 (0.190 : 1.075)	3	3.2 (2.2 : 4.4)	**0.192 (0.127 : 0.292)**	7	4.2 (1.8 : 6.6)	**0.338 (0.166 : 0.690)**
Wall type	mud	19	15.4 (9.4 : 21.4)	---	44	17.5 (11.8 : 23.2)	---	63	16.8 (12.5 : 21.2)	---
	cement	51	7.3 (5.3 : 9.3)	**0.475 (0.297 : 0.758)**	28	13.3 (7.4 : 19.2)	0.763 (0.445 : 1.308)	79	9.5 (7.0 : 12.1)	**0.618 (0.429 : 0.890)**

† : adjusted for differences between Villages, Significant effect size of a factor at 5% are bolded, and factor effect size with borderline significant (at 10% level) are underlined

Table 2.4.1 Factors associated with *Anopheles gambiae* s.l. density in Idete and Namwawala villages **(contd.)**

An. gambiae s.l.		Idete (n = 70)			Namwawala (n = 72)			Both villages combined (n = 142)		
Factor		**N**	**Mean (95% CI)**	**RR (95% CI)**	**N**	**Mean (95% CI)**	**RR (95% CI)**	**N**	**Mean (95% CI)**	**RR (95% CI)†**
Roof type	grass	24	13.8 (9.0 : 18.7)	---	53	18.6 (13.2 : 24.0)	---	77	17.1 (13.1 : 21.1)	---
	metal	46	7.1 (5.0 : 9.3)	**0.516 (0.326 : 0.815)**	19	8.4 (4.7 : 12.1)	**0.451 (0.269 : 0.759)**	65	7.5 (5.7 : 9.4)	**0.484 (0.343 : 0.683)**
Eaves gap	absent	24	4.8 (3.6 : 6.1)	---	10	2.8 (1.9 : 3.6)	---	34	4.2 (3.3 : 5.1)	---
	present	46	11.9 (8.6 : 15.2)	**2.455 (1.699 : 3.546**)	62	18.0 (13.4 : 22.7)	**6.501 (4.403 : 9.598)**	108	15.4 (12.4 : 18.5)	**3.300 (2.387 : 4.562)**
Eaves size										
small (<9 cm)		26	6.5 (3.8 : 9.2)	---	15	9.5 (2.3 : 16.7)	---	41	7.6 (4.6 : 10.8)	---
medium (9 – 15 cm)		30	12.3 (7.8 : 16.7)	**1.879 (1.091 : 3.236)**	34	23.5 (16.3 : 30.7)	**2.479 (1.109 : 5.541)**	64	18.3 (13.8 : 22.9)	**2.196 (1.381 : 3.491)**
large (>15 cm)		14	9.0 (5.6 : 12.5)	1.387 (0.797 : 2.414)	23	9.0 (6.0 : 11.1)	0.904 (0.405 : 2.014)	37	8.7 (6.7 : 10.8)	1.086 (0.682 : 1.727)
linear trend		--	---	1.022 (0.999 : 1.046)		---	0.986 (0.956 : 1.018)		---	1.007 (0.989 : 1.026)
Number of occupants										
up to 3		15	5.7 (3.4 : 8.0)	---	34	10.4 (6.8 : 13.9)	---	49	9.1 (6.4 : 11.7)	---
more than 3		55	10.3 (7.5 : 13.1)	**1.804 (1.117 : 2.914**)	38	20.5 (13.7 : 27.3)	**1.973 (1.233 : 3.157**)	93	14.5 (11.1 : 17.8)	**1.907 (1.354 : 2.687)**
linear trend		--	---	1.016 (0.911 : 1.133)		---	**1.199 (1.084 : 1.326**)		---	**1.125 (1.043 : 1.214)**
Bed net status										
untreated		47	10.6 (7.5 : 13.7)	---	6	12.3 (5.0 : 19.5)	---	53	10.8 (8.0 : 13.6)	---
Treated		23	6.5 (4.2 : 8.9)	**0.615 (0.393 : 0.966)**	66	16.1 (11.6 : 20.6)	1.315 (0.693 : 2.496)	89	13.9 (10.3 : 17.4)	0.744 (0.498 : 1.112**)**
House size	small	11	17.2 (8.3 : 26.0)	---	32	17.7 (10.5 : 25.0)	---	43	17.6 (11.8 : 23.4)	---
	large	59	8.0 (6.0 : 10.0)	**0.466 (0.266 : 0.818)**	40	14.3 (9.5 : 19.1)	0.805 (0.479 : 1.355)	99	10.6 (8.2 : 13.0)	**0.659 (0.446 : 0.974)**

† : adjusted for differences between Villages, Significant effect size of a factor at 5% are bolded, and factor effect size with borderline significant (at 10% level) are underlined.

House risk factors associated with* An. funestus *indoor abundance

The effect sizes (with confidence intervals) of the individual house risk characteristics, number of occupants and the bed-net status with their association with the mean catches for *An. funestus* for both villages, either individually or combined, are presented in Table 2.4.2. The density of mosquitoes changed with seasons, although the changes were not consistent across villages. While there was a reduction of 48% in mosquitoes collected during the dry season compared to the rainy season, (RR= 0.52 (0.30 - 0.89, $p < 0.05$)) in Idete village, in Namwawala mosquito catches increased slightly (1.4%) but the increase was not significant (RR = 1.01 (0.71 - 1.45), $p > 0.05$)). When both villages were combined, the mean catches of mosquitoes remained unchanged across seasons ($p > 0.05$). However, when the probability of significance was adjusted to 10% from 5%, a significant decrease of 27.4% in the mean *An. funestus* catch in the dry season compared to rainy season was observed when both villages were combined, (RR = 0.73 (0.54 - 1.00), $p < 0.1$)).

The presence of eave openings in the house correlated with increased mean catches of *An. funestus* at village level, approximately 4 times in Idete (RR = 4.34 (2.26 - 8.42)**,** 12 times more in Namwawala (RR = 12.04 (6.34 - 22.87), and 6 times when both villages were combined (RR = 5.55 (3.25 - 9.46)) compared to when eave gaps were absent ($p < 0.05$). Although increases in mosquito mean catches were significantly associated with medium (RR = 2.94 (1.04 - 8.35)) and large (RR = 3.33 (1.45 - 7.63)) eave sizes compared to houses with small eaves (< 9cm) in Idete village, the changes in mean catches in Namwawala village and when both villages were combined were not significant ($p > 0.05$). Houses of larger size and higher numbers of people inside, either at village level or both villages combined, did not affect *An. funestus* mean catches in those houses when compared to small houses and houses with fewer people respectively

($p > 0.05$). Similarly, increases in number of rooms or doors in the houses were correlated with decrease in mean catches of *An. funestus* in both villages either separately or combined ($p < 0.05$). In Idete and Namwawala, houses with more than four rooms had 78.7% and 89.7% reductions in mosquito mean catches respectively compared to the houses with one room ($p < 0.05$). Although the mean catches in houses with two rooms remained unchanged compared to houses with one room ($p > 0.05$), houses with three rooms had a 63.4% reduction in the mean catches when both villages were combined ($p < 0.05$) compared to houses with one room. Similarly, houses with two and more than three doors had 62.3% and 62.7% reduction respectively compared to houses with one door ($p < 0.05$) between study villages, either separately or combined.

Increases in numbers of windows were correlated with a decrease in mean catches of *An. funestus* in both villages either separately or combined ($p < 0.05$). Houses with > 5 windows had mean catches that were significantly ($p < 0.05$) lower than houses with 0 - 2 windows in Idete (77.3%), Namwawala (90.4%) and both (83.8%). However, there was no difference of mosquito mean catches between houses with 0 – 2 windows and those with 3 – 4 windows ($p > 0.05$). Furthermore, the presence of netting over windows, whether damaged or intact, was associated with reduction in the mean catches in both villages either separately or combined ($p < 0.05$). The mean catches of *An. funestus* were lower ($p < 0.05$) in the houses with cement-plastered walls in Idete village (85.5% reduction) and with both villages combined (81% reduction) than in houses made of mud walls. However, there was no difference in the mean catches in Namwawala village. Houses with metal roofs had lower mosquito mean catches of 83.7% in Idete, 63.4% in Namwawala, and 76.5% when both villages combined, compared to grass roofs.

Although the presence of treated bed nets did not impact mosquito catches when villages were separate ($p > 0.05$), when villages were combined, a reduction of approximately 56% was recorded in the presence of treated bednets when compared to the untreated bednets ($p < 0.05$). Furthermore, there was only a borderline reduction of 50.3% in the mean catches in Idete ($p < 0.1$) but not in Namwawala village ($p > 0.1$).

Table 2.4.2 Factors associated with *Anopheles funestus* density in Idete and Namwawala villages

An. funestus		Idete (n = 70)			Namwawala (n = 72)			Both villages combined (n = 142)		
Factor		**N**	**Mean (95% CI)**	**RR (95% CI)**	**N**	**Mean (95% CI)**	**RR (95% CI)**	**N**	**Mean (95% CI)**	**RR (95% CI)†**
Season	Rainy	70	2.00 (1.16 : 2.85)	---	72	0.94 (0.53 : 1.35)	---	142	1.47 (1.00 : 1.95)	---
	Dry	70	1.04 (0.10 : 1.99)	**0.520 (0.303 : 0.890)**	72	0.96 (0.42 : 1.49)	1.014 (0.709 : 1.451)	142	1.00 (0.47 : 1.52)	0.726 (0.526 : 1.002)
Number of rooms	1	11	2.30 (0.32 : 4.29)	---	18	1.26 (0.15 : 2.36)	---	29	1.63 (0.62 : 2.64)	---
	2	26	2.21 (0.15 : 4.28)	0.960 (0.275 : 3.348)	39	1.07 (0.44 : 1.70)	0.851 (0.300 : 2.415)	65	1.53 (0.61 : 2.45)	0.887 (0.396 : 1.989)
	3	21	0.89 (0.40 : 1.38)	0.386 (0.141 : 1.053)	8	0.48 (0.00 : 1.02)	0.383 (0.095 : 1.547)	29	0.77 (0.39 : 1.15)	**0.366 (0.158 : 0.847)**
	4+	12	0.49 (0.34 : 0.64)	**0.213 (0.087 : 0.523)**	7	0.13 (0.01 : 0.25)	**0.103 (0.030 : 0.357)**	19	0.36 (0.23 : 0.49)	**0.168 (0.080 : 0.351)**
	linear trend	70	---	**0.682 (0.554 : 0.839)**	72	---	**0.552 (0.398 : 0.768)**	142	---	**0.648 (0.537 : 0.782)**
Number of doors	1	30	2.44 (0.52 : 4.37)	---	51	1.14 (0.53 : 1.75)	---	81	1.62 (0.81 : 2.43)	---
	2	30	0.78 (0.35 : 1.20)	**0.318 (0.124 : 0.816**)	18	0.53 (0.12 : 0.95)	0.468 (0.185 : 1.183)	48	0.68 (0.38 : 0.99)	**0.377 (0.194 : 0.734)**
	3+	10	0.97 (0.17 : 1.77)	0.397 (0.129 : 1.219)	3	0.24 (0.08 : 0.40)	**0.210 (0.090 : 0.493)**	13	0.79 (0.16 : 1.42)	**0.372 (0.149 : 0.927)**
	linear trend	70	---	**0.640 (0.449 : 0.912)**	72	---	**0.490 (0.287 : 0.836)**	142	---	**0.605 (0.446 : 0.821)**
Number of windows	0-2	18	1.96 (0.77 : 3.16)	---	44	1.19 (0.50 : 1.88)	---	62	1.41 (0.81 : 2.01)	---
	3-4	26	2.27 (0.18 : 4.35)	1.153 (0.390 : 3.405)	17	0.92 (0.32 : 1.53)	0.777 (0.329 : 1.838)	43	1.73 (0.45 : 3.01)	0.891 (0.447 : 1.776)
	5+	26	0.45 (0.34 : 0.56)	**0.229 (0.121 : 0.436**)	11	0.11 (0.03 : 0.20)	**0.096 (0.038 : 0.244**)	37	0.34 (0.25 : 0.44)	**0.162 (0.094 : 0.281**)
	linear trend	70	---	**0.863 (0.805 : 0.926)**	72	---	**0.747 (0.620 : 0.901)**	142	---	**0.841 (0.779 : 0.909)**
Netting over window										
	absent	50	1.93 (0.72 : 3.13)	---	60	1.11 (0.58 : 1.64)	---	110	1.48 (0.87 : 2.10)	---
	present but damaged	16	0.50 (0.30 : 0.70)	**0.259 (0.125 : 0.536)**	9	0.21 (0.12 : 0.29)	**0.185 (0.100 : 0.342)**	25	0.39 (0.25 : 0.53)	**0.228 (0.135 : 0.385)**
	intact	4	0.48 (0.16 : 0.81)	**0.250 (0.101 : 0.616)**	3	0.07 (0 : 0.16)	**0.062 (0.015 : 0.246)**	7	0.31 (0.06 : 0.55)	**0.173 (0.076 : 0.392)**
Wall type	mud	19	4.10 (1.17 : 7.03)	---	44	1.16 (0.45 : 1.87)	---	63	2.04 (0.99 : 3.10)	---
	cement	51	0.59 (0.44 : 0.75)	**0.145 (0.069 : 0.306)**	28	0.64 (0.31 : 0.97)	0.548 (0.249 : 1.204)	79	0.61 (0.46 : 0.76)	**0.290 (0.165 : 0.509)**

† : adjusted for differences between Villages, Significant effect size of a factor at 5% are bolded, and factor effect size with borderline significant (at 10% level) are underlined.

Table 2.4.2 Factors associated with *Anopheles funestus* density in Idete and Namwawala villages (contd.)

An. funestus		Idete (n = 70)			Namwawala (n = 72)			Both villages combined (n = 142)		
Factor		**N**	**Mean (95% CI)**	**RR (95% CI)**	**N**	**Mean (95% CI)**	**RR (95% CI)**	**N**	**Mean (95% CI)**	**RR (95% CI)†**
Roof type	grass	24	3.38 (1.03 : 5.73)	---	53	1.15 (0.56 : 1.74)	---	77	1.85 (0.98 : 2.71)	---
	metal	46	0.55 (0.42 : 0.69)	**0.163 (0.079 : 0.337)**	19	0.42 (0.07 : 0.77)	**0.366 (0.141 : 0.954)**	65	0.51 (0.37 : 0.65)	**0.235 (0.127 : 0.434)**
Eaves gap	absent	24	0.48 (0.37 : 0.58)	---	10	0.09 (0.05 : 0.13)	---	34	0.36 (0.26 : 0.45)	---
	present	46	2.08 (0.77 : 3.40)	**4.359 (2.258 : 8.415)**	62	1.10 (0.58 : 1.61)	**12.04 (6.339 : 22.87)**	108	1.51 (0.88 : 2.14)	**5.549 (3.254 : 9.461)**
Eaves size										
small (<9 cm)		26	0.67 (0.37 : 0.97)	---	15	0.98 (0 : 2.15)	---	41	0.78 (0.31 : 1.26)	---
medium (9 – 15 cm)		30	1.96 (0.07 : 3.85)	**2.941 (1.036 : 8.354)**	34	1.10 (0.62 : 1.58)	1.116 (0.320 : 3.893)	64	1.49 (0.59 : 2.40)	1.866 (0.788 : 4.419)
large (>15 cm)		14	2.22 (0.63 : 3.81)	**3.327 (1.450 : 7.634)**	23	0.70 (0 : 1.62)	0.714 (0.125 : 4.087)	37	1.28 (0.42 : 2.14)	1.565 (0.586 : 4.181)
linear trend		--	---	1.045 (1.016 : 1.074)		---	0.993 (0.926 : 1.065)		---	1.023 (0.992 : 1.056)
Number of occupants										
up to 3		15	3.14 (0 : 7.09)	---	34	0.96 (0.24 : 1.67)	---	49	1.58 (0.32 : 2.84)	---
more than 3		55	1.13 (0.66 : 1.61)	0.360 (0.098 : 1.324)	38	0.94 (0.38 : 1.50)	0.980 (0.384 : 2.502)	93	1.05 (0.70 : 1.41)	0.637 (0.286 : 1.416)
linear trend		--	---	0.790 (0.655 : 0.953)		---	1.027 (0.871 : 1.211)		---	0.926 (0.822 : 1.043)
Bed net status										
Untreated		47	1.78 (0.56 : 3.01)	---	6	2.37 (0 : 5.40)	---	53	1.85 (0.72 : 2.98)	---
Treated		23	0.87 (0.58 : 1.20)	0.497 (0.233 : 1.061)	66	0.81 (0.44 : 1.19)	0.344 (0.090 : 1.313)	89	0.83 (0.54 : 1.12)	**0.443 (0.226 : 0.871)**
House size	small	11	2.42 (0.62 : 4.23)	---	32	1.23 (0.33 : 2.14)	---	43	1.54 (0.72 : 2.36)	---
	large	59	1.35 (0.38 : 2.33)	0.559 (0.202 : 1.551)	40	0.73 (0.37 : 1.09)	1.231 (0.597 : 2.535)	99	1.10 (0.51 : 1.69)	0.583 (0.300 : 1.133)

† : adjusted for differences between Villages, Significant effect size of a factor at 5% are bolded, and factor effect size with borderline significant (at 10% level) are underlined.

2.5 Discussion

Despite high coverage and extensive usage of insecticide treated nets in rural communities of southern Tanzania (Russell *et al.*, 2010), partly designed to deter and divert mosquitoes from entering houses (Okumu & Moore, 2011), a high number of malaria vectors are still found indoors with an average of 22.2 (CI=16.9 – 27.5) *An. gambiae* s.l. and 1.4 (CI= 1.1 – 1.6) An. *funestus* mosquitoes per trap night per house in Namwawala. In addition, an average of 13.1 (CI=10.9 – 15.3) *An.gambiae* s.l. and 2.1 (CI=1.6 – 2.6) *An. funestus* were collected in Idete per trap night in a house.

Small houses, constituting the minority of houses in the study area, characterized by relatively low numbers of windows, doors and rooms were associated with relatively high densities of malaria vectors. The correlation matrix also indicated a strong association between small houses (with low number of rooms, doors and windows) and higher indoor mosquito entry. This increase was associated with the possibility that smaller houses are likely to concentrate more human odours as result of poor air circulation and more warm, which would attract high mosquito numbers. Conversely, houses with more sleeping rooms had a lower density of vectors because they usually have more sleeping spaces, which is likely to encourage consistent use of bed nets by sleepers (Toe *et al.*, 2009; Iwashita *et al.*, 2010). Moreover, houses with many rooms are likely to have more nets, which collectively might reduce the number of mosquitoes indoors.

Houses made of mud walls and grass roofs had an increased risk of mosquito bites indoors. Such houses create cooler, darker conditions favoured by resting mosquitoes (Harbison *et al.*, 2006; Odiere *et al.*, 2007). Moreover, mud walls as well as grass roofs

often have crevices used by mosquitoes to enter the houses unlike cement walls and metal roofs (Kirby *et al.*, 2008). However, in Namwawala village, houses with cement-plastered walls had similar risk of indoor mosquito entry when compared to houses with mud walls. This might be due to cracks in the plastered walls that allowed *An. gambiae* s.l. and *An. funestus* entry, but which were not documented during the house characteristics survey.

In addition, the presence of intact screening over windows prevented indoor entry of *An. funestus* and *An. gambiae* s.l. Although damaged screening over windows appeared to significantly reduce the mean catches of *An. funestus* between study villages and when combined, there was no protective effect of damaged screening against *An. gambiae* s.l. While houses with damaged screening are likely to poses similar risk as those without screening, the observed protection of damaged screening against *An. funestus* might be unrealistic probably due to the very low numbers of *An. funestus* collected during the study compared to *An. gambiae* s.l.

Furthermore, houses with open eaves provided entry points that led to increase *An. gambiae* s.l. and *An. funestus* mosquito abundance inside the houses. The lack of correlation between different eave sizes and indoor densities of both *An. gambiae* s.l. and *An. funestus*, supports the argument that the presence of an eave gap, regardless of its size, poses an important risk for indoor mosquito entry. These findings are consistent with other studies (Smith & Hudson, 1972; Lindsay *et al.*, 2002; Sintasath *et al.*, 2005; Ernst *et al.*, 2006; Yé *et al.*, 2006; Kirby *et al.*, 2008; Njie *et al.*, 2009) which demonstrated that poorly constructed houses (with mud walls, grass roofs, lack of screening and with open eave tend to have increased human-vector exposure, resulting in a higher risk of malaria transmission.

It has been documented that houses with many occupants tend to attract vectors of disease (White, 1969; Konradsen *et al.*, 2003; Kirby *et al.*, 2008). In this study, the presence of many sleepers in a house exposed them to a higher risk of *An. gambiae* s.l. bites but not from *An. funestus*. Large amounts of human emanations from houses with more occupants tend to increase mosquito attractiveness towards that particular house compared to ones with fewer sleepers (Port *et al.*, 1980; Takken & Knols, 1999). The lack of relationship between *An. funestus* and number of occupants inside the house was unexpected and a challenge to deliver an explanation; however, it might have resulted from uneven distribution of *An. funestus* within the villages. Higher numbers of *An. funestus* collected during the dry season (Smith *et al.*, 1995) were mostly and consistently from a cluster of a few houses located in a particular village hamlet. Therefore, the majority of houses within the sampling area experienced none or low catches. Furthermore, significant impacts of house risk factors and house occupants on *An. funestus* indoor mean catches were not consistent between villages. While this observation remains inconclusive, one explanation might be the far lower numbers of *An. funestus* collected between villages compared to *An. gambiae* s.l.

Treated nets provided more protective advantages than untreated ones as also observed in previous studies (Lengeler, 2004; Killeen *et al.*, 2007; Russell *et al.*, 2010; Winskill *et al.*, 2012). However, the density of *An. gambiae s.l.* in Namwawala was higher compared to Idete despite 90% ITNs coverage in Namwawala. These results indicate that even at high coverage levels, ITNs still have limitations in reducing the number of malaria vectors entering the houses. Furthermore, recent studies (Pulford *et al.*, 2011; von Seidlein *et al.*, 2012) have indicated that poor compliance and usage of bed nets by communities in the tropics is associated with heat discomfort associated with poor airflow caused by bed nets. Although bed nets were procured individually and a distribution campaign was underway during the study period, the age of ITNs as well as frequency of usage were not systematically investigated in this study. However, the

results illustrate that a risk of transmission remains whenever people are not using treated nets in an optimal way. In addition, as shown by recent findings from western and south-eastern Uganda where there was no association between the far less endophagic *An. arabiensis* and surveyed house characteristics (Wanzirah *et al*, 2015), it is important to consider the diversity of malaria vectors before drawing conclusions on the impact of different house factors' effect size on indoor mosquito entry.

Improved house designs, and modifications to existing houses could substantially reduce the risk of mosquito-human contact. Although house improvement has been advocated as an efficient intervention for malaria control, the majority of houses in poor rural Africa are temporary and built with minimal material resources. This renders improvements expensive and/or impractical in most rural communities in the short term. Modern houses (Figure 2B) could be easily and cheaply modified by screening eaves, windows and doors accompanied by community sensitization towards intervention sustainability. Traditional houses (Figure 2A) are less amenable to modifications unless they are rebuilt as permanent structures. This would have to be addressed through a long-term strategy that sought to build better, inexpensive house models using better construction materials and sustainable financing initiatives, which can be adopted in poor settings. Such an intervention is likely to be beneficial in reducing vector borne diseases and other diseases linked to poor hygiene.

2.6 Conclusion

This study shows the impact of different housing characteristics on malaria vector density and the associated risk of indoor disease transmission. It also shows that even at high coverage levels of LLINs, there remains a high risk of human-mosquito contact and also that this transmission risk can be mitigated by changing the house structure.

Communities with modern, spacious and screened houses are at lower risk of indoor malaria transmission than temporary, small and unscreened houses.

CHAPTER 3

INCREASING ROLE OF *ANOPHELES FUNESTUS* AND *ANOPHELES ARABIENSIS* IN MALARIA TRANSMISSION IN THE KILOMBERO VALLEY, TANZANIA

The results reported in this chapter have been published in a slightly different form as Lwetoijera *et al. Malaria Journal 2014, Volume 13, Issue 1, 331*

3.1 Abstract

Background: In order to sustain the gains achieved by current malaria control strategies, robust surveillance systems that monitor dynamics of vectors and their roles in malaria transmission over time are essential. This longitudinal study demonstrates the trends in malaria vector dynamics and their relative contribution to malaria transmission in one hyperendemic transmission setting in Tanzania.

Methods: The study was conducted in two villages within the Kilombero Valley, in rural Tanzania for seven consecutive years (2008-2014). Seventy-two houses were selected per village and each house was sampled for mosquitoes monthly using a CDC light trap. Collected mosquitoes were assessed for species identity and sporozoite infection status using PCR and ELISA, respectively. *Anopheles funestus* and *Anopheles arabiensis* susceptibility to insecticides was assessed using WHO guidelines.

Results: A total of 196,685 malaria vectors were collected, of which 74% were *Anopheles gambiae* s.l. and 26% were *An. funestus*. Between 2008-2011, the proportion of the total catch of *An. gambiae* s.l. was higher than *An. funestus* in both villages. However, in 2012, the proportion of *An. funestus* 62% (32,228) exceeded *An. gambiae* s.l. 38% (19,926) in both villages. In 2013, the proportions remained similar between two species across villages, but in 2014 the proportions of *An. gambiae* s.l. was again higher than that of *An. funestus* in both villages combined.

Of 3,160 *An. funestus* samples that successful amplified with PCR, 98% were *An. funestus s.s.*, 1% were *Anopheles rivorulum* and 1% *Anopheles leesoni*. For *An. gambiae s.l.* (n = 9,117), 93% were *An. arabiensis* and 7% were *Anopheles gambiae s.s.* The proportion of *An. gambiae s.s.* identified by PCR (2,924) declined from 0.2% in the year 2008 to undetectable levels in 2012-2014. Malaria transmission intensity significantly decreased from an EIR of 78 infectious bites/person/year in 2008 to 35 ib/p/yr in 2011

but rebounded to 226 ib/p/yr in 2012, coinciding with an increased role of *An. funestus* in malaria transmission. Insecticide susceptibility tests indicated high levels of resistance in *An. funestus* against deltamethrin (87%), lambda cyhalothrin (74%), permethrin (65%), bendiocarb (65%), and DDT (66%). Similarly, *An. arabiensis* showed insecticide resistance to permethrin (77%), deltamethrin (64%) and lambda cyhalothrin (42%) in 2014.

Conclusion: The results indicate the continuing role of *An. arabiensis* and the increasing importance of *An. funestus* in malaria transmission, and pyrethroid resistance development in both species. Complementary vector control and surveillance tools are needed that target the ecology, behaviour and insecticide resistance management of these vector species, in order to preserve the efficacy of LLINs.

Keywords: Malaria, *Anopheles*, transmission, vector, surveillance, *gambiae*, *arabiensis*, *funestus*, season, insecticide, susceptibility, EIR, Kilombero, Tanzania.

3.2 Background

Malaria transmission in humans is sustained through vector-human interactions (Bruce-Chwatt *et al.*, 1966) and vector control interventions, such as long-lasting, insecticidal nets (LLINs), aim to break this interaction. Major promotion of LLINs in recent years has resulted in average household ownership rates and usage of LLINs of approximately 49% (range 44-54%) and 44% (range 39-48%), respectively, in sub-Saharan Africa (WHO, 2014). In mainland Tanzania, a recent report by the Tanzania HIV and Malaria Indicator Survey (THMIS) indicates that above average LLINs ownership and usage (approximately 90 and 66%, respectively) was associated with improved malaria control and overall reduction in malaria prevalence (Tanzania Commission for AIDS & ICF International Calverton, 2013).

One outcome of LLINs use is that, by limiting availability of human hosts (Bayoh *et al.*, 2010; Mwangangi *et al.*, 2013; Okumu *et al.*, 2013), vector species composition in any given area can change considerably after a long period of LLINs use. *Anopheles gambiae s.s*, *An. arabiensis* and *An. funestus* are the primary malaria vectors in sub-Saharan Africa (Mzilahowa *et al.*, 2012; Sinka *et al.*, 2012), often occurring sympatrically (Coetzee *et al.*, 2000). *Anopheles gambiae s.s.* is often regarded as the most important vector species across Africa (Gillies & Coetzee, 1987; Coetzee *et al.*, 2000; Russell *et al.*, 2010) and, because of its strongly anthropophagic and endophilic behaviour, it is the species that has been targeted most effectively by LLINs.

However, in some locations, populations of *An. gambiae s.s*. have developed insecticide resistance and it continues to be the dominant vector (Corbel *et al.*, 2004; N'Guessan *et al.*, 2007b). In other locations, *An. gambiae s.s*. populations have crashed and the

relative importance of the remaining vector species has shifted, with *An. arabiensis* becoming the major malaria vector (Bayoh *et al.*, 2010; Russell *et al.*, 2010; Mwangangi *et al.*, 2013).

Since single populations of *An. arabiensis* can exhibit a range of behaviours, biting and resting indoors as well as outdoors and feeding on both humans and animals, interventions that optimally target indoor resting and biting vectors often impact far less on this species (White *et al.*, 1972; Tirados *et al.*, 2006; Muriu *et al.*, 2008; Russell *et al.*, 2010; Kitau *et al.*, 2012). The primary vector of the *An. funestus* complex, *An. funestus* is also a very anthropophilic and endophilic mosquito and it too can be a highly efficient malaria vector (Gillies & de Meillon, 1968; Gillies & Coetzee, 1987; Mendis *et al.*, 2000).

Kilombero Valley in southern Tanzania has been subject to a large number of studies on malaria epidemiology, dating back many years, with malaria parasite prevalence rates of up to 70% and an entomological inoculation rate (EIR) of 300 infectious bites per person per year (ib/p/yr) being recorded in the 1990s, the period before the introduction of bed nets (Smith *et al.*, 1993). Following the scaling up of untreated nets in the early 2000s (Killeen *et al.*, 2007) and insecticide-treated bed nets (ITNs) and LLINs from 2004 to 2011 (Mulligan *et al.*, 2008; Alba *et al.*, 2011; Renggli *et al.*, 2013), a continuous decline in malaria vector numbers and malaria transmission has been seen (Russell *et al.*, 2010; Tanzania Commission for AIDS & ICF International Calverton, 2013). Although the populations of *An. gambiae s.s.* are significantly dwindling in southern and other parts of Tanzania (Russell *et al.*, 2010), the remaining populations of *An. arabiensis* and *An. funestus* appears to have shifted their blood-feeding periodicity by biting peoples outdoors to optimize their chances to obtain blood meal from their preferred hosts even

in the time of low LLINs coverage (Russell *et al.*, 2011b). It is however suggested that prolonged, widespread use of LLINs is likely to favour outdoor and early biting, either as an expression of the mosquito's innate phenotypic plasticity or possibly as a heritable, selectable trait that might be expected to increase in frequency (Russell *et al.*, 2011b; Killeen, 2014).

The malaria vector populations in this area are subject to ongoing rigorous monitoring and herein seven years of data to the end of 2014 are reported; describing changes in vector species composition and relative abundance, insecticide susceptibility and their contribution to malaria transmission following the years of widespread LLINs use since first introduced in 2004.

3.3 Methods and materials

Study site

The study was carried out in Namwawala (8.154425°S and 36.393005°E) and Idete (8.098190°S and 36.510350°E) villages (see figure 3.3.1) located in the flood plain of the Kilombero River (8.1°S and 36.6°E) in southeastern Tanzania. The epidemiology of malaria transmission and associated vector species composition within these villages has been documented over many years (Killeen *et al.*, 2007; Russell *et al.*, 2011b). Both villages experience an annual rainy season (January-May) and the main crops are rice and maize. Both villages are similar in size (Namwawala = 844 and Idete = 804) and approximately 92% of community members sleep under an ITN or LLIN (Russell *et al.*, 2010).

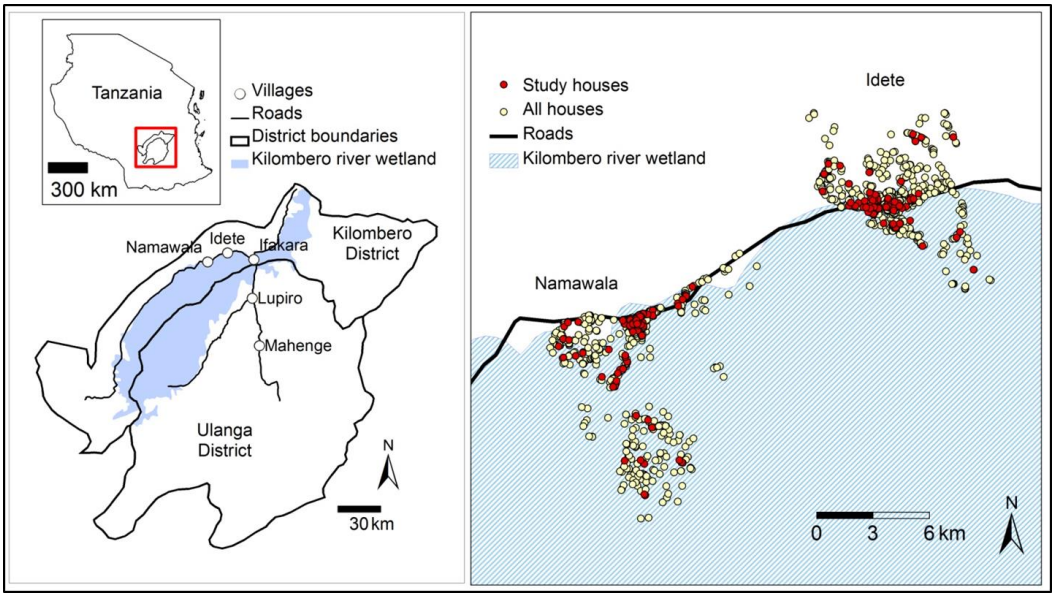

Figure 3.3.1: A map showing sentinel houses for mosquito sampling in Idete and Namwawala villages, in Kilombero Valley, Tanzania

Study design

This study was conducted over five years between January 2008 and December 2014. A total of 72 houses from each village were randomly selected from the Ifakara Health Institute (IHI) Demographic Surveillance System household list (Schellenberg *et al.*, 2001). All selected houses were geolocated using a handheld GPS (eTrex, Vista, Garmin, USA). Mosquitoes were sampled in every house each month during 2008, 2011 and 2014 and for six months from January to June in 2009 and July to December in 2010.

Mosquito sampling and processing

Mosquitoes were sampled using miniature Center for Disease Control (CDC) light traps (model 512, USA). One CDC light trap was used overnight per house, placed 1-1.5m from the fan above the ground close to the foot end of an occupied bed, and left to run for 12 h (19.00-07.00) (Lines *et al.*, 1991; Mboera *et al.*, 1998). For every participating house, one LLIN (Olyset, A to Z Textiles Mills, Arusha, Tanzania) was provided to protect the bed occupant where the CDC trap was set. The following morning, CDC light traps were collected and mosquitoes killed using chloroform, and identified in the field using a morphological key (Gillies & de Meillon, 1968). Female mosquitoes were classified as being unfed, partially fed, fully fed or gravid. Subsamples of five mosquitoes from each trap for *An. arabiensis* and *An. funestus* species were individually stored inside a tube containing cotton wool and silica gel beneath for further individual molecular species identification using polymerase chain reaction (PCR) assay for the *An. gambiae* complex (Scott *et al.*, 1993) and *An. funestus* group (Koekemoer *et al.*, 2002) and sporozoite infection status using enzyme-linked immune-sorbent assay (ELISA) (Burkot *et al.*, 1984) in the laboratory (species identification for the *An. funestus* group did not begin until 2009).

All the sorting information and laboratory analysis results were recorded using designated data collection forms for entomological studies (Kiware *et al.*, unpublished). In addition, variations in malaria transmission by different vector species over time were assessed and compared using the annual EIR calculated by biting rate (total collections/trap nights/year) and the proportion of females infected with sporozoites (Kelly-Hope & McKenzie, 2009). Monthly average rainfall data for 2008-2011 were obtained from the Kilombero Valley Teak Company (approximately 15 km from Idete

village), and data for year 2012 obtained using rain gauges installed in Namwawala village.

Insecticide susceptibility test

Following an increase in the *An. funestus* population in 2012 despite extensive usage of LLINs in the study area, it was unclear whether this was due to its reduced susceptibility to the insecticides used in LLINs. The insecticide susceptibility tests were conducted using WHO standard procedures and test kits for adult female mosquitoes of *An. arabiensis* and *An. funestus* (WHO, 2013c) in Namwawala villages from January to June 2013. Biossays were repeated in June 2014 for both species.

Five classes of insecticides currently recommended for vector control were tested using discriminating concentrations impregnated in pre-prepared test papers as follows: bendiocarb (0.1%), DDT (4%), deltamethrin (0.05%), lambda cyhalothrin (0.05%), and permethrin (0.75%). Unfed female wild *An. funestus* collected using CDC light traps were used for insecticide exposure bioassays, as recommended by WHO for this difficult-to-colonize species (WHO, 2013c). This method is limited by greater variation in susceptibility due to unknown age differences between test mosquitoes, it is though simple to carry in the field with minimal infrastructure and test mosquitoes highly representative of the natural population (WHO, 2013c).

Prior to exposure, morphologically identified mosquitoes were maintained on 10% glucose solution for at least five hours prior to testing; whereas, for *An. arabiensis*, F1 female mosquitoes two to three days old (recommended age group) were used for bioassays from reared *Anopheles* larvae collected from the breeding habitats in the study

sites (Chouaibou *et al.*, 2012; Jones *et al.*, 2012). Species identification was carried out after bioassays on dead mosquitoes using PCR.

A total of 100 mosquitoes were exposed per discriminating concentration in five replicates of 20 mosquitoes each, and compared to a control with same number of mosquitoes per replicate. In an exposure tube set in a vertical position, mosquitoes were held for a total of one hour in intervals of 10, 15, 20, 30, 40, 50, and 60 minutes. After the first hour of exposure, mosquitoes were transferred to non-insecticide treated, clean, holding tubes and observed for a further 20 minutes (WHO, 2013c). After 80 minutes (initial 60 min + further 20 min) of knockdown monitoring, all mosquitoes were transferred to non-insecticide treated, clean, holding tubes and kept for 24 h and provided with 10% glucose solution, after which mortality was monitored and recorded. All these procedures were performed in the field under average ambient temperatures of 26 ± 2°C and a relative humidity of 78 ± 3% in both bioassay rounds. Percentage knockdown in the observed mosquitoes was recorded immediately for each time interval, and mosquito mortality in each bioassay was expressed as the proportion of dead mosquitoes to total exposed, for each tested insecticide. Execution and interpretation followed recently updated WHO test procedures for insecticide resistance monitoring in malaria vector mosquitoes (WHO, 2013c).

Data analysis

Only data pertaining to *An. gambiae s.l.* and *An. funestus* were analysed, using SPSS version 20 (SPSS Inc, Chicago, USA). Data were fitted with generalized linear models (GLMs) using a negative binomial distribution with log-link function, and relative rates (RR) with 95% confidence intervals calculated to estimate yearly mean mosquito

catches, relative to the reference year. Species (*An. gambiae s.l.* and *An. funestus)* were treated as predictors and total number of mosquitoes as a dependent variable; the statistical differences in dependent variables was evaluated as a function of villages (Idete and Namwawala), seasons (wet and dry) and years (2008-2012).

Insecticide susceptibility test biossay data were considered for each diagnostic concentration and year of testing. Mortality was calculated as the percentage of mosquitoes dead post 24 hr' exposure to insecticide, and the results assessed according to WHO testing procedure for insecticide resistance monitoring in malaria vectors (WHO, 2013c). Mortality rates between 98 and 100% indicate full susceptibility, 90-97% is suggestive of resistance and requires further investigation, and mortality rates less than 90% confirm the existence of resistance.

Ethical clearance and protection of human participants

The study approval was granted by the Ifakara Health Institutional Review Board (IHRDC/IRB/No.A-32) and the National Institute of Medical Research (NIMR/HQ/R.8a/Vol. IX/764). On first visiting each house, the benefits and possible risks associated with the study were explained to the house occupants and informed consent to proceed was requested. After consenting, the head of the house was asked to sign two copies of the informed consent forms, (retained by the head of the house and the study investigator).

3.4 Results

Relative abundance of malaria vector species

During the seven consecutive years of sampling with CDC light traps in sentinel houses, a total of 196,685 malaria vectors were collected of which 74% were *An. gambiae sensu lato* and 26% were *An. funestus*. In each of the first four years (2008-2011), *An. gambiae* s.l. was the most abundant mosquito group, with significantly higher numbers than *An. funestus* in both study villages (p <0.0001): proportions in total catches in Namwawala were 93% (65,894) and 6% (4,754), and in Idete were 86% (49,344) and 14% (7,854) for *An. gambiae* s.l. and *An. funestus*, respectively.

In 2012, collections of *An. funestus* exceeded *An. gambiae* s.l. comprising 56% of the total catch (RR (95% CI) = 1.56 (1.33-1.69), p <0.0001)); higher proportions of *An. funestus* were recorded in Namwawala (58%, 15,334/26302) and Idete (65%, 16,894/25,852). In 2013, collections of *An. funestus* in Namwawala again were higher, (RR (95%CI) = 1.59 (1.38 -1.64), p <0.0001), with a proportion of total catches of 59% (2,265/3,852). Conversely, at Idete in 2013, the *An. gambiae* s.l. proportion was 63% (4,044/6,460), significantly higher than *An. funestus* (p <0.0001). When the 2013 catches from both villages were combined, the mean catches (±SD) of *An. gambiae* s.l., 3.5 (±13.4) and *An. funestus*, 3 (±13. 2) were similar (p > 0.05). In 2014, collections of *An. gambiae* s.l. were higher than *An. funestus* in both villages (p <0.0001), with higher proportions recorded in both Namwawala (63%, 2,206/3,489) and Idete (72% (2,067/2,884).

From a total of 10,847 *An. gambiae* s.l. processed by PCR for species identification, 84.05% were successfully identified, and comprised 93% *An. arabiensis* (n = 8,510) and 7% *An. gambiae* s.s. (n = 607). The relative proportions of the species were similar in

Idete (*An. arabiensis* 97% (n = 4,507), *An. gambiae* s.s. 3% (n = 151) and in Namwawala 90% (n = 4,122) *An. arabiensis*, 10% (n = 456) *An. gambiae* s.s. However, the relative proportion between the two sibling species changed over time, with a significant decrease seen in *An. gambiae* s.s. from approximately 14% (414/2,924) in 2008 to 0% (0/435) in 2014 (Table 3.4.1).

Of the 5,037 *An. funestus* samples that were analysed by PCR, 63% (3160) were successfully identified, of which 98% were *An. funestus s.s.* (n = 3,100), 1% were *An. rivorulum* (n = 33) and 1% *An. leesoni* (n = 33). The species composition of *An. funestus* in Idete was 97.69% (n = 1,776) *An. funestus s.s*, 1.43% (n = 26) *An. rivorulum* and 0.88% (n = 16) *An. leesoni.* In Namwawala it was 97.41% (n = 1,393) *An. funestus s.s.*, 0.56% (n = 8) *An. rivorulum* and 2.03% (n = 29) *An. leesoni*, (Table 3.4.1).

Table 3.4.1: Malaria vector composition, sporozoite prevalence (S), biting rate (B) and entomological inoculation rate (EIR) for *Anopheles gambiae s.s.*, *Anopheles arabiensis* and *Anopheles funestus* and their annual contribution to malaria transmission between 2008 and 2014 in the study area

Species	2008	2009/10	2011	2012	2013	2014
***An. gambiae sensu lato* species proportion**						
An. gambiae s.s.	0.14	0.15	0.002	0	0	0
An. arabiensis	0.86	0.85	0.998	1	1	1
No. of PCR amplifications	2,924	1,307	2,542	1,362	471	435
***An. funestus* group species proportion**						
An. funestus s.s.	-	0.887	0.956	1	1	0.968
An. rivulorum	-	0.013	0.021	0	0	0.002
An. leesoni	-	0	0.023	0	0	0.029
An. parensis	-	0	0.001	0	0	0
No. of PCR amplifications	-	330	880	1,527	185	442
Sporozoite prevalence (S ;%)						
An. gambiae s.s.	1.18	0.04	0	0	-	-
An. arabiensis	0.16	0.36	0.07	1.47	-	-
An. funestus	1.71	0	0.43	2.20	-	-
Biting rate (B; b/p/n)						
An. gambiae s.s.	8.52	6.05	0.04	0	0	0
An. arabiensis	52.37	35.51	59.74	20.70	5.849	5.771
An. funestus	1.74	12.84	10.09	14.31	2.231	1.251
Entomological Inoculation Rate (EIR; ib/p/y)						
An. gambiae s.s.	36.70	1.61	0	0	-	-
An. arabiensis	30.58	55.51	15.17	110.90	-	-
An. funestus	10.86	0	15.58	115.10	-	-
Total	**78.14**	**57.12**	**31.05**	**226.0**	-	-

Note: Sporozoite prevalence = Number of positive sporozoite mosquitoes/total tested; Biting rate = Total collections/trap nights/calibration factor, 0.3 for *An. gambiae* complex, and 0.68 for *An. funestus* (Killeen *et al.*, 2007), EIR = S × B × 365

Seasonal variation in vector abundance

Throughout the study, the period from January to May was categorized as the wet season, during which time the average (±SD) rainfall was 264 (±161) mm/month. June to December was the dry season, with an average rainfall of 23 (±57) mm/month (Figure 3.4.1). The abundance of both *An. gambiae s.l.* and *An. funestus* peaked in the wet season in both villages. The mean number (±SD) of *An. gambiae* s.l. caught per trap per night was 15 (±40) and 21 (±88) during the wet season, and 0.8 (±5) and 0.8 (±5) in the dry season at Idete and Namwawala, respectively. Furthermore, *An. gambiae* s.s. was present in the wet season only in the first three years (2008-2009/10) and was not detected in last four years of sampling (2011- 2014). In contrast, both *An. arabiensis*, and *An. funestus* s.s. were found in both seasons.

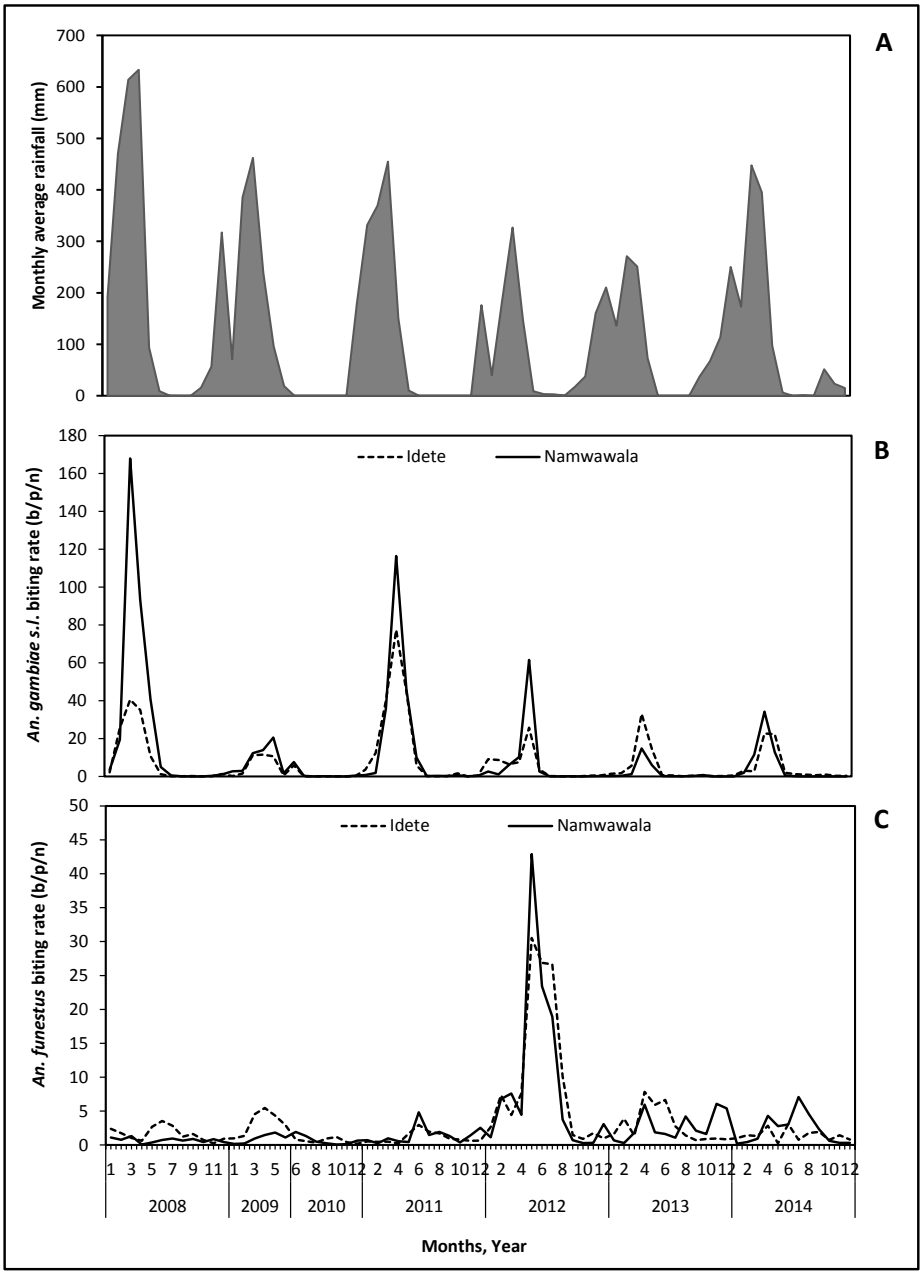

Figure 3.4.1: Monthly average rainfall in the Kilombero Valley (A), and abundance as estimated using CDC monthly biting rates, for *An. gambiae* s.l. (B) and *An. funestus* (C), in Idete and Namwawala villages from 2008 to 2014.

The mean number of *An. funestus* per trap per night in the wet and dry season of the first four years of study (2008-2011) was consistently similar in both villages. In the wet season, the mean catches (±SD) were 1.23 (±4.7) in 2008, 2.15 (±7.5) in 2009/10, 0.64 (±1.9) in 2011 compared to 1.15 (±5.2), 0.77 (±4.3) and 1.62 (±5.5) of the respective years in the dry season. In 2012, the mean catch of *An. funestus*, both in wet and dry seasons, was approximately six times higher than in the previous years (p <0.0001): 11.8 (±45.8) and 8.3 (±25.6) of wet and dry season, respectively. From 2013-2014, the mean catches ((±SD) of *An. funestus* in both wet and dry seasons, was lower than in 2012 (p < 0.0001). In the wet season, mean catches were 3.1 (±10.6) in 2013, 1.3 (±5.6) in 2014 compared to 3 (±14.6) and 2 (±7.9) in the dry season catches for the respective year.

Malaria transmission

A total of 10,138 mosquitoes (530 *An. gambiae* s.s., 7,130 *An. arabiensis* and 2,478 *An. funestus* s.s.) were screened for *Plasmodium falciparum* sporozoites of which 75 were positive (0.74% sporozoite prevalence). Although *An. gambiae* s.s. was the major malaria vector with a sporozoite prevalence of 1.2% in 2008, its dominance decreased with time to zero in 2011 and 2012, following its control to undetectable levels. Conversely, the importance of *An. arabiensis* and *An. funestus* s.s. increased with time from a sporozoite prevalence of 0.16% in 2008 to 1.5% in 2012 for *An. arabiensis*, and from 1.7% in 2008 to 2.2% in 2012 for *An. funestus* s.s. the EIR for the two consecutive years (2013-2014) were not calculated due to missing data.

Similarly, the EIR of *An. gambiae* s.s. decreased drastically from 30.70 ib/p/yr in 2008 to 0 ib/p/yr in 2012, whereas those of *An. arabiensis* increased approximately four times from 30.58 in 2008 to 110.9 in 2012 and that of *An. funestus* s.s. increased 11 times from 10.86 in 2008 to 115.10 in 2012.

Overall, the level of malaria transmission in the study villages markedly decreased with time from an EIR of 78.14 ib/p/yr in 2008 to 31.05 ib/p/yr in 2011 but increased sharply to 226 ib/p/yr in 2012, approximately seven times more than in the previous year (Table 3.4.1).

Anopheles arabiensis *and* Anopheles funestus *insecticide susceptibility tests*

In the WHO bioassay testing, as the results indicated (Figure 3.4.2), *An. funestus* was fully susceptible to deltamethrin (100% mortality) with reduced susceptibility to permethrin (93%), and lambda cyhalothrin (91%) and confirmed resistance to DDT (86%) in 2013. In 2014, *An. funestus* was resistant to permethrin (65%), lambda cyhalothrin (74%), bendiocarb (65%), and even to deltamethrin (87%) to which it was fully susceptible in 2013. Mortality in control tubes was 4% in both testing rounds. All tested mosquitoes were amplified as *An. funestus*, using PCR.

In 2013, *An. arabiensis* was fully susceptible to bendiocarb (100% mortality) and deltamethrin (98.3%), reduced susceptibility against DDT (97%), and confirmed resistance to permethrin (83.3%) and lambda cyhalothrin (78%), with a control mortality of 0% across all test concentrations. Similar levels of resistance were maintained across tested diagnostic concentrations in year 2014, whereby the mosquitoes were fully susceptible to bendiocarb (98% mortality) and resistant to deltamethrin (64%), permethrin (77%), and lambda cyhalothrin (42%), with a control mortality of 0% across all test concentrations.

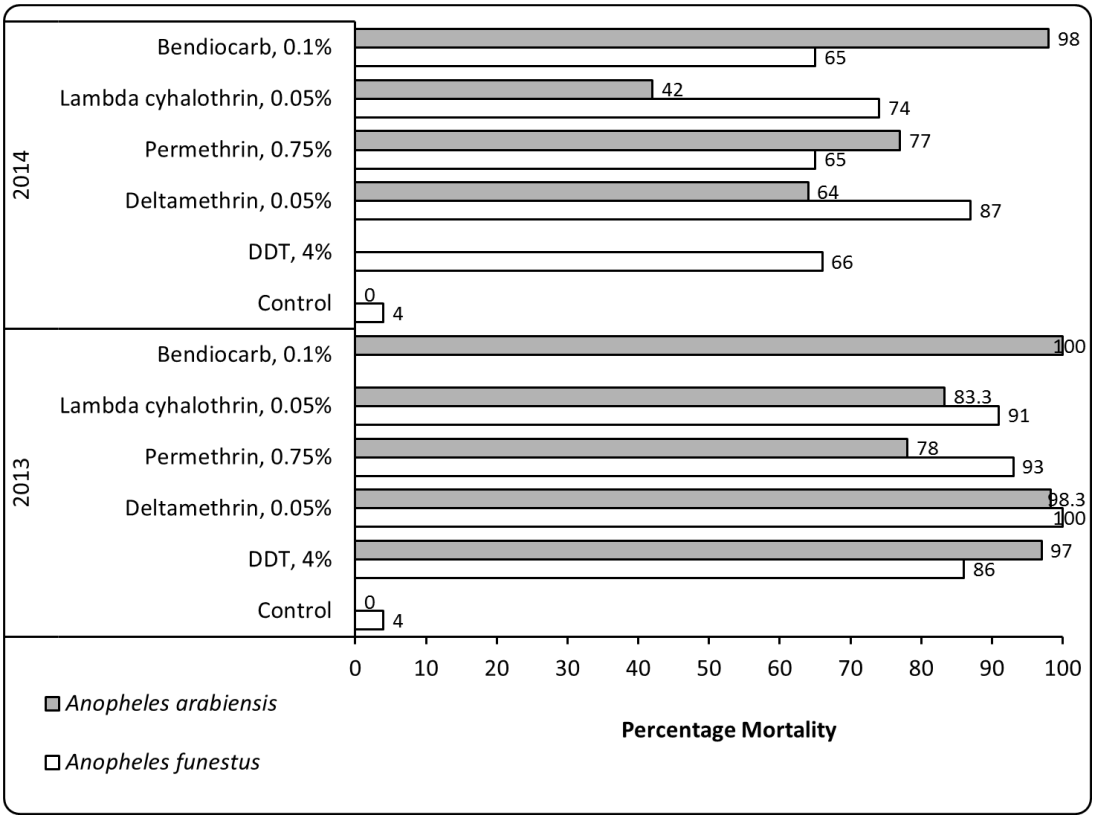

Figure 3.4.2: Results of WHO bioassay test for insecticide susceptibility status of wild female *Anopheles funestus* (white bars) and *Anopheles arabiensis* (grey bars) from the study sites in the Kilombero Valley, Tanzania, in January 2013 and June 2014; The graph shows percentage 24 h mortality rate after a one-hour exposure to the WHO diagnostic doses of insecticide. The minimum sample size for these assays was 100.

3.5 Discussion

This study provides substantial information on malaria vector dynamics and their contribution to malaria transmission in rural southern Tanzania over a seven years

period. Consistent with other studies, which have documented a shift in malaria vector composition and a change in malaria transmission dynamics seemingly as a result of extensive use of LLINs (Bayoh *et al.*, 2010; Derua *et al.*, 2012; Mwangangi *et al.*, 2013), this study reports a steady decrease to undetectable levels of *An. gambiae* s.s. with steady increase in the proportion of its sibling species *An. arabiensis* and a surge in the abundance of *An. funestus* s.s. in 2012 followed by its decrease in 2013 and 2014.

During species identification using PCR between 2010 and 2014, an average of 16% of *An. gambiae* s.l. and 37% *An. funestus* group did not amplify by PCR and were not identified to sibling species. Potentially, this might have resulted in either underestimation or over-estimation of the relative proportions of the various species. Causes of PCR non-amplification could have included inadequate optimization of standard operating practices in the molecular laboratory where samples were processed and analysed. Following optimization by 1) increasing primer concentration, 2) setting up and running PCR reaction immediately after DNA extraction to prevent loss of DNA quality because of over-storage and 3) running PCR reactions at appropriate and recommended temperatures in the laboratory to prevent background reactions before the actual PCR reactions, significantly improved the amplification rate for *An. gambiae* s.l. and *An. funestus* group submitted samples up to 91% in year 2014 (Mr Deogratius Roman, *personal communication*).

Anopheles gambiae s.s. preferentially feeds and rests inside houses. This makes it more vulnerable to insecticides applied to nets (LLINs) and walls (indoor residual spraying (IRS)) while *An. arabiensis*, with its opportunistic feeding behaviour both on humans and animals (Kelly-Hope & McKenzie, 2009; Russell *et al.*, 2010) and its potential to rest outside human dwellings, make it less affected by LLINs.

Early evening and outdoor feeding behaviour in *An. arabiensis* and *An. funestus* (Wilkes *et al.*, 1996; Russell *et al.*, 2011b) in order avoid contact LLINs have been recorded previously. Potentially, similar behavioural changes might have occurred in the present study and would have been detected if routine outdoor mosquito collections had been included in the design.

A significant increase in *An. funestus* abundance and EIR occurred 2012 followed by a decrease in year 2013-2014. This shift poses great concern in malaria control efforts due to its efficiency in transmitting malaria. Historically the control of *An. funestus s.s.* was successful through extensive IRS, taking advantage of its highly anthropophagic and endophilic behaviour, using dieldrin in Pare, Taveta, northern Tanzania (Gillies & Smith, 1960; Smith, 1966) Malindi on the coast of Kenya, using DDT (Gillies & Furlong, 1964) as well as in South Africa (Sharp *et al.*, 2007). This is partly because they spend a longer time on insecticide-treated materials (Davidson, 1953). However, the vector eventually resurged six years later due to a lack of IRS programme continuity and consolidation (Gillies & Furlong, 1964; The malERA Consultative Group on Vector Control, 2011). A similar scenario was expected in this particular region, where usage of LLINs is high (Koehler & Patterson, 1991; Tanzania Commission for AIDS & ICF International Calverton, 2013).

The steady increase in *An. funestus* population density, despite extensive usage of LLINs in the study area, was associated with reduced susceptibility to the insecticides used in LLINs. Recent findings from western Kenya have demonstrated similar phenomenon of resurging *An. funestus* populations, chiefly being due to resistance development to the pyrethroids used in LLINs (McCann *et al.*, 2014). However, decreasing in *An. funestus* mean catches in 2013 and 2014 compared to 2012 indicate the existence of other drivers

apart from resistance to insecticides which might have contributed to the observed resurgence of *An. funestus* in year 2012.

Preliminary findings from this study demonstrated resistance of *An. funestus* and *An. arabiensis* to pyrethroids, deltamethrin, lambda cyhalothrin and permethrin, used in Olyset LLINs, distributed in the study area in June 2011 (Tanzania Commission for AIDS & ICF International Calverton, 2013). Overall, there was great variation of the resistance status between 2013 and 2014 in both species tested; however, the variation was surprisingly large in *An. funestus*, more so *An. arabiensis*, which might be due to inconsistency in unknown age of the used *An. funestus* used in the bioassay (WHO, 2013c).

Due to the absence of organochlorine insecticide DDT and carbamate insecticide bendiocarb deployment for malaria vector control in the study area, the source of resistance in mosquitoes to these insecticides remains unknown. Although not tested in this particular study, pyrethroid (DDT and pyrethroid) carbamate cross-resistance was considered to be a probable cause of *An. funestus* resistance to DDT and bendiocarb, respectively, which has been proved to exist in malaria vectors elsewhere (Brooke *et al.*, 2001; Protopopoff *et al.*, 2013). In addition, the continuous and illegal use of DDT as a pesticide in agriculture in the region might have contaminated malaria vector breeding habitats and caused physiological resistance in mosquitoes (Nkya *et al.*, 2013).

Pyrethroid resistance in both species has been documented in multiple countries and regions of East Africa (Matambo *et al.*, 2007; Morgan *et al.*, 2010; McCann *et al.*, 2014), southern Africa (Hargreaves *et al.*, 2003; Casimiro *et al.*, 2006; Kloke *et al.*, 2011; Wondji *et al.*, 2012) and West Africa (Okoye *et al.*, 2008; Djouaka *et al.*, 2011; Ranson *et al.*, 2011). Further detailed studies are urgently required to establish current

vector control operational impacts associated with this level of resistance. These findings suggest an increased contribution of these vectors to malaria transmission and hence great threat to the future use of LLINs in controlling these vectors.

The other probable cause for the observed increase in *An. funestus* population in this study area, which is the limitation of this study and requires further investigation, might be a temporary shift of *An. funestus* to outdoor and early evening and daytime biting behaviours, which increased their chances to survive and reproduce by feeding on unprotected humans, as recently documented *An. funestus* behaviours in Benin (Moiroux *et al.*, 2012) and Senegal (Sougoufara *et al.*, 2014), West Africa.

The decrease in *An. funestus* proportions in catches from 2013-2014 compared to 2012 demonstrated the still unpredictable ecology of this vector. It must be assumed that the ecology of *An. funestus* is driven by ecological factors yet to be documented highlighting need for further research on the larval ecology and adult population dynamics of this important vector in different transmission settings. Although average annual rainfall was similar across years 2012-2014, the changes recorded might have been caused by varying in stability of *An. funestus* aquatic habitats as the result of changing in landscape due to agriculture and underground water movements during the study period (Hardy *et al.*, 2013).

In this study, both *An. funestus* and *An. gambiae s.l.* vector abundance varied with season. Although semi- and permanent aquatic habitats support development of *An. gambiae s.l.* (Fillinger & Lindsay, 2011), habitually, the increases in *An. gambiae s.l.* densities are facilitated by a wide range of ephemeral, sunlit, breeding habitats, such as hoof prints, rice puddles and ground depressions created during the rainy season (Gillies

& de Meillon, 1968; Minakawa *et al.*, 2004). The temporary nature of these habitats tends to reduce predation rate but also allows quick development of the juvenile stages, which results in *An. gambiae s.l.* dominating during the rainy season (Gillies & de Meillon, 1968). On the contrary, *An. funestus* prefer vegetated semi-permanent and permanent breeding habitats, such as spring-fed ponds and river channels lined with riparian vegetation, particularly trees, with dense canopies (Gillies & de Meillon, 1968; Hardy *et al.*, 2013). *Anopheles funestus* remained at a reasonable and detectable density across the rainy and dry seasons in the study areas and were significantly more abundant than *An. gambiae s.l.* in the dry season, probably due to their aquatic habitat stability against desiccation sustained by streams or stream margins and groundwater-fed natural springs (Gillies & de Meillon, 1968; Charlwood *et al.*, 2000; Hardy *et al.*, 2013).

Irrespective of seasonal variation in vector abundance, *An. funestus s.s.*, *An. gambiae s.s.* and *An. arabiensis* were all-important malaria vectors in the study area (Russell *et al.*, 2010). Despite high abundance of *An. arabiensis* and a higher EIR between 2008 and 2010, *An. funestus* contributed a relatively higher or equal EIR in 2011 and 2012. Historically, *An. funestus* has displayed high sporozoite prevalence (Charlwood *et al.*, 1995) similar to that observed in this study and in a recent study conducted in neighbouring villages within the valley (Kaindoa *et al.*, unpublished). This trend of increase in abundance and high sporozoite prevalence of *An. funestus* has been also observed in Asembo district, western Kenya (McCann *et al.*, 2014) and so appears to represent a trend across several regions of East Africa.

The huge increase in potential malaria transmission in 2012 (EIR = 226) coincided with an increase in abundance and sporozoite rates in *An. funestus* as it did in a neighbouring village in the valley (EIR = 156) (Kaindoa *et al.*, unpublished). The unpredictable changing patterns involving substantial increase in *An. funestus* and its reduced

susceptibility to pyrethroids poses a serious threat that needs attention from vector control stakeholders. A separate study in West Africa also reported a rebound in malaria transmission partly being caused by resistance development in *An. gambiae* to pyrethroids (Trape *et al.*, 2011).

A previous study has shown that despite high coverage and usage of LLINs, a high proportion of mosquitoes still enters houses (Gatton *et al.*, 2013). Therefore, the increase in *An. funestus*, particularly in the dry season, is likely to exacerbate the problem. Therefore, new strategies to address resistance and outdoor biting behaviour in the early part of the evening as displayed by *An. funestus* and *An. arabiensis* are required. This can be achieved through improving the LLINs; for instance, recent development of nets which can target multiple resistant mosquitoes, Olyset® Plus (Pennetier *et al.*, 2013), and by targeting vectors while outdoors using non-resistant compounds, either through larval source management in the dry season via autodissemination of insect juvenile hormone, e.g., pyriproxyfen (Devine *et al.*, 2009; Lwetoijera *et al.*, 2014, See Chapter 5, Section 5.4), or by mosquito sterilization with pyriproxyfen (Lwetoijera *et al.*, 2014, See Chapter 5, Section 5.4), and killing them with toxic sugar-baited traps (Muller & Schlein, 2008), non-chemical electric grid (Majambere *et al.*, 2013) and odour-baited traps (Matowo *et al.*, 2013).

3.6 Conclusion

This study showed the importance of *An. funestus* and *An. arabiensis* in sustaining residual malaria transmission. A substantial increase in *An. funestus* coincided with a dramatic reduction in *An. gambiae s.s.* in the year 2012. Malaria transmission significantly declined from 2008 to 2011 and rebounded in 2012 coinciding with the

increased role of *An. arabiensis* and *An. funestus* in malaria transmission. Although fully susceptible to deltamethrin, *An. arabiensis* and *An. funestus* were found to be resistant and with reduced susceptibility respectively, to Permethrin, one of the pyrethroids used on LLINs. These findings call for thorough ecological studies of *An. funestus* and, in the longer term, for complementary vector control tools, robust vector surveillance systems, and an insecticide resistance management plan to complement and preserve the efficacy of LLINs.

CHAPTER 4

EFFECTIVE AUTODISSEMINATION OF PYRIPROXYFEN TO BREEDING SITES BY THE EXOPHILIC MALARIA VECTOR *ANOPHELES ARABIENSIS* IN SEMI-FIELD SETTINGS IN TANZANIA

The results reported in this chapter have been published in a slightly different form as Lwetoijera *et al. Malaria Journal 2014, Volume 13, Issue 1, 161*

4.1 Abstract

Background: Malaria vector control strategies that target adult female mosquitoes are challenged by the emergence of insecticide resistance and behavioural resilience. Conventional larviciding is restricted by high operational costs and inadequate knowledge of mosquito-breeding habitats in rural settings that might be overcome by the juvenile hormone analogue, pyriproxyfen (PPF). This study assessed the potential for *Anopheles arabiensis* to pick up and transfer lethal doses of PPF from contamination sites to their breeding habitats (*i.e.* autodissemination of PPF).

Methodology: A semi-field system (SFS) with four identical separate chambers was used to evaluate PPF-treated clay pots for delivering PPF to resting adult female mosquitoes for subsequent autodissemination to artificial breeding habitats within the chambers. In each chamber, a tethered cow provided blood meals to laboratory-reared, unfed female *An. arabiensis* released in the SFS. In PPF-treated chambers, clay pot linings were dusted with 0.2 – 0.3 g AI PPF per pot. Pupae were removed from the artificial habitats daily, and emergence rates calculated. Impact of PPF on emergence was determined by comparing treatment with an appropriate control group.

Results: The mean proportion (95%CI) of adult emerged from PPF-treated section were fewer, 0.21 (0.09 – 0.51) than adult emerged in the untreated control chamber 0.95 (0.56 – 1.34, $p < 0.0001$), resulting to 76.5% reduction in adult emergence in the treatment section. Laboratory bioassay of water samples from artificial habitats in these experiments resulted in lower number of adults emerged in treated chambers, with mean proportion of 0.62 (0.16 – 1.07) compared to controls, 0.99 (0.95 – 1.03, $p < 0.003$). In experiments where no mosquitoes introduced, there were no significant differences in

the number of adults emerged between control and treatment, indicating that transfer of PPF to breeding sites only occurred when mosquitoes were present; *i.e.* that autodissemination had occurred.

Treatment of a single clay pot resulted in 58% reduction in adult emergence in six habitats, with mean proportion of 0.34 (0.21 – 0.45) compared to the controls, 0.98 (0.96 – 1.00, $p < 0.0001$), showing a high level of habitats coverage amplification of the autodissemination event.

Conclusion: The study provides proof of principle for the autodissemination of PPF to breeding habitats by malaria vectors. These findings highlight the potential for this technique for outdoor control of malaria vectors and call for the testing of this technique in field trials.

4.2 Introduction

Malaria remains one of mankind's leading public health challenges and a major economic burden for the developing nations where it is endemic. Disproportionately, 90% of all malaria deaths globally occur in Africa mostly in children under 5 years of age who account 78% of all deaths occurring in Africa (WHO, 2014). The World Health Organization (WHO) continues to recommend a range of combined strategies for malaria prevention with vector control, primarily through the use of long lasting insecticide-treated nets (LLINSs) and indoor residual insecticide spraying (IRS), a key component of those strategies (Mwangangi *et al.*, 2013; Okumu *et al.*, 2013; WHO, 2014). Despite great progress in reducing malaria transmission in Africa over the past decade, the future use of both of these interventions, and indeed any approach that relies on chemical insecticides, is seriously threatened by the emergence and ongoing spread

of insecticide resistance (Ranson *et al.*, 2011; WHO, 2011b; Coetzee & Koekemoer, 2013; Sokhna *et al.*, 2013). Moreover, LLINs and IRS target only vectors that are active indoors, and even in areas where this has been successful, malaria transmission by outdoor biting and outdoor resting vector populations of *Anopheles arabiensis* and *Anopheles funestus* remains a serious public health challenge (Russell *et al.*, 2011b; Reddy *et al.*, 2012). Effective sustainable tools or approaches with proven impact on outdoor biting and resting vector populations have yet to be developed.

Targeting the aquatic larval stages of the vector with conventional insecticides (larviciding), as a complement to LLINs and IRS, can be an effective method to suppress vector density (Fillinger *et al.*, 2009: Geissbuhler *et al.*, 2009; Tusting *et al.*, 2013), but it is limited by the difficult task of identifying and treating sufficient mosquito breeding habitats to impact the vector population (Majambere *et al.*, 2007; Gu *et al.*, 2008). WHO recommendations limit the use of larviciding to settings where larval habitats are few, findable, and easy to map and treat; typically this restricts larviciding to urban and rural settings at times when these habitats are restricted in number size and numbers (WHO, 2012b). In rural settings where breeding habitats are abundant in number and character, this is a far greater challenge for which novel approaches are urgently needed.

Pyriproxyfen (PPF) is a juvenile hormone analogue (JHA) that interrupts normal development and metamorphosis of targeted mosquitoes (Dhadialla *et al.*, 1998). Highly potent in terms of activity and specificity, it has low toxicity and a high margin of safety to non-target organisms (Mulla *et al.*, 1989). To date, there has been no evidence of PPF resistance in any mosquito (Invest & Lucas, 2008), however the possibility of resistance development in mosquitoes is highly likely to occur (Schaefer & Mulligan III, 1991), as

it has been documented in other insects (Crowder *et al.*, 2008; Shah *et al.*, 2015). For effective mosquito control, WHO recommends a PPF dosage limit of 50 ppb, an extremely low level considering the maximum permissible level in drinking water is 300 ppb (WHO, 2004b). PPF can be delivered in formulations that persist in treated aquatic habitats for up to six months under field conditions (Kawada *et al.*, 1988; Yapabandara & Curtis, 2002). PPF also has an additional unique benefit, termed autodissemination, which is defined as the ability of adult mosquitoes to pick up PPF from treated solid surfaces, retain and transfer it to breeding habitats in sufficient quantities to contaminate those habitats, rendering them unproductive either by killing larvae or preventing pupae from emerging to adults (Devine *et al.*, 2009).

The few studies demonstrating the potential of autodissemination of PPF in vector control have been limited to the *Aedes* vectors of dengue and chikungunya viruses (Devine *et al.*, 2009; Caputo *et al.*, 2012). Small field trials in urban settings in Peru and Italy, against *Aedes aegypti* (Devine *et al.*, 2009) and *Aedes albopictus* (Caputo *et al.*, 2012) respectively, resulted in significant adult emergence inhibition in treated areas. Many aspects of the biology of these *Aedes* species, such as their aggressive feeding, skip-oviposition (distributing portions of each egg batch in multiple habitats) and preference for relatively small volume man-made containers as breeding habitats, undoubtedly contribute to the prospect for exploiting autodissemination in urban control programs for dengue and chikungunya (Kawada *et al.*, 1988; Devine *et al.*, 2009; Caputo *et al.*, 2012) and fabrication of efficient PPF contamination sites/stations (Gaugler *et al.*, 2011; Caputo *et al.*, 2012). The outdoor-active *Anopheles* spp. that transmit malaria in rural Africa breed in a wide variety of breeding habitats, ranging in size and character and across much larger areas (Fillinger & Lindsay, 2006) and are a much greater challenge for this approach.

This study reports on the first experiments undertaken in a large semi-field system in Tanzania, evaluating the potential of PPF autodissemination for control of *An. arabiensis* and probably other African malaria vectors. Here, the results of controlled experiments quantifying the efficacy of clay pots, a simple inexpensive PPF contamination station, for delivering PPF to resting adult female *Anopheles arabiensis* at levels that prevent emergence at untreated breeding habitats are presented, demonstrating for the first time that, in principle autodissemination of PPF can occur at operationally effective rates in an *An. arabiensis*, an efficient African malaria vector.

4.3 Methods and materials

Study site

This study was carried out at Kining'ina village (8.11417°S, 36.67484°E), in rural southern Tanzania, between May 2012 and October 2013 inside a semi-field system (SFS). Details of the design and use of this SFS have been provided previously (Ferguson *et al.*, 2008; Ng'habi *et al.*, 2010). Briefly, the SFS is an outdoor construction with mesh walls 4.53 m high, measuring 552.96 m^2 in total area but partitioned into six separate chambers each measuring 9.6 × 9.6 m. The concrete floors of the chambers were filled to a depth of 40 cm with local soil, and vegetation growing naturally from the seeds therein. Although the SFS had six chambers, only four chambers were used for the experiments. A simple mud hut (1.75m x 1.5 m, 2m high) was built within each chamber to provide a shelter for a tethered cow bait, and possible resting location for mosquitoes. The simple mud hut was built to mimic the shelters used by communities to keep cows and not to represent an indoor set up.

Mosquitoes

All experiments were performed using insectary-reared unfed mated *An. arabiensis* females aged 3 – 9 days post eclosion. It was assumed that mosquitoes at this age would have mated (Charlwood & Jones, 1979). The *An. arabiensis* colony was established in March 2010, originating from individuals collected in Lupiro village within the Kilombero valley. It is reared routinely inside under natural temperature and 12: 12 h light: dark photoperiod of that area. Larvae were fed Tetramin® fish food and adults maintained on 6% glucose solution and human blood (on voluntary basis). Mosquitoes were starved of sugar and water six hours prior to release in the experiments.

Experimental procedures and study design

Five experiments were conducted between May 2012 and September 2013: first, to investigate the existence of PPF autodissemination from PPF-treated clay pots to the breeding habitats by contaminated mosquitoes; second, to confirm that the observed PPF contamination at the experimental breeding habitats was mosquito-borne; third, to investigate mosquito' resting site preferences inside the SFS; fourth, to measure the proportion of mosquitoes resting inside the clay pots that were subsequently able to contaminate oviposition sites; fifth, to measure amplification of autodissemination from limited numbers of treatment points to a greater number of breeding sites.

Experiment 1: Evaluation of PPF-treated clay pots for the delivery of pyriproxyfen to resting adult female mosquitoes for subsequent autodissemination

In every replicate of this experiment, 1500 – 5000 adult female *An. arabiensis* were released inside an SFS chamber, where a cow tethered inside the mud hut was provided for blood feeding, clay pots as resting sites during egg development, and water

containers as oviposition sites. Clay pots have been used for sampling wild *An. arabiensis*, as adult females of this and other species will rest within these and similar vessels (Odiere *et al.*, 2007; Wong *et al.*, 2013).

Eight 10 L clay pots were placed on the ground: 5 around the perimeter of the SFS chamber and the other 3 around the walls of the mud hut. Each pot was lined with black cotton that had been dampened with water and dusted with PPF powder (0.2 – 0.3 g AI per clay pot; Sumilarv®, Sumitomo Chemical Co. Ltd., Japan). Dusting was done by unevenly sprinkling 2 – 3 g of 10% AI PPF powder over all surface of dampen cotton cloth using a makeup/painting brush. The cotton cloth was treated with PPF after being attached inside around the circumference of clay pot using 3 mm aluminium wire to ensure maximum containment of PPF powder (Fig 4.3.1C). Pots were dried for 24 h, facilitating the PPF powder to attach lightly to the fabric while not hindering its pickup by mosquitoes that contacted it. Two identical artificial breeding habitats (2.5 L plastic basins, 21 cm in diameter; filled with 250 g of soil and 2L of water; water levels were replenished as required) were buried with the rim at ground level, 5 m apart and between 1 and 8 m from clay pots (Fig 4.3.1).

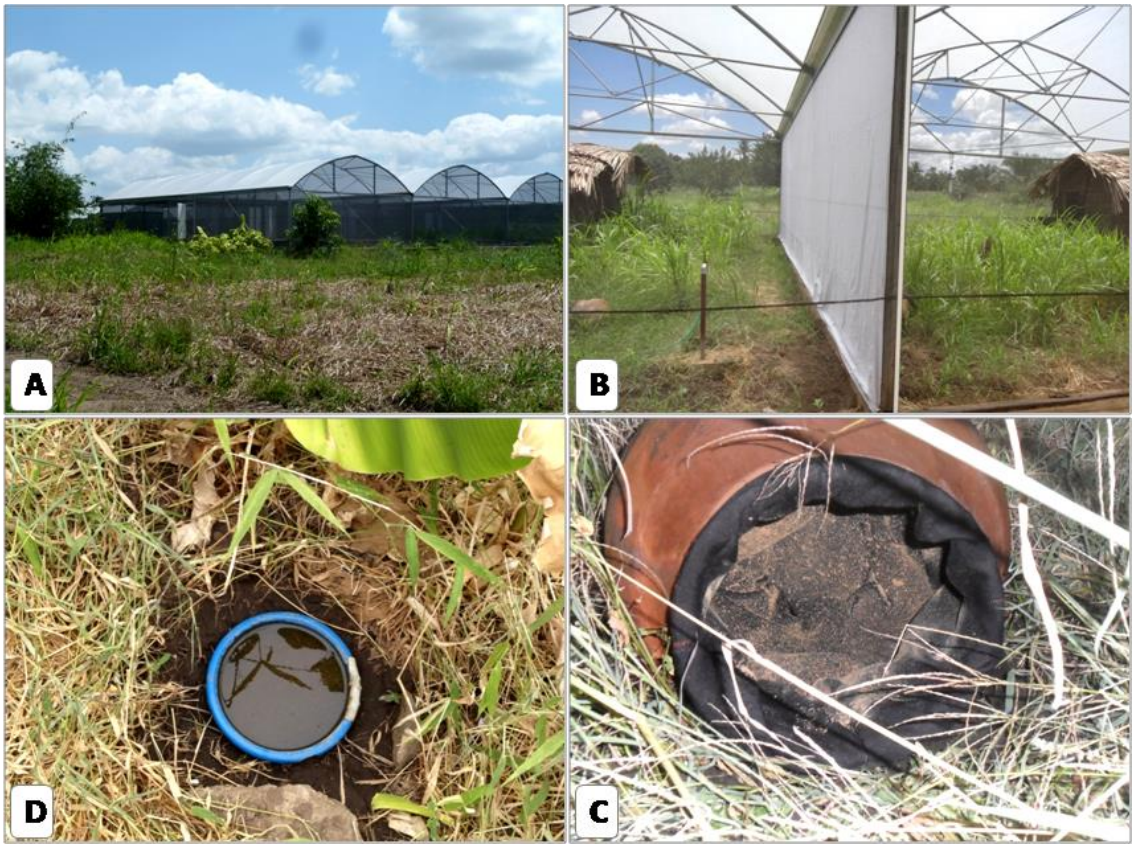

Figure 4.3.1: (A) The semi-field system used in experiments; (B) adjoining chambers with huts for housing bait cows visible in each; (C), pyriproxyfen-treated cloth interior of a clay resting pot placed on the ground within a chamber; (D) plastic basin sunk in the ground within a chamber to provide the artificial habitat for egg laying.

The experiment was run for 25 days following release of the mosquitoes, to allow 10 days until the first pupae developed and a further 15 days to harvest all pupae from the artificial aquatic habitats that successively developed from eggs laid by released mosquitoes. The breeding habitats were visually examined daily for the presence of eggs and larvae to confirm if mosquitoes visited the habitats. Each day, pupae developed from deposited eggs were removed, counted and transferred to an insectary

where they were maintained under the cage in cups containing water from the habitats until they emerged as adults or died.

Control experiments were run simultaneously in a separate chamber using an identical protocol but without any PPF application to the cotton lining of the clay pots. A total of 6 replicates of both treatment and control experiments were run, over a period of 6 months. Treatment and control chambers were separated by a distance of 3.2 m and, to avoid PPF contamination of the control chamber, the same SFS chambers were used in all replicates for treatment and control. Of importance, control and treatment were not rotated but fixed between chambers, when one replicate was on-going in a pair of control and treatment chambers; the other pair of control and treatment chambers was put into use. Where control and treatment chambers were adjacent to each other, a panel of white cloth was mounted on one side of partition net to prevent movement of PPF particles between chambers. A break of at least seven days between replicates minimized the chance of any mosquitoes surviving from the previous replicate. PPF contamination between replicates was minimized by spraying the chamber structure, the hut and vegetation with water, new plastic basins were used and cow were thoroughly cleaned by washing with only water without soap before each replicate. Successful contamination and dissemination was evaluated by comparing the differences in pupal mortality and emergence inhibition from the basins between treated experiments and controls.

PPF contamination of water in the experimental breeding habitats was investigated further by two methods. First, immediately after recording first stage larvae in the breeding habitats (typically 5-8 days after mosquito release), three 150 ml water sub-samples were collected from each habitat and transferred to separate 250 ml glass

beakers. Twenty 2nd or 3rd instar *An. arabiensis* larvae taken from the laboratory colony (*i.e.* fresh uncontaminated individuals) were placed in each beaker and daily mortality and emergence rates recorded until all were dead or had emerged as adults. The procedures were repeated twice, i.e. only in two consecutive experiments of the 6 experimental replicates.

In a second bioassay, at the termination of each experimental replicates (*i.e.* day 25 following initial introduction of adult females) and after pupation of all larvae and removal of all pupae, 250 second or third instar *An. arabiensis* larvae taken from the laboratory colony (*i.e.* fresh uncontaminated individuals) were introduced in each breeding habitat (assumed to be contaminated with PPF from previously released adults) and daily mortality and emergence recorded until all were dead or had emerged as adults. The procedures were repeated twice, i.e. only in two consecutive experiments of the 6 experimental replicates.

Experiment 2: Confirmation that pyriproxyfen contamination of breeding habitats was mosquito-borne

To examine whether the PPF impact on adult emergence from the breeding habitats observed in the previous experiment might have resulted from passive carriage by wind currents, or by other organisms (*e.g.* other invertebrates, amphibians, rodents, etc.), two tests were conducted using the setup of experiment 1.

In the first test, 250 second or third instar *An. arabiensis* larvae taken from the laboratory colony were introduced in the two breeding habitats with fresh water and soil in both treatment and control SFS chambers, which had been prepared exactly as

described for Experiment 1. No adult mosquitoes were released in either chamber. The daily pupation, mortality and emergence rates were recorded until all pupae were dead or had emerged as adults. The experiment was allowed to run until all had pupated.

In the second test, the chambers used for treatment and control were reversed, *i.e.* the control was run in the chamber previously used for treatment and *vice versa*. A total of 5000 adult female mosquitoes were released in each chamber and two replicates of the second test were conducted and breeding habitats productivity were monitored as described in experiment 1.

Experiment 3: Mosquito resting site preference inside the semi field systems

To determine the proportions of released mosquitoes that rested inside the clay pots in the experimental setup, adult female mosquitoes were released inside treated and control SFS chambers, as described for experiment 1. On each morning over the following three days (an average period for eggs development before mosquito visits the habitats to lay eggs), all mosquitoes found resting inside clay pots and walls and ceiling of the cattle hut were collected using mouth aspirators, counted and recorded as either fed or unfed. The experiment was repeated twice, first with 2000 mosquitoes and then with 4000 mosquitoes released in each chamber (released mosquitoes were increased in the second replicate to increase the proportion of mosquitoes to be recaptured).

Experiment 4: Determining contamination rates of the* Anopheles arabiensis *population resting inside clay pots

To estimate the proportion of *An. arabiensis* contaminated with PPF in this setup, 5000 unfed adult female mosquitoes were released inside both treated and control SFS

chambers, where only clay pots were treated with PPF as described in experiment 1. On each of the three mornings after release, a maximum of 60 mosquitoes (30 from each of the resting sites) were collected inside all clay pots and mud huts (walls and ceiling) and assessed for their feeding status. Following resting behaviour in mosquito after acquiring a blood meal, mosquitoes were collected 36 h after release to ensure that high proportion was blood-fed. Individual mosquitoes were collected with separate mouth aspirators and held in a plastic cup (approximately 30 – 60 minutes) to avoid cross-contamination until use. Mosquitos were killed by refrigeration and each mosquito was suspended in 50 ml of water containing 10 third stage larvae of laboratory-reared *An. arabiensis* to monitor larval mortality and pupa emergence inhibition, over 12 days. In addition, the plastic collection aspirators were rinsed with water to remove any possible PPF particles and clean water added to a total volume of 50 ml in which 10 third-stage larvae were suspended, and followed up as just described. The experiment was repeated twice.

To calculate the proportion contaminated, a maximum mortality threshold above an upper 95% CI from a control section was set. Thus an observed larval or pupal mortality in a bioassay cup above the set threshold in the treatment arm, implied that the suspended mosquito was contaminated. The estimated contamination in the treatment section was corrected using Abbot's formula (Abbott, 1925), where the control larval mortality was greater than 5%. Corrected contamination = [% Contamination – % mortality in control) / (100 – % mortality in control)] × 100.

Experiment 5: Determination of autodissemination efficiency with fewer treatment points and more breeding habitats

The impact of only 1 – 2 treated clay pots with PPF to deliver PPF contamination to resting mosquitoes was determined in two tests. In the first test, only two of the eight

pots were treated with PPF and compared to a control section where all eight pots remained untreated. A batch of 5000 unfed female *An. arabiensis* was released once in a control and treatment chambers.

In the second test, only one pot was treated with PPF in treatment section, and 5000 unfed female *An. arabiensis* were released in a control and treatment chambers, in three consecutive batches of 2000, then 2000 and lastly 1000, with an interval of one day between releases. The rationale of releasing different mosquito batches was to facilitate multiple visiting events of mosquitoes to the habitats, which were likely to occur when mosquitoes are released in different batches rather than single batch. This also mimic what is likely to happen in nature where different mosquitoes are likely to transit in the same clay pots over time. In both tests, six breeding habitats were provided, and pupae collected from individual habitat were monitored as described until all were dead or had emerged as adults.

Data analysis

All data were analysed using R v2.12.2 (R Core Team, 2013) and the lme4 package (Bates *et al.*, 2013) for generalized linear mixed effects models. A visual inspection of the plots of error versus fitted values distribution was used to determine the best model fit. The model was then tested with each nested parameter separately to determine the underlying variation. SFS section was found to count for a lot of variation and therefore, required the full nested model to be retained. The differences in the total number of pupae collected and proportion emerged between control and treatment SFS chambers were determined with Poisson and binomial distribution respectively using a best-fit generalized linear mixed effect model. While treatment groups (with/without PPF) were classified as fixed effect in the model, experimental replicates, numbers of mosquito

released, numbers of larvae, total numbers of pupae collected per control and treatment chambers, and numbers of breeding habitats per control and treatment chambers were assigned as random effects for the autodissemination of PPF and larval bioassay data.

4.4 Results

Experiment 1: Evaluation of PPF-treated clay pots for the delivery of pyriproxyfen to resting adult female mosquitoes for subsequent autodissemination

The results of the experiments measuring the impact of PPF-treated resting pots on emergence from nearby breeding habitats are summarized in Figure 4.4.1. In the 6 replicates carried out, there was no difference in the mean number (95%CI) of pupae collected from the treatment group 717 (94 – 1340) compared with the control group 590 (369 – 811, $p = 0.579$) [Fig 4.4.1A], suggesting that oviposition behaviour of mosquitoes after PPF treatment was not affected by the treatment. The mean (95%CI) number of adult emerged from collected pupae were high in the control group, 558 (356 – 760) compared with the treatment group, 131 (25 – 286, $p < 0.0001$) [Fig 4.4.1B]. Similarly, an average proportion (95%CI) of adult emerged per experimental replicate was higher in the control group, 0.95 (0.56 – 1.34) compared to the PPF treatments group, 0.21 (0.09 – 0.51, $p < 0.0001$) [Fig 4.4.1C]. Low adult emergence rate observed in the treatment chambers strongly suggest the occurrence of PPF autodissemination events mediated by gravid female mosquitoes attempting to oviposit.

In the laboratory bioassay measuring the effect of breeding habitat water on development of larvae, an average proportion (95%CI) of 0.99 (0.95 – 1.03) emerged to

adults in water from the controls, while only 0.62 (0.16 – 1.07) emerged from the treatment group (p = 0.003), [Fig. 4.4.1D].

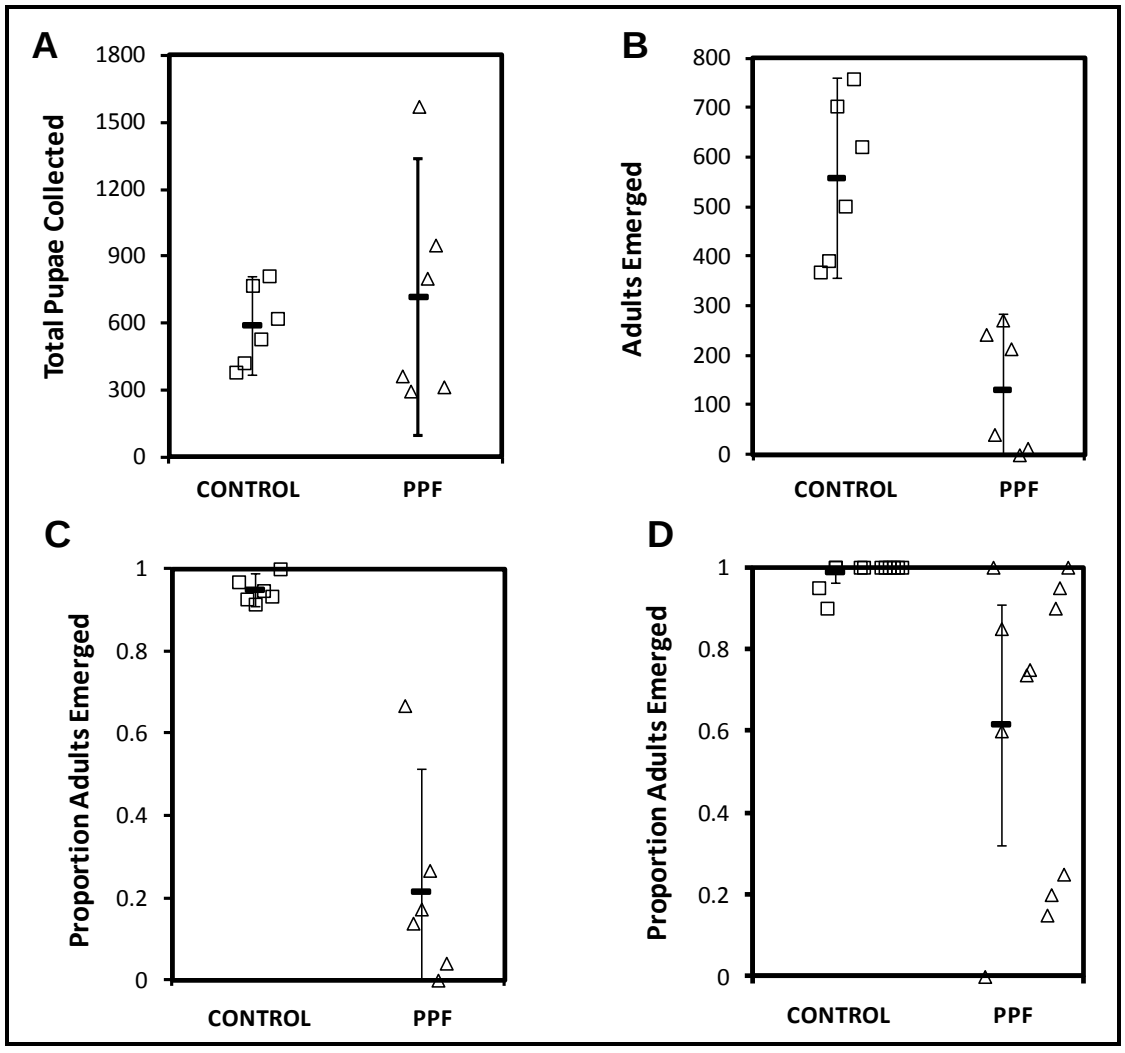

Figure 4.4.1: Number of pupae produced (A), adults emerged (B), proportion of adult emerged (C) in the breeding habitats and proportion of adult emerged from larval

bioassay on water samples from control and PPF - treated sections (D). The line represents 95%CI across the mean.

In the second larval bioassay, laboratory-reared larvae placed in the breeding habitats after the clay pot experiment ended, had lower average (95%CI) emergence proportion in the treatment chamber, 0.16 (0.07 – 0.39) compared to the control chamber, 0.97 (0.92 – 1.02, $p < 0.0001$), which confirm auto dissemination of PPF to the breeding sites. Attrition of introduced larvae due to predation and other natural causes were similar in both groups (315/500 and 359/500 larvae accounted for in control and treated groups respectively) and there was no evidence of any increase in larval mortality due to PPF ($p = 0.773$). All introduced larvae emerged successfully or died within 20 days of the start of the experiment.

Experiment 2: Confirmation that pyriproxyfen contamination of breeding habitats was mosquito-borne

In the first test of experiment 2 carried out, laboratory-reared larvae were placed in the breeding habitats of control and treatment chambers, prepared as described for experiment 1, except that here, no mosquitoes were released. The result of the single replicate showed that there was no difference in average (95%CI) proportion adult emergence per day between treatment, 0.63 (0.39 – 0.87) and control sections, 0.69 (0.37 – 1.01, $p < 0.0001$). The total number of pupae collected from breeding habitats in the control (n= 379) and treatment (n= 392) chambers were not different ($p>0.05$).

In the second test of experiment 2, the design of experiment 1 was repeated by releasing 5000 adult female mosquitoes in each experimental chamber except here, the control

was run in an SFS chamber previously used for PPF treatment, and *vice versa* for the treatment. Average (95%CI) adult mosquito proportion emergence were higher in the control group, both before, 0.95 (0.56 – 1.34) and after, 0.72 (0.38 – 1.06) the locations were switched compared to the treatment, 0.21 (0.09 – 0.51) and 0.05 (0.02 – 0.12, $p < 0.0001$). The results of both experiments demonstrated that reductions in emergence rates in the breeding habitats occurred only when adult mosquitoes were present in the PPF-treated chamber.

Experiment 3: Mosquito resting site preference inside the semi-field systems

All recaptured mosquitoes from different resting sites were blood fed. A mean (95%CI) recapture rate of 0.39 (0.37 – 0.41) was achieved in all replicates, with no difference seen between control, 0.38 (0.37 – 0.39) and treatment groups, 0.39 (0.37 – 0.41, $p = 0.266$). Although, total number of mosquitoes recaptured increased when the number of mosquitoes released was greater ($p = 0.006$), the proportion of mosquito recaptured remains similar between replicates ($p = 0.543$). As figure 3 shows, the majority of mosquitoes were collected from the ceiling and walls within the hut with 17% found within the resting pots.

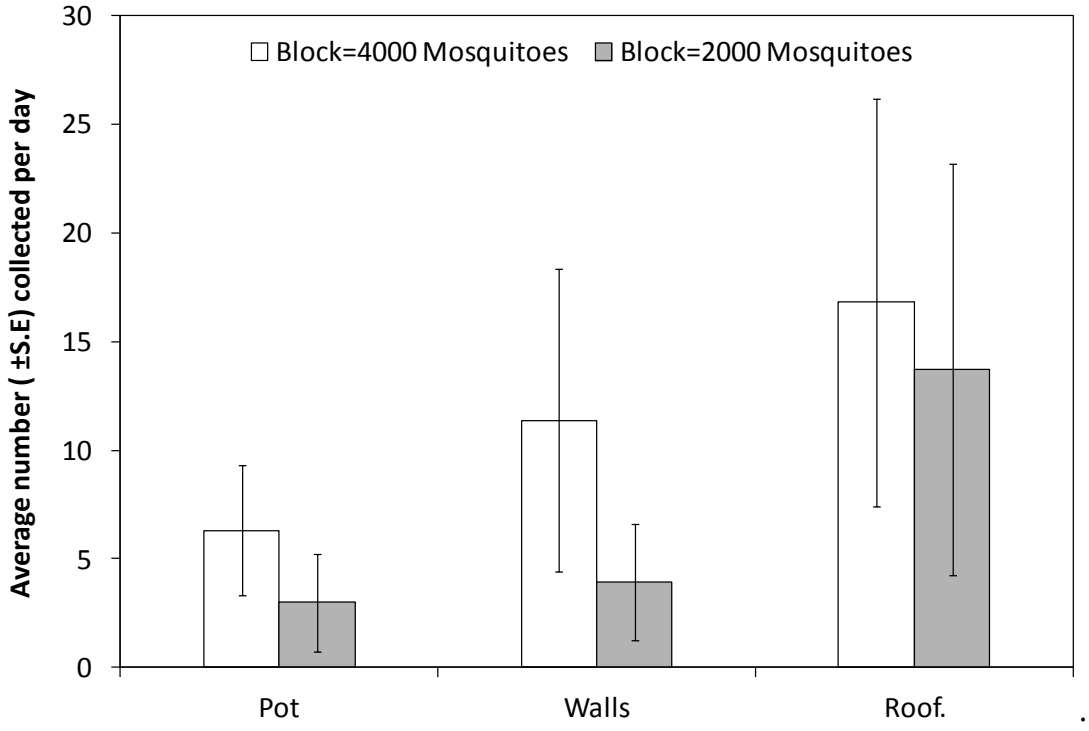

Figure 4.4.2: Average number (±SE) of mosquitoes collected at different resting sites inside the Semi-field systems.

Experiment 4: Determining contamination rates of* Anopheles arabiensis *population resting inside clay pots

As determined by their ability to inhibit adult emergence in a laboratory bioassay, all mosquitoes collected inside treated clay pots were PPF-contaminated, while approximately 72% of those found resting in the hut within the treated chamber, were contaminated. Average mosquitoes from PPF treated clay pots and huts caused 0.01 (0 – 0.02) and 0.52 (0.46 – 0.58) average ((95%CI) adult emergence proportion from exposed larvae respectively in larval bioassay. In the control chamber, an average (95%CI) of 0.93 (0.85 – 1.01) of all larvae successful emerged to adults during larval

bioassay using mosquitoes collected from clay pots and cattle shed in the control chamber.

Experiment 5: Determination of autodissemination efficiency with fewer treatment points and more breeding habitats

In both tests, impacts of PPF on pupal emergence were observed in all habitats in the treated chambers. When two clay pots were tested, the mean (95%CI) pupae collected from all breeding habitats were similar between control (52.57 ± 26.98) and treatment (62.92 ± 34.15) chambers, (p = 0.522). Similarly, the mean number of pupae collected was not different between control (100.34 ± 19.65) and treatment (104.88 ± 23.66) chambers when one clay pot was tested (p = 0.883). There was reduction in mean proportion (95%CI) of emerged adults in the treated chambers when two, 0.33 (0.15 – 0.51) or only one clay pots, 0.34 (0.21 – 0.47) clay pots were treated compared with the respective controls, 0.82 (0.70 – 0.94); 0.98 (0.96 – 1.00, $p < 0.0001$). Furthermore, the mean (95%CI) number of adult emerged from collected pupae was higher in the control group, 97 (60.3 – 133.7) than in a treatment group, 41 (13.7 – 68.3) when one clay pot was tested, $p < 0.05$). Similarly, when two clay pots were tested, control group had higher adult emergence, 44 (17.6 – 60.4) compared to the treatment group, 28 (11.4 – 44.6, $p < 0.05$).

4.5 Discussion

Previous field studies have demonstrated the potential for the autodissemination technique when applied to free flying population *Aedes* mosquito species under field settings (Devine *et al.*, 2009; Caputo *et al.*, 2012). In this study, we also proved the occurrence of PPF autodissemination using captive populations of malaria vector *An.*

arabiensis under semi-field settings. Overall, autodissemination of PPF by *An. arabiensis* inhibited 76.5% of adult emergence, which is approximately to the control level of 80% recommended by WHOPES for controlling malaria vector juvenile stages (WHO, 2005) under semi-field conditions. In some cases, for example experiment 1, Fig. 2C, total emergence inhibition in PPF-treated sections was achieved with no single adult mosquito emerging from these habitats. Larval bioassays showed a lower adult emergence rate in the treatment sections compared to the control further confirming the delivery of PPF to the breeding habitats in all experiments. More importantly, by introducing insectary larvae directly in the habitats, an even lower emergence rate was observed compared to the control sections. This could be due to the presence of organic matter in the breeding habitats that would allow PPF adsorption and could prolong its persistence in aquatic habitats (Schaefer *et al.*, 1988).

Though not clearly elucidated by the data presented here, it remains as a limitation of current study, that wide range and many number of mosquito released (1500 – 5000) in relation to number and size of breeding habitats might have affected the productivity of the habitats provided (pupae as a proxy indicator) by causing high larval mortality in the habitats due to overcrowding factors (Ye-Ebiyo *et al.*, 2003), and result in relative small number of pupae collected. However, the reason for a wide age range of mosquito released was due to a shortage of mosquitoes of the same age whereas many mosquitoes were released to make sure that our experiments were not confounded by shortage of mosquitoes following natural mortality and scavenging natural enemies such as ants, spiders and others which were likely to be present in the SFS.

Surprisingly, variations in the numbers of mosquitoes released did not affect the proportions of adults that ultimately emerged from the pupae in the contaminated

aquatic habitats, the inclusion of the numbers of mosquitoes released resulted in the best model. Since the numbers of mosquitoes visiting contamination stations would have differed between experiments and replicates, variation in mosquito numbers released and pupae collected were described as random rather than fixed factors.

Importantly, similar emergence rates recorded in the absence of mosquitoes between control and treatment chambers indicated that passive transfer of PPF (which might have confounded or potentially artificially enhanced any observed impact) did not occur at any stage in these studies. In addition, similar impact of PPF on adult emergence observed as the results of released mosquitoes before and after switching locations of control and treatment chambers confirmed that dissemination by ovipositing mosquitoes alone was responsible for transfer of the effective dosages of PPF to the breeding habitats.

In assessing potential mosquito resting sites for targeting with PPF inside SFS, similar number of mosquitoes recaptured between control and treatment groups indicated that PPF does not repel resting mosquitoes. Overall, the proportions of recaptured adult female mosquitoes were few; this might have been caused by restricted collections from few designated places, and missed those resting in the vegetation grown inside the experimental chambers. High number of mosquitoes collected from the wall and ceiling of the mud hut compared to the clay pots, highlight the potential of targeting these sites with PPF.

In the experiment to assess autodissemination efficiency with fewer treatment points and more aquatic habitats, it was demonstrated that only one treated resting pot competing

with alternative untreated resting sites including seven clay pots and resting sites within the mud hut was sufficient to inhibit 58% adult emergence in six breeding habitats via ovipositing mosquitoes alone. These findings are very promising and highlight the potential that autodissemination offers for amplification of limited numbers of treatment points to significant levels of effective breeding habitat treatment coverage. Clearly, field-based experiments and mathematical modelling should now be designed to investigate this further and establish the relationship between contamination stations and habitats coverage.

The mechanism of PPF delivery to mosquitoes is crucial for the overall success of the autodissemination technique (Devine *et al.*, 2009; Gaugler *et al.*, 2011; Caputo *et al.*, 2012). In this study, the use of clay pots as a point source for PPF application effectively delivered PPF to the mosquitoes resting within and at rates sufficient to enable autodissemination. The attractiveness and usefulness of clay pots as an outdoor and indoor sampling tool for malaria and other disease vectors as well as a delivery tool for entomopathogenic fungi has been described elsewhere (Odiere *et al.*, 2007; Farenhorst *et al.*, 2008; van den Bijllaardt *et al.*, 2009). Although absolute numbers of mosquitoes resting inside clay pots are relatively low, these tools are considered to be efficient for sampling blood fed mosquitoes compared to many other sampling techniques (Wong *et al.*, 2013). The results presented here indicate that this simple and affordable method has additional potential in vector control.

When aquatic habitats are limited, mosquitoes that are contaminated in clay pots and then carry lethal doses of PPF to their aquatic habitats also affect the offspring of uncontaminated mosquitoes. Thus, contaminated adults amplify the impact of their own contamination by affecting the offspring of all mosquitoes that share the contaminated

mosquito's breeding site (Fillinger *et al.*, 2004; Harris *et al.*, 2011). Although not investigated in this study, field deployment of autodissemination approach is predicted to be affected by number of mosquitoes visiting the habitats, size of the breeding habitats and distance of the habitats from PPF contamination stations and this necessitate detailed assessment of these parameters.

Moreover, targeting only the clay pots with PPF resulted in the effective contamination of mosquitoes that were ultimately collected from the huts, suggesting that blood-fed mosquitoes move between resting sites during that phase of their gonotrophic cycle. This is clearly an advantage in terms of optimizing the effect of PPF through further "coverage amplification of the habitats" whereby PPF is likely to be delivered to many breeding habitats by PPF-contaminated mosquitoes using few habitats, and potentially might act to reduce the number and costs of contamination stations required (Devine & Killeen, 2010). Clay pots, by providing shelter from rain and sunlight, might also prolong the lifespan of single PPF treatments, an important consideration in any 'insecticide'-based program. However, it should be noted that this experimental design provides only estimates, rather than actual numbers, of mosquitoes that rest or pass through clay pots and of whether they are contaminated or not.

The impact of PPF varies at different stages of the mosquito's life cycle. Previous work has shown that mosquitoes that are contaminated within 24 h of a bloodmeal become sterilized and do not lay eggs (Harris *et al.*, 2013; Lwetoijera *et al.*, 2014, See Chapter 5, Section 5.4) but this sterilization effect does not occur when exposure to PPF occurs beyond 24 h after the bloodmeal. However, in the experiments reported here, the test mosquitoes produced large numbers of developing offspring in the artificial habitats provided, suggesting that the clay pots set outside the cattle sheds, were not visited by

blood-fed mosquitoes until sometime after completion of feeding when egg-maturation was underway. If so, then it was while resting outdoors after the bloodmeal that these mosquitoes were contaminated, and targeting this stage of the gonotrophic cycle (i.e. >24 h after blood feeding) may maximize delivery of PPF to the breeding habitats (Gaugler *et al.*, 2011). Alternatively, if PPF-contamination occurred immediately after or within 24 h of bloodfeeding, then it suggests that these PPF-sterilized mosquitoes, despite not being gravid, went on to visit the breeding habitats where they prevented emergence of the next generation of mosquitoes from the eggs laid by uncontaminated adults.

Although a key necessity for its success is the development of efficient contamination stations, a role performed well by the clay pots in the experiments reported here, the autodissemination technique. This can potentially target both indoor and outdoor biting mosquitoes, susceptible and pyrethroid resistant mosquito strains at their larval habitat, with impacts on adult mosquito density and malaria transmission (Macdonald, 1957; Devine & Killeen, 2010; WHO, 2012b). The integration of this method of control with current vector control measures (LLINs and IRS) could help in the control of outdoor biting vectors such as *An. arabiensis* as well as providing an approach to managing insecticide resistance (Haji *et al.*, 2013). The autodissemination of insecticides by adult mosquitoes for the control of malaria is likely to work better in the dry season when the breeding habitats are few and stable with reduced water flushing (Fillinger *et al.*, 2004; Devine & Killeen, 2010). With recent development of highly potent formulations up to 10% AI PPF dust, it might be possible to effectively contaminate greater volumes than current possible using malaria vectors and other mosquitoes that share the habitats with anophelines mosquitoes.

This is the first study to investigate the potential for using PPF autodissemination for the control of *An. arabiensis*, one of the efficient African malaria vectors. The results are very promising and indicate that this approach offers an opportunity to be considered amongst future malaria control strategies in Africa. Before its full potential can be assessed, further vector studies will be required in key areas: 1) the effectiveness seen in these semi-field experiments must be demonstrated under full field conditions; 2) quantitative studies on 'amplification' are required to determine the numbers and densities of treatment points required to deliver effective control at breeding sites; 3) investigations of impacts on other species sharing the breeding sites, including other vectors, nuisance mosquitoes and non-target species.

CHAPTER 5

STERILIZATION OF MALARIA VECTORS *ANOPHELES ARABIENSIS* USING PYRIPROXYFEN UNDER SEMI-FIELD SETTINGS

The results reported in this chapter have been published in a slightly different form as Lwetoijera *et al. American Journal of Tropical Medicine and Hygiene 2014, Volume 90, Issue 5, 852-855*

5.1 Abstract

Introduction: One of the main challenges to malaria elimination is the resilience of vectors, such as *Anopheles arabiensis*, that evade lethal exposure to insecticidal control measures or express resistance to their active ingredients. This study investigated a novel technology for population control that sterilizes mosquitoes using pyriproxyfen, a juvenile hormone analogue.

Methodology: Females of *An. arabiensis* were released in a semifield system divided into four equal sections, and each section had a mud hut sheltering a tethered cow providing a blood source for mosquitoes. In all sections, the inner mud hut walls and roofs were lined with black cotton cloth. In one-half of the sections, the cloth was dusted with pyriproxyfen.

Results: 96% (89.3 – 102.7%) reduction in adult production was achieved in pyriproxyfen-treated sections compared with control sections.

Conclusion: This high level of control can be exploited to design new vector control strategies that particularly target existing behaviourally resilient and insecticide-resistant populations.

5.2 Introduction

Current frontline malaria vector control interventions, such as long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) have contributed greatly to the recent successes in malaria control (WHO, 2014). However, these tools are more effective against vector species that primarily feed indoors on humans and rest indoors. They are less effective against outdoor feeding and resting mosquitoes. *Anopheles arabiensis*, currently mediating most of the residual malaria transmission in east Africa, (Bayoh *et al.*, 2010; Russell *et al.*, 2011b) is not optimally controlled by LLINs and IRS, because it commonly feeds outdoors on humans or cattle, rests outdoors, and can enter but then rapidly exit houses containing these products without exposure to lethal doses of their active ingredients (AIs) (Russell *et al.*, 2011b; Okumu *et al.*, 2013). Another challenge to malaria vector control is the development of resistance in malaria vectors against all classes of insecticides currently used for LLINs and IRS, particularly pyrethroids, the most widely used and the only class approved for use in bednets (WHO, 2012a).

Pyriproxyfen (PPF) is a juvenile hormone analogue that traditionally has been used in aquatic habitats to prevent mosquito larvae and pupae from developing into adults. However, it can also sterilize adult mosquitoes on contact (Ohashi *et al.*, 2012; Harris *et al.*, 2013; Ohba *et al.*, 2013, Mbare *et al.*, 2014). This study builds from our previous work performed in laboratory conditions showing that *An. arabiensis* mosquitoes were particularly vulnerable to sterilization immediately after blood feeding (Harris *et al.*, 2013). Adult mosquitoes can also transfer PPF from resting sites to breeding sites to interfere with immature development (Devine *et al.*, 2009; Caputo *et al.*, 2012). Here, we show, an operational practicable means of controlling a free-flying captive population of *An. arabiensis* using PPF.

5.3 Methods and materials

This study was carried out at Kining'ina village (8.11417°S, 36.67484°E), rural southern Tanzania inside a semi-field system (SFS) with walls consisting of netting only, and therefore, the microclimate inside it closely resembled the natural environment outside of it (Ferguson *et al.*, 2008). The SFS was divided into four equal sections, with a space volume of approximately 417.5 m^3 each. In each section, a mud hut sheltering a tethered cow, eight clay pots, and four plastic basins with soil and water were designed to provide blood, resting, and oviposition sites for mosquitoes (Figure 5.3.1). In all sections, the inner mud hut walls and roofs were lined with black cotton cloth, and in one-half of the sections, the cloth was dusted with PPF powder (0.6–0.8 g AI/m2). In total, 5,000 unfed 3 to 9 days old insectary-reared *An. arabiensis* females, previously caged with equivalent numbers of males, were released per section, with a cow to provide blood for the first 3 days only. Mosquitoes used in the experiments were starved 6 h before release. Therefore, they fed on the cow, and after 3 days, a solution of 6% glucose was set up at multiple locations inside the SFS for sugar feeding.

Figure 5.3.1: Semi-field system set up: Semi-field system (A) with mud huts built inside each section to shelter a cow (B), and breeding habitats (C). Mud huts were lined with black cloth and dusted with PPF in treatment sections (D), (Lwetoijera *et al.*, 2014, See Chapter 4, Section 4.3).

These mosquitoes remained in the SFS to complete their gonotrophic cycle. All pupae that subsequently developed from the aquatic habitat were removed, counted, and reared in small cages to monitor the numbers of emerging adults and therefore, the impact of PPF exposure on the mosquitoes' ability to produce viable offspring. Seven days after larvae were observed in the habitats, 150 mL water were collected from every habitat using a glass beaker to determine whether PPF had been transferred to these habitats by contaminated mosquitoes during oviposition (Devine & Killeen, 2010). To assess the presence of PPF in each beaker, larval bioassays were conducted using second and third

instar larvae from the insectary. Twenty *An. arabiensis* larvae were introduced in each beaker and monitored daily until all larvae and pupae had either died or developed and emerged to adults.

Five replicates each of the control and treatment were completed in three separate experiments in the following setup. In the first experiment, two replicates (treatment and control) were run (5,000 × 4= 20,000 mosquitoes); in the second experiment, two replicates (treatment and control) were run (5,000 × 4 = 20,000 mosquitoes), and in the third experiment, one replicate (treatment and control) was run (5,000 × 2 = 10,000 mosquitoes), making a total of 50,000 mosquitoes reared and released.

Data analysis

All statistical analyses were conducted in R v2.12.2 (R Core Team, 2013) using the lme4 package for generalized linear mixed effects models (Bates *et al.*, 2013). To determine any differences in the numbers of pupae or adults produced between treated and control sections, a generalized linear mixed effects model with a Poisson distribution and a log link function for count data was performed. The treatment group (control or PPF) was classified as a fixed effect, whereas SFS section nested within experiment was put in as a random effect as per the experimental design. A visual inspection of the plots of error versus fitted values distribution was used to determine the best model fit by To determine the best model, we performed a model reduction by comparing the full nested (including all parameters from the experiment) model with reduced model (i.e. trying different combinations with one parameter removed at each time). The model was then tested with each nested parameter separately to determine the underlying variation. SFS section was found to count for a lot of variation and therefore, required the full nested model to be retained. The differences in pupal emergence rates in both SFS habitats and

the bioassays experiments were compared by fitting a generalized linear mixed effects model with binomial error structure and logit link function for proportion data. The data were fitted to a model including treatment as a fixed effect and breeding habitat nested within SFS section nested within experiment as a random effect as per the experimental design. Visual inspection of the plots of error versus fitted values distribution was used to determine the best model fit. Model reduction was conducted by removing nested parameters one by one; however, the full nested model was retained.

5.4 Results

Experiments lasted between 11 and 16 days from release of adult mosquitoes to collection of the last pupae in the breeding habitats. An overwhelming 95% reduction in pupal production and 96% reduction in adult production were achieved in PPF-treated sections compared with control sections (Figure 5.4.2A and B). In four of five replicates, exposure to this juvenile hormone analogue completely sterilized all mosquitoes; not a single pupae or new adult was seen. The few adults emerging from a PPF-treated section in the fifth replicate probably resulted from mosquitoes that had been contaminated with PPF but were not completely sterilized and managed to lay eggs. The pupae collected in the PPF-treated section showed a lower emergence rate (82%; 164/201) compared with the control (95%; 4,132/4,349; χ^2 [1] = 65.6, P < 0.001) (Figure 2C). This result suggested possible PPF-autodissemination to the breeding habitats by contaminated mosquitoes. However, bioassays with insectary larvae reared in water from the control and PPF-treated habitats showed similar emergence rates (Figure 5.4.2D). A similar pattern has been observed in recent studies (Lwetoijera *et al.*, 2014, See Chapter 4, Section 4.3), where PPF activity is more pronounced in breeding habitats with organic material than water samples kept in glass beakers.

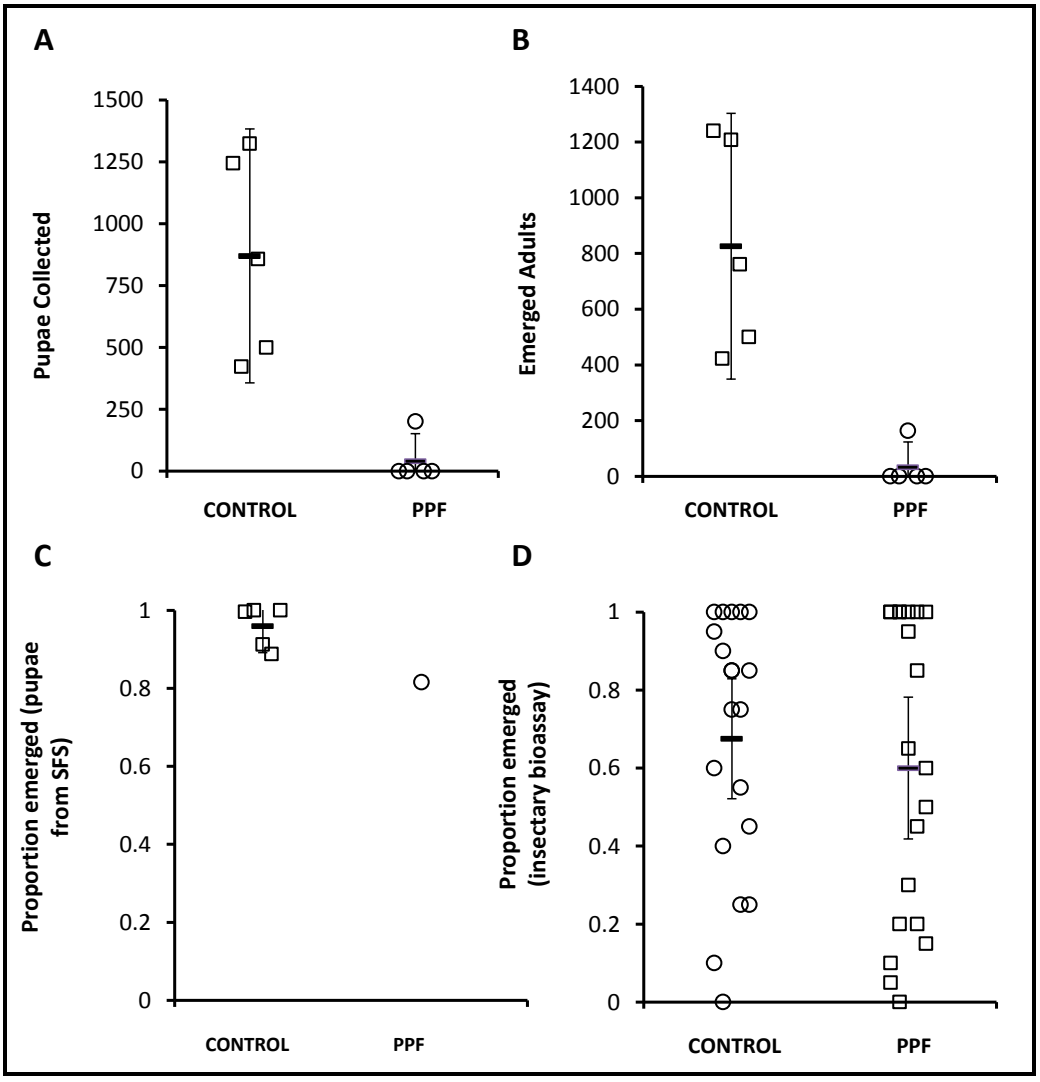

Figure 5.4.2: Impact of pyriproxyfen on adult mosquito emergence: Number of pupae produced (A) and adults emerging (B) from control and treated sections; and the proportion of adult emergence in SFS (C) and insectary bioassays (D).

5.5 Discussion

The high level of sterilization seen in this key malaria vector reveals an exciting new opportunity for malaria vector control. This technology is a practical, novel technology for population control that sterilizes mosquitoes rather than killing them. It offers the chance to develop new tools that are not compromised by existing resistance mechanisms. New paradigms in vector control are in great demand, especially for vectors such as *An. arabiensis* (Kitau *et al.*, 2012; Okumu *et al.*, 2013) and other anophelines (Elliott, 1972) that exhibit flexibility in feeding and resting indoors and outdoors and minimize their contact with conventional adulticides applied indoors.

The findings reported here have limitations given that the experiments were conducted within an enclosed environment on insectary-reared mosquitoes that had never been subjected to insecticide pressure but also PPF exposed mosquitoes were not dissected to assess abdominal egg retention and egg viability. However, this technology can be readily adapted in natural conditions to assess its impact on wild populations of *An. arabiensis*. Treating walls and roof linings with PPF comprehensively sterilizes captive populations of free-flying *An. arabiensis*, making it a potential control tool and complement to LLINs and IRS. For IRS formats, durable wall linings with PPF can be designed, for controlling indoor resistant malaria vectors. PPF-treated materials could be deployed outdoors in areas where mosquitoes rest or transit, such as areas where people gather in the early hours of the evening and inside and outside of cattle sheds. These treated materials could also be specifically designed to attract resting mosquitoes. Similar substrates are already exploited for the delivery of conventional insecticides (Messenger *et al.*, 2012).

Our prototype (PPF-treated wall and roof linings) uses a safe and registered insecticide class that has yet to be deployed against adult malaria vectors. Alternatives to conventional adulticides are desperately needed. The physiological resistance to pyrethroids, recently characterized in populations of *An. arabiensis* from Zanzibar, precipitated the substitution of pyrethroids for a carbamate compound with a history of resistance development in malaria vectors (Okoye *et al.*, 2008; Haji *et al.*, 2013). No resistance to PPF has been reported in mosquitoes, and no cross-resistance has been observed between PPF and other classes of insecticides of public health interest (Invest & Lucas, 2008). As with other insecticides, the possibilities of malaria vectors developing resistance against this compound in future should not be dismissed (Schaefer & Mulligan III, 1991). PPF could be applied in combination, mosaics, or rotations with current insecticides to mitigate the emergence of resistance (WHO, 2012a). It is remarkably stable in the shade and available in a variety of commercial formulations that fit this new application. The indication that the few mosquitoes that managed to lay eggs from the PPF-treated section also transferred PPF to their breeding habitats and significantly reduced subsequent mosquito emergence is a welcome development. The autodissemination of PPF by adult mosquitoes has been already observed in Aedes species (Devine *et al.*, 2009; Caputo *et al.*, 2012), and we have proven same phenomenon in malaria vectors (Lwetoijera *et al.*, 2014, Chapter 4, Section 4.4).

CHAPTER 6

DISCUSSION

6.1 Research findings

This thesis aimed at evaluating the potential of exploiting adult mosquito behaviour to disseminate pyriproxyfen (PPF) into mosquito' breeding habitats for malaria' vector control. In this chapter, present and future implications of these findings in controlling malaria vectors are comprehensively discussed.

The first objective of this research was to conduct a robust mosquito surveillance system to establish the association between house characteristics and abundance of malaria vectors indoors, as well as for an in-depth understanding of malaria vector population dynamics and recent changes in transmission patterns of the disease in the study area. The outcome of this surveillance helped to establish baseline information for future evaluation of the impact of pyriproxyfen-based vector control strategies on malaria vectors in this area with high bed net coverage. It was demonstrated that even in the communities with high coverage of long lasting insecticide treated nets (LLINs), the density of *An. arabiensis* and *An. funestus* malaria vectors remained high inside houses. While poor housing structures (with many openings and no screening) and high human biomass per house were associated with an increase of indoor malaria vector densities and hence high malaria risk to communities in this locality; surprisingly the presence of LLINs on the other hand appeared not to negatively affect these populations. These findings emphasize not only the insufficiency of LLINs to control indoor biting

exposure via its repellence effect but also the benefit of improving house design to prevent indoor vector abundance.

Furthermore, in describing changes in vector species composition and relative abundance, insecticide susceptibility and their contribution to malaria transmission, it was clear that indoor densities of *An. gambiae* s.s. were controlled with LLINs undoubtedly due to its anthropophagic and endophilic tendencies. However, the remaining population of *An. arabiensis* and *An. funestus* were resistant to pyrethroids, carbamates and organochlorines that are approved for use in malaria vector control. The proportion of *An. funestus* in this area has been fluctuating sometimes higher than other malaria vectors, and their level of *Plasmodium* infection rates suggest that transmission by *An. funestus* was >6 times higher than the more widespread *An. arabiensis*.

These observations provide evidence that in addition to *An. arabiensis, An. funestus* was emerging as the greatest malaria challenge to vector control efforts (McCann *et al.*, 2014) (Lwetoijera *et al*, 2014, See Chapter 3, Section 3.4) in terms of increasing in densities with high sporozoite rates, insecticide resistance, and this calls for urgent development of novel and complementary approaches to sustain the gains already achieved with continuous use of LLINs (Hemingway, 2014; Killeen, 2014; Strode *et al.*, 2014; WHO, 2014).

Lack of outdoor mosquito collections was a limitation of this surveillance in explaining outdoor biting patterns. However, previous and recent findings showed that *An. arabiensis* and *An. funestus* feed outdoors in early morning and evening hours as a means to avoid lethal LLINs contacts, a behaviour that might be heritable or due to

phenotypic plasticity (Coluzzi *et al.,* 1977; Russell *et al.*, 2011b; Moiroux *et al.*, 2012; Gatton *et al.,* 2013; Sougoufara *et al.*, 2014). Therefore, outdoor based interventions, such as larviciding may have the potential of controlling immature stages at the aquatic habitat, and prevent build-up of adult mosquito population that would have preferred either to feed indoors or outdoors.

The second objective was to design and evaluate an efficient mechanism of contaminating mosquitoes with PPF that has potential for field implementation and that could be integrated in existing vector control strategies. This process exclusively relied on vector behaviours, namely host seeking, feeding, resting and oviposition that might be exploited to disseminate or target PPF.

Using laboratory' reared captive populations of *An. arabiensis*, it was proved for the first time under controlled conditions that *An. arabiensis* were capable of disseminating lethal dose of PPF to their aquatic habitats from PPF treated clay pots (i.e. Autodissemination) and render them unproductive. The autodissemination of PPF to aquatic habitats has been extensively studied using *Aedes* mosquitoes and demonstrated to be effective in controlling juvenile stages of the targeted *Aedes* mosquitoes under laboratory and field settings (Devine *et al.*, 2009; Caputo *et al.*, 2012; Suman *et al.*, 2014), but was not yet proven for malaria vectors under semi-field and field settings.

Because they are attractive to resting mosquitoes (Odiere *et al.,* 2007; Farenhorst *et al.,* 2008), clay pots were selected as the autodissemination tool for contaminating resting mosquitoes with PPF. Although absolute numbers of mosquitoes resting inside clay pots are relatively low (van den Bijllaardt *et al.,* 2009), clay pots have potential of attracting

blood fed mosquitoes to rest compared to many other sampling techniques (Wong *et al.*, 2013). This is likely to: 1) make it more efficient in ensuring long PPF-mosquito contact during blood digestion, 2) target mosquitoes later after blood feeding and closest to the egg-laying time, 3) target mosquitoes at the time the eggs have been formed and about to be laid, and hence maximise the chances for dissemination of picked up PPF to the habitats during egg laying events. In addition to its internal cool and humid microclimate which is preferred by resting mosquitoes, the clay pots protect PPF against destructive UV-light and extend their lifetime, and their small internal surface area for PPF application is advantageous in minimizing the human contact as well as intervention cost. For example, PPF powder applied inside clay pots was still effective in causing up to 80% mosquito emergence inhibition two months post treatment (Nzumbi, *personal communication*). Despite all these desirable characteristics of clay pots for delivering PPF to resisting mosquitoes, in the field settings, it is most likely to compete with surrounding vegetation for resting mosquitoes which might reduce the proportion of mosquitoes to be contaminated with PPF.

Of importance, one treated resting pot competing with alternative untreated resting sites including seven clay pots and resting sites within the mud hut was sufficient to inhibit > 65% adult emergence in six breeding habitats via ovipositing mosquitoes alone. These findings are very promising and highlight the potential that autodissemination offers for amplification of limited numbers of treatment points to significant levels of effective breeding habitat treatment coverage. In addition, when aquatic habitats are limited, the minority of mosquitoes that are contaminated in clay pots and then carry lethal doses of PPF to their aquatic habitats also affect the offspring of uncontaminated mosquitoes. Thus, contaminated adults amplify the impact of their own contamination by affecting the offspring of all mosquitoes that share the contaminated mosquito's breeding site (Fillinger *et al.*, 2013; Harris *et al.*, 2013).

Recently, the chemosterilant effect of PPF on adult *Anopheles* mosquitoes i.e. its ability to negatively impact mosquito fecundity and fertility has been demonstrated (Ohashi *et al.*, 2012; Harris *et al.*, 2013; Mbare *et al.*, 2014). Similarly, captive populations of susceptible *An. arabiensis* were sterilized using PPF treated ceiling and wall linings of cattle shelters inside the semi-field systems (Lwetoijera *et al.*, 2014, See Chapter 5, Section 5.4). The high preference of blood-fed mosquitoes to rest on the ceiling and walls of cattle shelter made these ideal vehicles for contamination with PPF.

The sterilization of malaria vectors was indirectly recorded by monitoring the absence of eggs/larva/pupae in the breeding habitats. One limitation of this study was that PPF exposed mosquitoes were not dissected to assess abdominal egg retention and egg viability. However, a separate study performed in the laboratory using mosquitoes from the same colony, demonstrated complete sterilization (100% reduced fertility, confirmed via dissection) when mosquitoes were contaminated within 24 h of a blood meal (Harris *et al.*, 2013). This, and the absence of eggs, larvae and pupae in the available aquatic habitats show that there were profound sterilization effect taking place inside the SFS.

6.2 Future perspectives

The future implementation of PPF-autodissemination as a potential larviciding method against immature mosquitoes, together with the chemosterilant effect of PPF on adult mosquitoes in interrupting malaria transmission would be governed by its uniqueness for controlling susceptible and resistant mosquitoes (Ngufor *et al.*, 2014; White *et al.*, 2014). Despite continuous development of mosquito resistance against the different insecticides recommend by WHO for malaria vector control (WHO, 2014), to date, there is no established evidence of resistance and/or cross resistance development in malaria

vectors against PPF (Invest & Lucas, 2008). However, following recent evidence of PPF resistance in houseflies and whiteflies (Crowder *et al.*, 2008; Shah *et al.*, 2015), the possibility of targeted malaria mosquitoes to develop resistance against PPF should not be dismissed. PPF has low toxicity to mammals and non-target organisms and is already approved by the WHO for some public health uses (WHO, 2008).

The study of PPF autodissemination in controlling *Aedes* mosquitoes has progressed from proof of principle to effective implementation in the field (Devine *et al.*, 2009; Caputo *et al.*, 2012, Suman *et al.*, 2014), and its potential for controlling vectors of malaria is promising, although at a more preliminary stage. This thesis describes the first steps towards malaria vector control with autodissemination of PPF. Further experiments will evaluate its efficacy using a self-propagating captive population, and will describe the field settings under which the technique might be applied.

The evaluation of interventions in the field experiments are usually costly, take a long time to implement and are complicated by variables that cannot be accounted and controlled for. However, semi-field experiments using a self-propagating colony in conditions closest to the natural environment of wild type population can provide a better early-stage system to evaluate multiple factors that might be of importance in optimizing pyriproxyfen-based control strategies. In the process of proving the autodissemination and sterilization principles, these studies utilized exceptionally large numbers of mosquito and small artificial aquatic habitats. The impact of PPF was evaluated via a single gonotrophic cycle and contamination event of released mosquitoes and was not necessarily representing the ecology of malaria vector under field settings.

In such experimental settings, it is therefore indispensable to establish self-propagating populations of *An. arabiensis*, which will be used to:- 1) assess the proportion of mosquitoes that could be contaminated with clay pots treated with PPF and the proportion of contaminated mosquitoes that are actually visiting the breeding habitats, and how many of these are enough to render a habitat unproductive, 2) estimate a minimum number of clay pots treated with PPF and how often it should be re-treated to completely crash the established self-propagating *An. arabiensis* population. Although, it is envisaged that the impact of PPF on the established population of *An. arabiensis* would be mainly due to autodissemination of PPF to the habitats, it would remain imperative to also assess the contribution of adult sterilization to the overall mosquito population reduction.

6.2.1 Field evaluation

Before any new vector control tool, in this context PPF, is adopted for large scale implementation it has to be evaluated under field settings. Variation in malaria vectors distribution and abundance is mainly associated with season. Dry season could be the prime time for deployment of PPF-autodissemination strategy due to the stable and limited number of mosquito breeding habitats compared to the rainy season (Charlwood *et al.*, 2000; Fillinger *et al.*, 2004). Although this season would be associated with low mosquito abundance and wide range of existing natural resting sites which might compete with target contamination sites, it is envisaged that the effective contamination and transfer of lethal PPF dosage to the aquatic habitats would be derived from attractiveness of the contamination stations to resting mosquitoes and PPF accumulation from multiple visit during the mosquito's gonotrophic cycles (Devine & Killeen, 2010). Importantly, *Culex* mosquitoes which occur relatively in higher numbers throughout the year and visit the same breeding habitats as *Anopheles* may also enhance the

autodissemination process (Robert *et al.*, 1998; Keating *et al.*, 2003; Muturi *et al.*, 2008; Russell *et al.*, 2010; Kudom *et al.*, 2012; Mbare *et al.*, 2013).

The efficacy and sustainability of PPF-autodissemination strategies will depend on the: 1) optimization of contamination methods such as the PPF formulations with increased active ingredient and longer shelf life, attractiveness to resting mosquitoes, electrostatic substrates that can easily offload maximum PPF onto mosquitoes upon contact (Andriessen *et al.*, 2015), exploitation of male mosquitoes to transfer PPF to the breeding habitats and to cross-contaminate females (Mains *et al.*, 2015), 2) assessing the role of non-target vehicles such as *Culex* species in the autodissemination of PPF and 3) assessment of residual activity of PPF in the field.

6.2.2 Impact on resistant vectors

Extensive use of LLINs and IRS are not only changing the distribution and abundance of malaria vectors but also imposing considerable selection pressure for the evolution of resistance against the chemicals they use (Strode *et al.*, 2014). Currently all vector species of malaria across Africa are resistant to all pyrethroids used in LLINs and to more than 80% of pyrethroids used for IRS (Hemingway, 2014; Strode *et al.*, 2014). Because malaria vectors are still fully susceptible to pyriproxyfen it could be applied either directly in semi/permanent breeding habitats or effectively combined with existing insecticidal interventions to help control resistant vector populations of both *An. arabiensis* and *An. funestus* (Ohashi *et al.*, 2012; Harris *et al.*, 2013; Kawada *et al.*, 2014; White *et al.*, 2014).

Recent empirical evidence of the impact of PPF on pyrethroid-resistant *An. gambiae* (Kawada *et al.*, 2014; Ngufor *et al.*, 2014) together with a mathematical model (White *et al.*, 2014) demonstrate the capacity of PPF / pyrethroid mixtures to reduce the frequency of pyrethroid-resistant alleles in a population. These support the potential of integration of pyriproxyfen-based strategies, in managing resistant malaria vectors and improving malaria control sustainability. For example, in the agricultural sector, PPF has been successfully used as a rotational alternative with other insecticides in control programmes targeting resistant whitefly, *Bemisia tabaci*, a pest that has shown the potential to cause financial loss in cotton damage and lost yields in North America (Ellsworth & Jones, 2001; Ellsworth & Martinez-Carrillo, 2001).

With the increasing importance of *An. arabiensis* in sustaining residual malaria transmission due to its feeding flexibility mainly on cattle apart from human but also its physiological resistance against insecticides used for LLINs and IRS; targeting cattle with PPF could potentially control both susceptible and pyrethroid-resistant *An. arabiensis*. Similar to the treatment of cattle with pyrethroids which was proven effective but challenged by resistance to the pyrethroids (Rowland *et al.*, 2001), cattle could be sprayed with PPF to sterilize hosting seeking *An. arabiensis* and hence prevent the propagation of pyrethroid-resistant alleles in its population. Of importance, cattle treated with PPF have shown neither to repel nor inhibit mosquitoes from feeding (Lwetoijera *et al*, unpublished).

6.3 Conclusions

For the first time, using clay pots as contamination tools, it was demonstrated that adult *An. arabiensis* can disseminate PPF to their aquatic habitats resulting in substantial

reduction (76.5%) in number of adult produced. Similarly, using cattle shelters (walls and ceilings) treated with PPF, *An. arabiensis* were almost completely sterilized resulting in > 95% reduction in adult production. These findings provide a range of options for future field evaluation.

PPF-autodissemination can potentially control pyrethroid resistant and susceptible malaria vectors indoors and outdoors, in their aquatic habitats and by imposing a huge reproductive fitness cost. It can also target mosquitoes of other diseases which share aquatic habitats with malaria vectors, such as Culex mosquitoes. The treatment of walls and ceilings of cattle shelters with PPF could be further optimised into an IRS format for areas known to harbour large numbers of resting mosquitoes.

Surveillance of wild malaria vectors populations dominated by *An. arabiensis* and *An. funestus*, showed increasing levels of resistance to all classes of insecticides recommended for mosquito control. This hampers the effectiveness of existing malaria interventions and hence the increased risk for malaria transmission. PPF-autodissemination and sterilization of adult females could complement existing vector control measures to target these problematic mosquito populations.

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APPENDIX

The pages below indicate the list of published papers in the following order:-

1. Lwetoijera *et al.* 2013. A need for better housing to further reduce indoor malaria transmission in areas with high bed net coverage. *Parasites & Vectors* 2013, 6:57

2. Lwetoijera *et al.*2014. Increasing role of *Anopheles funestus* and *Anopheles arabiensis* in malaria transmission in the Kilombero Valley, Tanzania. *Malaria Journal* 2014, 13:331

3. Lwetoijera *et al.* 2014. Effective autodissemination of pyriproxyfen to breeding sites by the exophilic malaria vector *Anopheles arabiensis* in semi-field settings in Tanzania. *Malaria Journal* 2014, 13:161

4. Lwetoijera *et al.* 2014. Comprehensive sterilization of resilient malaria vectors: a step closer to malaria elimination. *American Journal of Tropical Medicine and Hygiene*, 90(5).

Parasites & Vectors

RESEARCH Open Access

A need for better housing to further reduce indoor malaria transmission in areas with high bed net coverage

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Abstract

Background: The suppression of indoor malaria transmission requires additional interventions that complement the use of insecticide treated nets (ITNs) and indoor residual spraying (IRS). Previous studies have examined the impact of house structure on malaria transmission in areas of low transmission. This study was conducted in a high transmission setting and presents further evidence about the association between specific house characteristics and the abundance of endophilic malaria vectors.

Methods: Mosquitoes were sampled using CDC light traps from 72 randomly selected houses in two villages on a monthly basis from 2008 to 2011 in rural Southern Tanzania. Generalized linear models using Poisson distributions were used to analyze the association of house characteristics (eave gaps, wall types, roof types, number of windows, rooms and doors, window screens, house size), number of occupants and ITN usage with mean catches of malaria vectors (*An.gambiae s.l.* and *An. funestus*).

Results: A total of 36490 female *An. gambiae s.l.* were collected in Namwawala village and 21266 in Idete village. As for *An. funestus* females, 2268 were collected in Namwawala and 3398 in Idete. Individually, each house factor had a statistically significant impact ($p < 0.05$) on the mean catches for *An. gambiae s.l.* but not *An. funestus*. A multivariate analysis indicated that the combined absence or presence of eaves, treated or untreated bed-nets, the number of house occupants, house size, netting over windows, and roof type were significantly related ($p < 0.05$) to *An. gambiae s.l.* and *An. funestus* house entry in both villages.

Conclusions: Despite significant reductions in vector density and malaria transmission caused by high coverage of ITNs, high numbers of host-seeking malaria vectors are still found indoors due to house designs that favour mosquito entry. In addition to ITNs and IRS, significant efforts should focus on improving house design to prevent mosquito entry and eliminate indoor malaria transmission.

Keywords: House risk factors, *Anopheles gambiae s.l.*, *Anopheles funestus*, ITNs, Malaria

Background

The *Anopheles gambiae* and *Anopheles funestus* complexes comprise the major and most efficient malaria vectors in sub-Saharan Africa [1]. Their transmission efficiency is mediated by their behavioural adaptation to feed indoors on humans [2]. To date, insecticide treated nets (ITNs) and indoor residual spraying (IRS) are the mainstay for controlling malaria vectors and associated malaria transmission [3,4]. Despite the huge success of these interventions, residual malaria transmission cannot be addressed by ITNs and IRS alone, even at very high coverage [5,6]. Moreover, their sustainability is threatened by a widespread increase in insecticide resistance in the target species [7,8]. In Senegal, the initial successes of an ITN distribution program were partially confounded by an increase in insecticide resistance and a consequent rebound in malaria incidence [9] and in

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northern Tanzania the predominant vector *An. arabiensis* has been reported to display avoidance behaviour against ITNs [10]. The integration of existing interventions with environmental management and socio-economic development through house improvement and screening offers a non-insecticidal, complementary approach to increasing protection against mosquito bites [11,12]. These additional interventions could enhance the interruption of malaria transmission through the reduction and prevention of human-vector contacts inside human dwellings. It has long been established that the transmission of many vector-borne diseases is facilitated by house designs that favour mosquito entry [13-15] and that housing improvements and screening have made substantial contributions to the control and elimination of malaria vectors in many richer countries [16]. Therefore, understanding house risk factors that are associated with reduction of indoor mosquito bites and disease transmission in different settings is crucial for disease vector control and elimination.

Several studies have identified and documented various house characteristics associated with mosquito entry. Presence of eave gaps, lack of a ceiling and lack of screening over windows and doors proved to be the major contributors to mosquito entry [16-20]. Furthermore, it has been shown in a randomised control trial that blocking all potential house entry points for mosquitoes substantially reduces vector densities and entomological inoculation rates (EIR) [19]. Other than protection against malaria mosquitoes, the use of screened houses offers protection against nuisance bites and other mosquito borne diseases [15,21].

While this strategy is deemed efficient in reducing indoor biting and disease morbidity in low malaria transmission settings [16], its impact is yet to be examined in areas experiencing moderate to high malaria transmission and with high ITN coverage such as the Kilombero valley in south-eastern Tanzania.

A recent study in Northern Tanzania has shown a strong association between houses, individual and behavioural risk factors and malaria transmission [22]. However, the authors argued that it was important to complement these findings with entomological data in order to have a fuller understanding of malaria transmission inside human dwellings [22]. This study therefore assessed the impact of house characteristics on indoor vector abundance in communities with a high coverage of ITNs.

Methods

Study site

The study was carried out in Namwawala and Idete villages located in the flood plain of the Kilombero River (8.1°S and 36.6°E) in south-eastern Tanzania (Figure 1). The epidemiology of malaria transmission and associated disease vector species composition within these villages has been well studied and documented over the past years [23,24]. Both villages experience an annual rainy season (Dec – May) and the main crops are rice

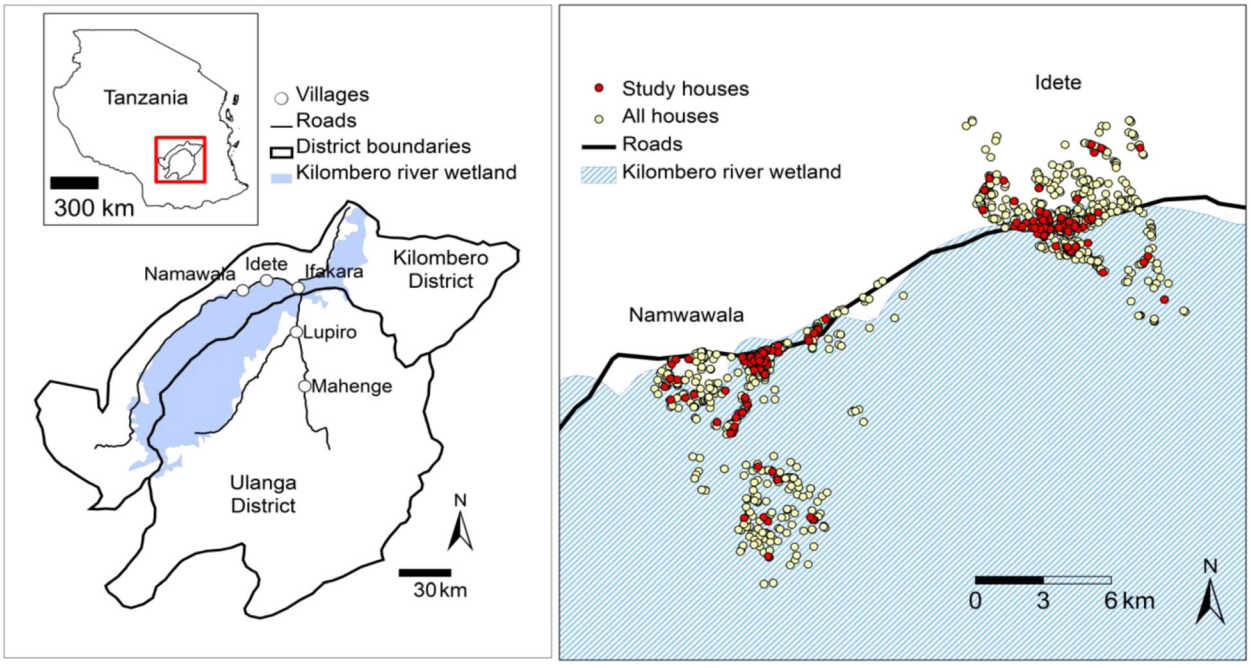

Figure 1 Kilombero and Ulanga districts (8.1°S and 36.6°E) in Tanzania showing Namwawala and Idete villages (left) and spatial distribution of sentinel houses used for mosquito sampling (right) [25].

and maize. However, both villages have a relatively similar number of houses (Namwawala = 804 and Idete = 844), Namwawala has a high number of households (3909) compared to Idete (2932). Houses in Idete are built on relatively elevated areas compared to Namwawala. Approximately 92% of community members sleep under a treated net [23].

Study design

This longitudinal study was conducted over four years. A total of 72 houses from each village were randomly selected from Ifakara Health Institute (IHI) Demographic Surveillance System household list [26]. All selected houses were geo-located using a handheld GPS (eTrex, Vista, Garmin, USA). Each of the 72 houses was sampled monthly (i.e. 6 houses per day, 4 days per week and 3 weeks per month). This longitudinal study was carried out between January 2008 and December 2011, during which mosquitoes were sampled every month during 2008 and 2011, for 6 months of the wet/rain season (January to June) in 2009 and for 6 months of the dry season (July to December) in 2010. This totals 36 months of sampling.

House risk factors

Structured questionnaires were used to record ownership, number and status of bed nets (either treated or untreated) including the one LLIN provided by the research team in this study, and the number of house occupants. The house characteristics which were recorded include house size, number of sleeping rooms, presence and size of eave gaps, number of windows, presence of window screening, number of doors, presence of ceiling, wall and roof types. These factors were correlated with mosquito densities indoors (an indicator of human biting rate) over time in both villages, at house level and were monitored yearly to accommodate any significant changes. Representative house types, which are commonly found in the study area are shown in Figure 2.

Mosquito sampling and processing

Mosquitoes were sampled using miniature Centre for Disease Control (CDC) light traps (model 512, USA). One CDC light trap was set per house, placed 1–1.5 m above the ground close to the foot of a bed with an occupant sleeping under a treated net, and left to run for 12 hours (7 pm–7 am). For every participating house, one LLIN (Olyset, A to Z Textiles Mills, Arusha, Tanzania) was provided to protect the bed occupant where the CDC trap was set. Each morning of a sampling night, mosquitoes were collected and killed using chloroform and were morphologically identified in the field. Furthermore, female mosquitoes were classified as being unfed, partially fed, fully fed or gravid [2]. Subsamples of five mosquitoes from each trap were individually stored inside a tube containing cotton wad and silica gel beneath. Polymerase chain reaction (PCR) was used for identification of *Anopheles gambiae* [27] and *An. funestus* Giles [28] complexes, whereas an enzyme-linked immunosorbent assay (ELISA) was used to determine sporozoite infection in malaria vectors [29]. Unprocessed mosquito samples were stored on silica gel at room temperature.

Ethics

The study approval was granted by the Ifakara Health Institute Institutional Review Board (IHRDC/IRB/No. A-32) and the National Institute of Medical Research (NIMR/HQ/R.8a/Vol. IX/764). The benefits and possible risks associated with the study were explained to the house occupants before commencement. After consenting, the head of the house was asked to sign two copies of the informed consent forms, of which, one remained with the head of the house and the other copy was kept by the study investigator.

Data analysis

The analysis was performed using generalized linear models (GLM) (MATLAB R2012a, Poisson distribution, 95% confidence interval) to assess the impact of each

Figure 2 Representative house types commonly available in Idete and Namwawala villages. A temporary house (**a**) and a permanent house (**b**).

individual house factor on the mean catches of *An. gambiae s.l.* and *An. funestus* for both villages. Model estimate (ME) value was generated for each factor in comparison to a reference category. If the sum of ME for a factor and reference category was more than that of ME for a reference category then a factor increases indoor mean catches of mosquitoes, otherwise it decreases. Thus, ME value for a factor indicates by how much a factor increases or decreases the indoor mean catches when compared to a reference category. We categorized the house factors as follows: Eave gap: present or absent, eave gap size (small: <9 cm, medium: 9–15 cm, large >

Table 1 Parameters associated with *Anopheles gambiae s.l.* density in Idete and Namwawala villages

An.gambiae	Idete (N = 70)			Namwawala (N = 72)		
Factor	**N**	**Estimate**	**P value**	**Estimate**	**N**	**P value**
Number Of Rooms						
[a]One	37	2.31	0.0000	2.89	57	0.0000
More than One	33	-0.24	0.0035	-0.76	15	<0.0001
Number Of Doors						
[a]One	30	2.45	0.0000	2.93	51	0.0000
More than One	40	-0.47	<0.0001	-0.64	21	<0.0001
Number Of Windows						
[a]Up to 3	26	2.50	0.0000	2.85	53	0.0000
More than 3	44	-0.51	<0.0001	-0.29	19	<0.0001
Netting Over Window						
[a]Absent	50	2.35	0.0000	2.85	60	0.0000
Present but damaged	16	-0.59	0.0004	-1.65	9	<0.0001
Intact	4	-085	<0.0001	-0.35	3	0.0005
House Status						
[a]Small	12	2.76	0.0000	2.88	34	0.0000
Large	58	-0.71	<0.0001	-0.19	28	0.0008
Wall Type						
[a]Mud	52	2.65	0.0000	2.88	32	0.0000
Cement	18	-0.66	<0.0001	-0.28	40	<0.0001
Roof Type						
[a]Grass	46	2.58	0.0000	2.94	19	0.0000
Metal	24	-0.65	<0.0001	-0.81	53	<0.0001
Eave Status						
[a]Absent	46	1.50	0.0000	1.06	62	0.0000
Present	24	0.94	<0.0001	1.84	10	<0.0001
Eave Size						
[a]Small	26	1.80	0.0000	2.89	22	0.0000
Medium	14	0.67	<0.0001	-0.05	27	0.4452
Large	30	0.36	0.0028	-6.24	23	<0.0001
Number of Occupants						
[a]Up to 3	15	1.76	0.0000	2.35	34	0.0000
More than 3	55	0.54	<0.0001	0.70	38	<0.0001
Bed-net Status						
[a]Untreated	47	2.33	0.0000	2.58	6	0.0000
Treated	23	-0.45	<0.0001	0.22	66	0.0588

[a] reference category, N = number of observations.
Note: Model estimate (ME) value for a factor indicates by how much a factor increases or decreases the indoor mean catches when compared to a reference category.

15 cm), roof type: grass or metal roofs, wall type: mud or cement, number of occupants: up to three or more than three, windows: up to three or more than three, netting over window: intact, present but damaged or absent, doors: one or more than one, rooms: one or more than one, house size: small or large (small house considered to be the one with 1 room and/or 1 door and less than 37.4 m^3), bed nets: treated or untreated. All houses had nets, and they were considered treated if the number of treated nets divided by the total number of nets in the house was greater than 0.5, otherwise untreated.

Results

Mosquito collections

A total of 36490 female *An. gambiae s.l.*, were collected in Namwawala village compared to 21266 from Idete village. Of these, approximately 98% were non-blood fed, 1.7% were blood fed and the remaining 0.3% were gravid. Namwawala had fewer female *An. funestus* 2268 than Idete village 3398. Although there were variations in catches, changes in vector abundance patterns between villages were similar over time. A PCR analysis of 6755 mosquitoes of the *Anopheles gambiae* complex yielded 607 (9%) *An.gambiae s.s.* and 6148 (91%) *An. arabiensis* mosquitoes. Furthermore, a sub-sample of 3025 *An. funestus* analyzed for species identification comprised 2805 (93%) *An. funestus s.s.*, 120 (4%) *An. rivulorum*, and 100 (3%) *An. leesoni.*

House risk factors associated with *An. gambiae s.l.* indoor abundance

Table 1 provides parameter estimates of each house risk characteristic when run individually in a univariate model and their significance on the mean catches for *An. gambiae s.l.* All factors in both villages had a statistically significant impact ($p < 0.05$) on the indoor mosquito mean catches except bed net status in Namwawala ($p > 0.05$). Houses where an eave gap was present had significantly higher *An. gambiae s.l.* mean catches (ME 0.94 in Idete and 1.84 in Namwawala) compared to when it was absent (ME 1.50 in Idete and 1.06 in Namwawala). Mosquito density increased with more people inside the house but decreased with large houses (more rooms, windows, and doors). Compared to a window with no netting, a house with a damaged net on the window had lower mean catches of *An.gambiae s.l.* and the catches decreased further for houses with an intact net. Furthermore, houses with either mud walls or grass/thatch roofing had higher numbers of mosquitoes when compared to cement plastered walls and metal roofing.

The presence of bednets was significantly correlated to lower mean catches in Idete village ($p < 0.05$). However, this was not the case in Namwawala village ($p > 0.05$). The ownership rate of nets in Namwawala village was 89% for treated and 11% for untreated nets, whereas in Idete village it was 50% for treated and 50% for untreated nets.

House risk factors associated with *An. funestus* indoor abundance

The model estimates and *p*-values of each of the individual house risk characteristics, number of occupants and the bed-net status with their association with the mean catches for *An. funestus* for both villages are presented in Table 2. The presence of eave gap in the house was significantly correlated with increased mean catches of *An. funestus* (ME 1.42 in Idete, 2.48 in Namwawala, $p < 0.05$) compared to when eave gaps were absent (ME -0 .73 in Idete, -2.39). House size did not significantly affect mean catches in Namwawala ($p > 0.05$) but in Idete mean catches for *An. funestus* decreased with large houses (ME -0.60, $p < 0.05$), when compared to small houses (ME 0.85). Similarly, houses with more than one room or door had lower mean catches in both villages. Increase in number of windows did not significantly affect the *An. funestus* mean catches ($p > 0.05$), however, the mean catches of *An. funestus* significantly decreased with increased number of people in the houses in Idete ($p < 0.05$) but not in Namwawala ($p > 0.05$). Netting over windows did not reduce the mean catches in both villages. The mean catches of *An. funestus* were significantly lower ($p < 0.05$) in the houses with cement plastered walls (ME -1.52 Idete, -0.55 Namwawala) compared to mud walls, as well as where metal roofs were present (ME -1.78 Idete, -0.89 Namwawala), compared to grass roofs. Mosquito catches decreased significantly ($p < 0.05$) in the presence of treated bednets (ME -0.52 Idete, -1.03 Namwawala) when compared to the untreated bednet (ME 0.52 Idete, ME 0.85 Namwawala).

Multivariate analysis

A correlation matrix for all of the parameters was created to analyse the relationship among the house risk characteristics but no clear conclusion could be drawn. Thus, a multivariate analysis was performed using a 'stepwise regression approach' in which at each step the best variable (i.e. a house risk characteristic) with a significant level ($p < 0.05$) is added. This analysis indicated that the presence of an eave gap, bednet status, number of occupants, house size and wall type had a significant impact on the mean catches of *An.gambiae* in both Namwawala and Idete. In Namwawala, also roof type and number of doors had a significant impact on the mean catches of *An.gambiae*.

Bednet status, number of occupants, house size, roof type and number of windows had a significant impact on the mean catches of *An. funestus* in Idete while netting over windows, presence of eave gap, bednet status,

Table 2 Parameters associated with *Anopheles funestus* density in Idete and Namwawala villages

An.funestus	Idete (N = 70)			Namwawala (N = 72)		
Factor	**N**	**Estimate**	**P value**	**Estimate**	**N**	**P value**
Number Of Rooms						
[a]One	37	0.76	0.0000	0.11	57	0.3485
More than One	33	-1.10	<0.0001	-1.25	15	0.0081
Number Of Doors						
[a]One	30	0.86	0.0000	0.13	51	0.3207
More than One	40	-1.11	<0.0001	-0.79	21	0.0167
Number Of Windows						
[a]Up to 3	26	0.52	0.0003	0.07	53	0.5748
More than 3	44	-0.26	0.1997	-0.55	19	0.0868
Netting Over Window						
[a]Absent	50	0.61	0.0000	0.10	60	0.3877
Present but damaged	16	-1.29	0.0003	-2.76	9	0.2057
Intact	4	-1.40	0.0603	-1.59	3	0.0251
House Status						
[a]Small	12	0.85	0.0000	0.12	34	0.4408
Large	58	-0.60	0.0065	-0.34	28	0.1534
Wall Type						
[a]Mud	52	1.25	0.0000	0.14	32	0.3365
Cement	18	-1.52	<0.0001	-0.55	40	0.0439
Roof Type						
[a]Grass	46	1.17	0.0000	0.13	19	0.3283
Metal	24	-1.78	<0.0001	-0.89	53	0.0131
Eave Status						
[a]Absent	46	-0.73	0.0124	-2.39	62	0.0222
Present	24	1.42	<0.0001	2.48	10	0.0183
Eave Size						
[a]Small	26	-0.42	0.0778	0.12	22	0.4612
Medium	14	1.04	0.0001	-0.36	27	0.1860
Large	30	1.21	<0.0001	-0.24	23	0.4563
Number of Occupants						
[a]Up to 3	15	1.05	0.0000	-0.04	34	0.8015
More than 3	55	-0.96	<0.0001	-0.00	38	0.9968
Bed-net Status						
[a]Untreated	47	0.52	0.0000	0.85	6	0.0014
Treated	23	-0.52	0.0273	-1.03	66	0.0005

[a] reference category, N = number of observations.
Note: Model estimate (ME) value for a factor indicates by how much a factor increases or decreases the indoor mean catches when compared to a reference category.

and number of doors had a significant impact on the mean catches of *An. funestus* in Namwawala.

Discussion

Despite high coverage and extensive usage of insecticide treated nets in rural communities of southern Tanzania [23], partly designed to deter and divert mosquitoes from entering houses [30], a high number of malaria vectors are still found indoors with an average of 22.22 (CI = 16.93 – 27.51) *An. gambiae s.l.* and 1.35 (CI = 1.07 – 1.63) *An. funestus* mosquitoes per trap night per house in Namwawala. In addition, an average

of 13.12 (CI = 10.94 – 15.30) *An.gambiae s.l.* and 2.09 (CI = 1.56 – 2.63) An. *funestus* were collected in Idete per trap night in a house.

Small houses, constituting the majority of houses in the study area, characterized by relatively low numbers of windows, doors and rooms were associated with relatively high densities of malaria vectors. Although the association of house size and indoor mosquito density remains unknown, it was, however, assumed that smaller houses are likely to concentrate more human odours, which would attract high mosquito numbers. Conversely, houses with more sleeping rooms had a lower density of vectors because they usually have more sleeping spaces, which is likely to encourage consistent use of bed nets by sleepers [31,32]. Moreover, houses with many rooms are likely to have more nets, which collectively might reduce the number of mosquitoes indoors.

Houses made of mud walls and grass roofs had an increased risk of mosquito bites indoors. Such houses create cooler, darker conditions favoured by resting mosquitoes [33,34]. Moreover, mud walls as well as grass roofs often have crevices used by mosquitoes to enter the houses unlike cement walls and metal roofs [18]. In addition, lack of or damaged screening over windows as well as open eaves provided entry points and led to increased mosquito abundance inside the houses. These findings are consistent with other studies [16,35-38] which demonstrated that poorly constructed houses (with mud walls, grass roofs, lack of screening and with eave gaps tend to have increased human-vector exposure), resulting in a higher risk of malaria transmission.

It has been documented that houses with many occupants tend to attract vectors of disease [39,40]. In this study, the presence of many sleepers in a small house exposed them to a higher risk of *An.gambiae s.l.* bites but to a lower risk from *An. funestus.* Large amounts of human emanations from houses with more occupants tend to increase mosquito attractiveness towards that particular house compared to ones with fewer sleepers [41,42]. The observed inverse relationship between *An. funestus* and number of occupants inside the house was unexpected; however, it might be due to uneven distribution of *An. funestus* within the villages. Higher numbers of *An. funestus* collected during the dry season [43] were mostly and consistently from a cluster of a few houses located in a particular village hamlet. Therefore, the majority of houses within the sampling area experienced none or low catches.

Furthermore, significant impacts of house risk factors on *An. funestus* indoor mean catches were not consistent between villages. While this observation remains inconclusive, we postulated the cause to be exceedingly low numbers of *An. funestus* collected between villages compared to *An. gambiae s.l.*

Treated nets provided more protective advantages than untreated ones as also observed in previous studies [22,23,44,45]. However, the density of *An. gambiae s.l.* in Namwawala was higher compared to Idete despite 90% ITN coverage in Namwawala. These results indicate that even at high coverage levels, ITNs still have limitations in reducing the number of malaria vectors entering the houses. Furthermore, recent studies [46,47] have indicated that poor compliance and usage of bed nets by communities in the tropics is associated with heat discomfort associated with poor airflow caused by bed nets. Although bed nets were procured individually and there was a distribution campaign during the study period, the age of nets as well as usage of ITNs was not systematically investigated in this study, our results illustrate that a risk of transmission remains whenever people are not using treated nets in an optimal way. Improved house designs, and modifications to existing houses could substantially reduce the risk of mosquito-human contact. Although house improvement has been advocated as an efficient intervention for malaria control, the majority of houses in poor rural Africa are temporary and built with minimal material resources. This renders improvements expensive and/or impractical in most rural communities in the short term. Permanent houses (Figure 2b) could be easily and cheaply modified by screening eaves, windows and doors accompanied by community sensitization towards intervention sustainability. Temporary houses (Figure 2a) are less amenable to modifications unless they are rebuilt as more permanent structures. This would have to be addressed through a long-term strategy that sought to build better, inexpensive house models using better construction materials and sustainable financing initiatives, which can be adopted in poor settings. Such an intervention is likely to be beneficial in reducing vector borne diseases and other diseases linked to poor hygiene.

Conclusions

This study shows the impact of specific housing characteristics on malaria vector density and the associated risk of indoor disease transmission. It also shows that even at high coverage levels of ITNs, there remains a high risk of human-mosquito contact and also that this transmission risk can be mitigated by changing house structure. Communities with permanent, spacious and screened houses are at lower risk of indoor malaria transmission.

Competing interests

The authors have declared no competing interests.

Authors' contributions

DWL and SM proposed the study hypothesis. DWL and SSK performed statistical analysis and wrote the first draft of the manuscript. DWL supervised the study data collections. ZDM, CH, SD & GD contributed to writing of the manuscript. All authors read and approved the final manuscript.

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Increasing role of *Anopheles funestus* and *Anopheles arabiensis* in malaria transmission in the Kilombero Valley, Tanzania

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Abstract

Background: In order to sustain the gains achieved by current malaria control strategies, robust surveillance systems that monitor dynamics of vectors and their roles in malaria transmission over time are essential. This longitudinal study demonstrates the trends in malaria vector dynamics and their relative contribution to malaria transmission in hyperendemic transmission settings in Tanzania.

Methods: The study was conducted in two villages within the Kilombero Valley, in rural Tanzania for five consecutive years (2008–2012). Seventy-two houses were selected per village and each house was sampled for mosquitoes monthly using a CDC light trap. Collected mosquitoes were assessed for species identity and sporozoite infection status using PCR and ELISA, respectively. *Anopheles funestus* and *Anopheles arabiensis* susceptibility to insecticides was assessed using WHO guidelines.

Results: A total of 100,810 malaria vectors were collected, of which 76% were *Anopheles gambiae s. l.* and 24% were *An. funestus*. Of all *An. funestus* samples that amplified with PCR (n = 2,737), 97% were *An. funestus s.s.*, 2% were *Anopheles rivorulum* and 1% *Anopheles leesoni*. Whereas for *An. gambiae s.l.* (n = 8,117), 93% were *An. arabiensis* and 7% were *Anopheles gambiae s.s.* The proportion of *An. gambiae s.s.* identified by PCR (2,924) declined from 0.2% in the year 2008 to undetectable levels in 2012. Malaria transmission intensity significantly decreased from an EIR of 78.14 infectious bites/person/year in 2008 to 35 ib/p/yr in 2011 but rebounded to 226 ib/p/yr in 2012 coinciding with an increased role of *An. funestus* in malaria transmission. Insecticide susceptibility tests indicated high levels of resistance in *An. funestus* against deltamethrin (87%), permethrin (65%), lambda cyhalothrin (74%), bendiocarb (65%), and DDT (66%). Similarly, *An. arabiensis* showed insecticide resistance to deltamethrin (64%), permethrin (77%) and lambda cyhalothrin (42%) in 2014.

Conclusion: The results indicate the continuing role of *An. arabiensis* and the increasing importance of *An. funestus* in malaria transmission, and pyrethroid resistance development in both species. Complementary vector control and surveillance tools are needed that target the ecology, behaviour and insecticide resistance management of these vector species, in order to preserve the efficacy of LLINs.

Keywords: Malaria, *Anopheles*, Transmission, Vector, Surveillance, *Gambiae*, *Arabiensis*, *Funestus*, Season, Insecticide, Susceptibility, EIR, Kilombero, Tanzania

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Background

Malaria transmission in humans is sustained through vector-human interactions [1] and vector control interventions, such as long-lasting, insecticidal nets (LLINs), aim to break this interaction. Major promotion of LLINs in recent years has resulted in average household ownership rates and usage of LLINs of approximately 42 and 36%, respectively, in sub-Saharan Africa [2]. In mainland Tanzania, a recent report by the Tanzania HIV and Malaria Indicator Survey (THMIS) indicates that above average LLIN ownership and usage (approximately 90 and 66%, respectively) was associated with improved malaria control and overall reduction in malaria prevalence [3].

One outcome of LLIN use is that, by limiting availability of human hosts [4-6], vector species composition in any given area can change considerably after a long period of LLIN use. *Anopheles gambiae sensu stricto, Anopheles arabiensis* and *Anopheles funestus* are the primary malaria vectors in sub-Saharan Africa [7,8], often occurring sympatrically [9]. *Anopheles gambiae s.s.* is often regarded as the most important vector species across Africa [9-11] and, because of its almost entirely anthropophagic and endophilic behaviour, it is the species that has been targeted most effectively by LLINs.

However, in some locations, populations of *An. gambiae s.s.* have developed insecticide resistance and it continues to be the dominant vector [12,13]. In other locations, *An. gambiae s.s.* populations have crashed and the relative importance of the remaining vector species has shifted, with *An. arabiensis* becoming the major malaria vector [4,5,11].

Since single populations of *An. arabiensis* can exhibit a range of behaviours, biting and resting indoors as well as outdoors and feeding on both humans and animals, interventions that optimally target indoor resting and biting vectors often impact far less on this species [11,14-17]. The primary vector of the *An. funestus* complex, *An. funestus* is also a very anthropophilic and endophilic mosquito and it too can be a highly efficient malaria vector [10,18,19].

Kilombero Valley in southern Tanzania has been subject to a large number of studies on malaria epidemiology, dating back many years, with malaria parasite prevalence rates of up to 70% and an entomological inoculation rate (EIR) of 300 infectious bites per person per year (ib/p/yr) being recorded in the 1990s, the period before the introduction of bed nets [20]. Following the scaling up of untreated nets in the early 2000s [21] and insecticide-treated bed nets (ITNs) and LLINs from 2004 to 2011 [22-24], a continuous decline in malaria vector numbers and malaria transmission has been seen [3,12]. Although the populations of *An. gambiae s.s.* are significantly dwindling in southern and other parts of Tanzania [12], the remaining populations of *An. arabiensis* and *An. funestus* appears to have shifted their blood-feeding periodicity to optimize their chances to obtain blood meal from their preferred hosts even in the time of low LLIN coverage [25]. It is however suggested that prolonged, widespread use of LLINs is likely to favour outdoor and early biting, either as an expression of the mosquito's innate phenotypic plasticity or possibly as a heritable, selectable trait that might be expected to increase in frequency [25].

The malaria vector populations in this area are subject to ongoing rigorous monitoring and herein five years of data to the end of 2012 are reported; describing changes in vector species composition and relative abundance, insecticide susceptibility and their contribution to malaria transmission following the years of widespread LLIN use since first introduced in 2004.

Methods

Study site

The study was carried out in Namwawala (8.154425°S and 36.393005°E) and Idete (8.098190°S and 36.510350°E) villages (Figure 1) located in the flood plain of the Kilombero River (8.1°S and 36.6°E) in southeastern Tanzania. The epidemiology of malaria transmission and associated vector species composition within these villages has been documented over many years [21,25]. Both villages experience an annual rainy season (January-May) and the main crops are rice and maize. Both villages are similar in size (Namwawala = 844 and Idete = 804) and approximately 92% of community members sleep under an ITN or LLIN [12].

Study design

This study was conducted over five years between January 2008 and December 2012. A total of 72 houses from each village were randomly selected from the Ifakara Health Institute (IHI) Demographic Surveillance System household list [26]. All selected houses were geolocated using a handheld GPS (eTrex, Vista, Garmin, USA). Mosquitoes were sampled in every house each month during 2008, 2011 and 2012 and for six months from January to June in 2009 and July to December in 2010.

Mosquito sampling and processing

Mosquitoes were sampled using miniature Center for Disease Control (CDC) light traps (model 512, USA). One CDC light trap was used overnight per house, placed 1–1.5 m from the fan above the ground close to the foot end of an occupied bed, and left to run for 12 hours (19.00-07.00) [27,28]. For every participating house, one LLIN (Olyset, A to Z Textiles Mills, Arusha, Tanzania) was provided to protect the bed occupant where the CDC trap was set. The following morning, CDC light traps were collected and mosquitoes killed

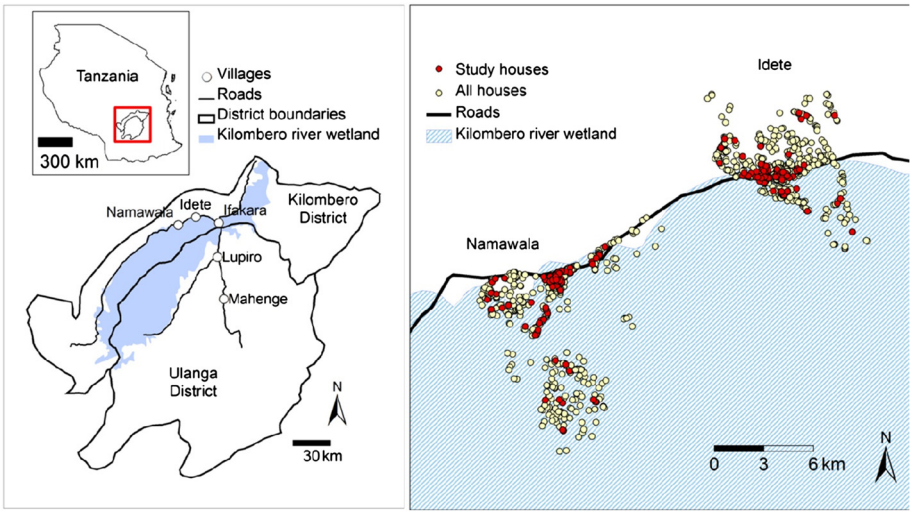

Figure 1 A map showing sentinel houses for mosquito sampling in Idete and Namwawala villages, within Kilombero Valley, in Kilombero district, Tanzania.

using chloroform, and identified in the field using a morphological key [18]. Female mosquitoes were classified as being unfed, partially fed, fully fed or gravid. Subsamples of five mosquitoes from each trap for *An. arabiensis* and *An. funestus* species were individually stored inside a tube containing cotton wool and silica gel beneath for further individual molecular species identification using polymerase chain reaction (PCR) assay for the *An. gambiae* complex [29] and *An. funestus* group [30] and sporozoite infection status using enzyme-linked immunosorbent assay (ELISA) [31] in the laboratory (species identification for the *An. funestus* group did not begin until 2009).

All the sorting information and laboratory analysis results were recorded using designated data collection forms for entomological studies (Kiware *et al.*, unpublished). In addition, variations in malaria transmission by different vector species over time were assessed and compared using the annual EIR calculated by biting rate (total collections/trap nights/year) and the proportion of females infected with sporozoites [32]. Monthly average rainfall data for 2008–2011 were obtained from the Kilombero Valley Teak Company (approximately 15 km from Idete village), and data for year 2012 data were obtained using rain gauges installed in Namwawala village.

Insecticide susceptibility tests

Following significant increase in *An. funestus* population in 2012, despite extensive usage of LLIN in the study area, it was unclear whether this was due to its reduced susceptibility to the insecticides used in LLINs. The tests were conducted using WHO standard procedures and test kits for adult female mosquitoes of *An. arabiensis* and *An. funestus* [33] in Namwawala villages from January to June 2013. As the confirmatory process, the biossays were repeated in June 2014 for both species.

Five classes of insecticides currently recommended for vector control were tested using discriminating concentrations impregnated in pre-prepared test papers as follows: deltamethrin (0.05%), permethrin (0.75%), lambda cyhalothrin (0.05%), bendiocarb (0.1%), and DDT (4%). Unfed female wild *An. funestus* collected using CDC light traps were used for insecticide exposure bioassays, as recommended by WHO for this difficult-to-colonize species [33]. However, this method is limited by greater variation in susceptibility due to unknown age differences between test mosquitoes, it is simple to carry in the field with minimal infrastructure and test mosquitoes highly representative of the natural population [33].

Prior to exposure, morphologically identified mosquitoes were maintained on 10% glucose solution for at least five hours prior to testing; whereas, for *An. arabiensis*, F1 female mosquitoes two to three days old (recommended age group) were used for bioassays from reared *Anopheles* larvae collected from the breeding habitats in the study sites [34,35]. Species identification was carried out after bioassays on dead mosquitoes using PCR.

A total of 100 mosquitoes were exposed per discriminating concentration in five replicates of 20 mosquitoes each, and compared to a control with same number of mosquitoes per replicate. In an exposure tube, mosquitoes were held for a total of one hour in intervals of 10, 15, 20, 30, 40, 50, and 60 minutes. After the first hour of exposure, mosquitoes were transferred to non-insecticide treated, clean, holding tubes and observed for a further 20 minutes [33]. After 80 minutes (initial 60 min + further 20 min) of knockdown monitoring, all mosquitoes were transferred

to non-insecticide treated, clean, holding tubes and kept for 24 hours and provided with 10% glucose solution, after which mortality was monitored and recorded. All these procedures were performed in the field under average ambient temperatures of 26 ± 2°C and a relative humidity of 78 ± 3% in both bioassay rounds. Percentage knockdown in the observed mosquitoes was recorded immediately for each time interval, and mosquito mortality in each bioassay was expressed as the proportion of dead mosquitoes to total exposed, for each tested insecticide. Execution and interpretation followed recently updated WHO test procedures for insecticide resistance monitoring in malaria vector mosquitoes [33].

Statistical analyses

Only data pertaining to *An. gambiae s.l.* and *An. funestus* were analysed, using SPSS version 20 (SPSS Inc, Chicago, USA). Data were fitted with generalized linear models (GLMs) using a negative binomial distribution with log-link function, and relative rates (RR) with 95% confidence intervals calculated to estimate yearly mean mosquito catches, relative to the reference year. Species (*An. gambiae s.l.* and *An. funestus)* were treated as predictors and total number of mosquitoes as a dependent variable; the statistical differences in dependent variables was evaluated as a function of villages (Idete and Namwawala), seasons (wet and dry) and years (2008–2012).

Insecticide susceptibility test biossay data were considered for each diagnostic concentration and year of testing. Mortality was calculated as the percentage of mosquitoes dead post 24 hours' exposure to insecticide, and the results were assessed according to WHO testing procedure for insecticide resistance monitoring in malaria vectors [33]. Mortality rates between 98 and 100% indicate full susceptibility, 90-97% is suggestive of resistance and requires further investigation, and mortality rates less than 90% confirm the existence of resistance.

Ethical considerations

The study approval was granted by the Ifakara Health Institutional Review Board (IHRDC/IRB/No.A-32) and the National Institute of Medical Research (NIMR/HQ/R.8a/Vol. IX/764). On first visiting each house, the benefits and possible risks associated with the study were explained to the house occupants and informed consent to proceed was requested. After consenting, the head of the house was asked to sign two copies of the informed consent forms, (retained by the head of the house and the study investigator).

Results

Relative abundance of malaria vector species

During the five consecutive years of sampling with CDC light traps in sentinel houses, a total of 100,810 malaria vectors were collected of which 76% were *Anopheles gambiae sensu lato* and 24% were *An. funestus*. In each of the first four years (2008–2011), the proportion of *An. gambiae s.l.* was significantly higher than *An. funestus* in both study villages (p <0.0001): proportions in total catches in Namwawala were 94% (40,028) and 6% (2,398), and in Idete were 87% (24,869) and 13% (3,730) for *An. gambiae s.l.* and *An. funestus*, respectively. However, in 2012, the proportion of total catch of *An. funestus* was significantly higher than *An. gambiae s.l.* in both villages: 42% (6,622) and 58% (8,953) in Namwawala and 35% (4,479) and 65% (8,447) in Idete for *An. gambiae s.l.* and *An. funestus*, respectively, (RR (95% CI) = 1.35 (1.23-1.49), p <0.0001).

A total of 8,117 *An. gambiae s.l.* were successfully identified by PCR and comprised 93% *An. arabiensis* (n = 7,549) and 7% *An. gambiae s.s.* (n = 568). The relative proportions of the species were similar in Idete (*An. arabiensis* 96% (n = 3,610), *An. gambiae s.s.* 4% (n = 151) and in Namwawala 90% (n = 3,900) *An. arabiensis*, 10% (n = 456) *An. gambiae s.s.*. However, the relative proportion between the two sibling species was changing over time, with significant decrease of *An. gambiae s.s.* from 14% (409/2,924) in year 2008 to disappearance 0% (0/1,362) in year 2012, compared to *An. arabiensis* increasing from 86% in 2008 to 100% in 2012 (Table 1).

Of the 2,737 *An. funestus* samples that were identified by PCR, 97% were *An. funestus s.s.* (n = 2,655), 2% were *Anopheles rivorulum* (n = 55) and 1% *Anopheles leesoni* (n = 27). The species composition of *An. funestus* in Idete was 98% (n = 1,554) *An. funestus s.s,* 1.5% (n = 23) *An. rivorulum* and 0.4% (n = 6) *An. leesoni.* In Namwawala it was 98% (n = 1,133) *An. funestus s.s.,* 0.6% (n = 7) *An. rivorulum* and 1.2% (n = 14) *An. leesoni,* (Table 1).

Seasonal variation in vector abundance

During the study, the period from January to May was categorized as the wet season, receiving an average (+SD) of rainfall of 281 + 178 mm/month, and June-December as the dry season, with an average of rainfall of 24 + 66 mm/month (Figure 2). The abundance of both *An. gambiae s.l.* and *An. funestus* peaked in the wet season in both villages. The mean number (+SD) of *An. gambiae s.l.* caught per trap per night during the wet season was 19 + 48 and 32 + 110, whereas in the dry season it decreased to 0.86 + 5.7 and 1.1 + 5.8 at Idete and Namwawala, respectively. Furthermore, *An. gambaie s.s.* was only present in the wet season in the first three years (2008-2009/10) before its disappearance in 2011/12, compared to its sibling species *An. arabiensis*, which was found to exist in both season, similar to *An. funestus s.s.*, a dominating member of *An. funestus* group.

The mean number of *An. funestus* per trap per night in the wet and dry season of the first four years of study

Table 1 Malaria vector composition, sporozoite prevalence (S), biting rate (B) and entomological inoculation (EIR) for *Anopheles gambiae s.s., Anopheles arabiensis* and *Anopheles funestus* and their overall estimated yearly contribution to malaria transmission from year 2008–2012 in the study area

Species	2008	2009/10	2011	2012
***An. gambiae complex* sibling species proportion**				
An. gambiae s.s.	0.14	0.15	0.002	0
An. arabiensis	0.86	0.85	0.998	1
No. of PCR amplifications	2,924	1,307	2,542	1,362
***An. funestus* group sibling species proportion**				
An. funestus s.s.	-	0.887	0.956	1
An. rivulorum	-	0.013	0.021	0
An. leesoni	-	0	0.023	0
An. parensis	-	0	0.001	0
No. of PCR amplifications	-	330	880	1,527
Sporozoite prevalence (S;%)				
An. gambiae s.s.	1.18	0.04	0	0
An. arabiensis	0.16	0.36	0.07	1.47
An. funestus	1.71	0	0.43	2.20
Biting rate (B; b/p/n)				
An. gambiae s.s.	8.52	6.05	0.04	0
An. arabiensis	52.37	35.51	59.74	20.70
An. funestus	1.74	12.84	10.09	14.31
Entomological Inoculation Rate (EIR; ib/p/y)				
An. gambiae s.s.	36.70	1.61	0	0
An. arabiensis	30.58	55.51	15.17	110.90
An. funestus	10.86	0	15.58	115.10
Total	**78.14**	**57.12**	**31.05**	**226.0**

Note: Sporozoite prevalence = Number of positive sporozoite mosquitoes/total tested; Biting rate = Total collections/trap nights/calibration factor, 0.3 for *An. gambiae* complex, and 0.68 for *An. funestus* [21]; EIR = S × B × 365.

(2008–2011) was consistently similar in both villages. In the wet season, the mean catches (+SD) were 1.23 + 4.7 in 2008, 2.15 + 7.5 in 2009/10, 0.64 + 1.9 in 2011 compared to 1.15 + 5.2, 0.77 + 4.3 and 1.62 + 5.52 of the respective years in the dry season. In 2012, the mean catch of *An. funestus*, both in wet and dry seasons, was approximately six times significantly higher than in the previous years (p <0.0001): 11.75 + 45.8 and 8.3 + 25.6 of wet and dry season, respectively.

Malaria transmission

A total of 10,138 individual mosquitoes (530 *An. gambiae s.s.,* 7,130 *An. arabiensis* and 2,478 *An. funestus s.s.*) were screened for *Plasmodium falciparum* sporozoites of which 75 were positive (0.74% sporozoite prevalence). Although *An. gambiae s.s.* was the major malaria vector with a sporozoite prevalence of 1.18% in 2008, its dominance decreased with time to zero in 2011 and 2012, following its control to undetectable levels. Conversely, the importance of *An. arabiensis* and *An. funestus s.s* was increasing with time from a sporozoite prevalence of 0.16% in 2008 to 1.47% in 2012 for *An. arabiensis*, and from 1.71% in 2008 to 2.2% in 2012 for *An. funestus s.s.*

Similarly, the EIR of *An. gambiae s.s.* decreased drastically from 30.70 ib/p/yr in 2008 to 0 ib/p/yr in 2012, whereas those of *An. arabiensis* increased approximately four times from 30.58 in 2008 to 110.9 in 2012 and that of *An. funestus s.s.* increased 11 times from 10.86 in 2008 to 115.10 in 2012.

Overall, the level of malaria transmission in the study villages markedly decreased with time from an EIR of 78.14 ib/p/yr in 2008 to 31.05 ib/p/yr in 2011 but overwhelmingly increased to 226 ib/p/yr in 2012, approximately seven times more than in the previous year (Table 1).

Anopheles arabiensis and Anopheles funestus insecticide susceptibility tests

In the WHO bioassay testing, as the results indicated (Figure 3), *An. funestus* was fully susceptible to deltamethrin (100% mortality) with reduced susceptibility to permethrin (93%), and lambda cyhalothrin (91%) and confirmed resistance to DDT (86%) in year 2013. In 2014, *An. funestus* was resistant to permethrin (65%), lambda cyhalothrin (74%), bendiocarb (65%), and even to deltamethrin (87%) to which it was fully susceptible in 2013. Mortality in control tubes was 4% in both testing rounds. All tested mosquitoes were amplified as *An. funestus,* using PCR.

In year 2013, *An. arabiensis* was fully susceptible to bendiocarb (100% mortality) and deltamethrin (98.3%), reduced susceptibility against DDT (97%), and confirmed resistance to permethrin (83.3%) and lambda cyhalothrin (78%), with a control mortality of 0% across all test concentrations. Similar levels of resistance were maintained across tested diagnostic concentrations in year 2014, whereby the mosquitoes were fully susceptible to bendiocarb (98% mortality) and resistant to deltamethrin (64%), permethrin (77%), and lambda cyhalothrin (42%), with a control mortality of 0% across all test concentrations.

Discussion

This study provides substantial information on malaria vector dynamics and their contribution to malaria transmission in rural southern Tanzania over a five year period. Consistent with other studies, which have documented a shift in malaria vector composition and a change in malaria transmission dynamics seemingly as a result of extensive use of LLINs [4,5,36], this study reports a steady decrease to undetectable levels of *An.*

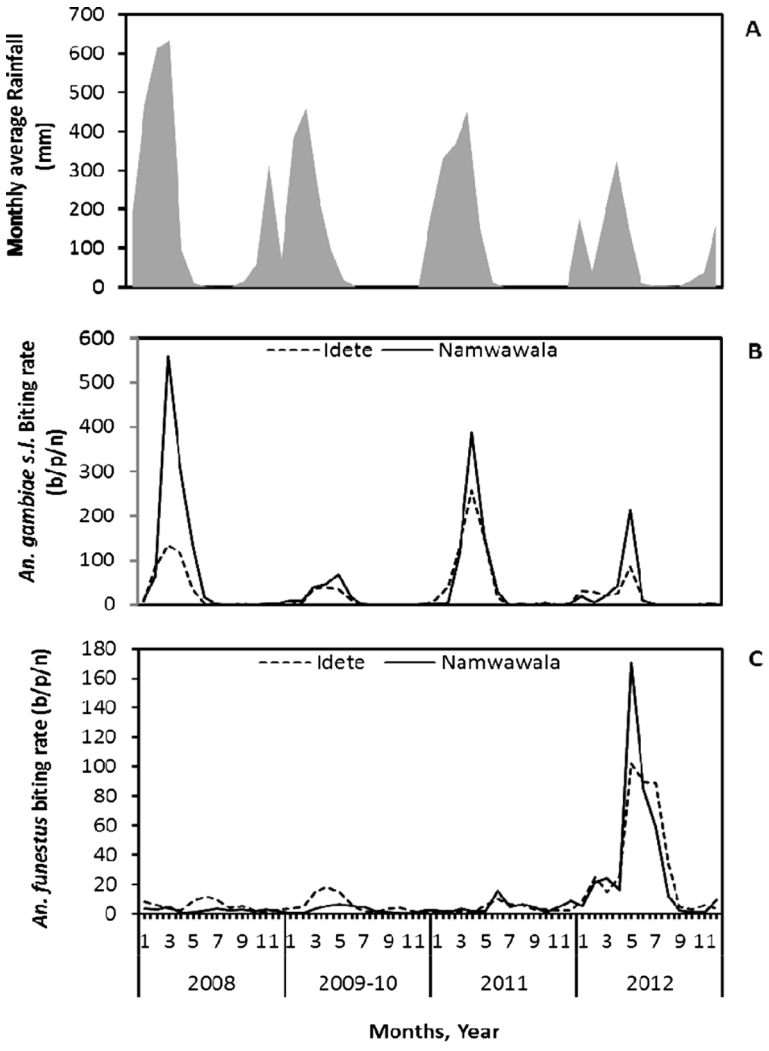

Figure 2 Monthly average rainfall in the Kilombero Valley. (A) estimated using CDC monthly biting rates, adjusted by dividing by species-specific relative efficiency of 0.3 and 0.68 for *An. gambiae s.l.* **(B)** and *An. funestus* **(C)**, respectively [21], in Idete and Namwawala villages over time.

gambiae s.s. with steady increase in the proportion of its sibling species *An. arabiensis* and a surge in the abundance of *An. funestus s.s.* in year 2012.

Anopheles gambiae s.s. prefers to feed and rest inside houses. This makes it more vulnerable to insecticides applied to nets (LLINs) and walls (indoor residual spraying (IRS)) while *An. arabiensis*, with its opportunistic feeding behaviour both on humans and animals [12,32] and its potential to rest outside human dwellings, make it less affected by LLINs. Although, lack of outdoor mosquito collections was a major limitation of this study in explaining the shift in biting periodicity and outdoor biting, it has been recently documented elsewhere that *An. arabiensis* and *An. funestus* [25,37] display a behavioural avoidance to contact LLINs by feeding outdoors in early part of the evening which might increase its chance to survive current interventions.

A significant increase in *An. funestus* abundance and EIR in 2012 is demonstrated. This shift poses great concern in malaria control efforts due to its efficiency in transmitting malaria. Historically the control of *An. funestus s.s.* was successful through extensive IRS, taking advantage of its highly anthropophagic and endophilic behaviour, using dieldrin in Pare, Taveta, northern Tanzania [38,39] Malindi on the coast of Kenya, using DDT [40] as well as in South Africa [41]. This is partly because they spend a longer time on insecticide-treated materials [42]. However, the vector eventually resurged six years later due to a lack of IRS programme continuity and consolidation [40,43]. A similar scenario was expected in this particular region, where usage of LLINs is high [3,44].

The steady increase in *An. funestus* population density, despite extensive usage of LLINs in the study area, may

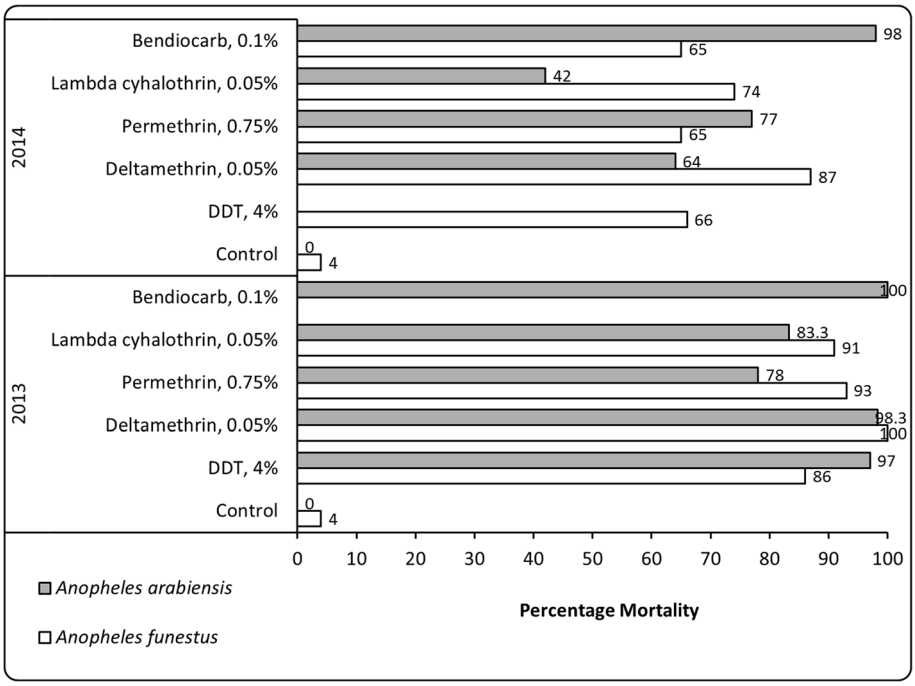

Figure 3 Results of WHO bioassay test for insecticide susceptibility status of wild female *Anopheles funestus* (white bars) and *Anopheles arabiensis* (grey bars) from the study sites in the Kilombero Valley, Tanzania, in January 2013 and June 2014. The graph shows percentage 24 hours mortality rate after a one-hour exposure to the WHO diagnostic doses of insecticide. The minimum sample size for these assays was 100.

be due to its reduced susceptibility to the insecticides used in LLINs. Recent findings from western Kenya have demonstrated similar phenomenon of resurging *An. funestus* populations, chiefly being due to resistance development to the pyrethroids used in LLINs [45].

Preliminary findings from this study demonstrated high resistance of *An. funestus* and *An. arabiensis* to pyrethroids, deltamethrin, lambda cyhalothrin and permethrin, used in Olyset LLINs, distributed in the study area in June 2011 [3]. Overall, there was great variation of the resistance status between 2013 and 2014 in both species tested; however, the variation was surprisingly huge in *An. funestus* than *An. arabiensis*, which might be due to inconsistency in unknown age of the used wild mosquito females [33].

Due to the absence of organochlorine insecticide DDT and carbamate insecticide bendiocarb deployment for malaria vector control in the study area, the source of resistance in mosquitoes to these insecticides remains unknown. Although not tested in this particular study, pyrethroid (DDT and pyrethroid) carbamate cross-resistance was considered to be a probable cause of *An. funestus* resistance to DDT and bendiocarb, respectively, which has been proved to exist in malaria vectors elsewhere [46,47]. In addition, the continuous and illegitimate use of DDT as a pesticide in agriculture in the region might have contaminated malaria vector breeding habitats and caused physiological resistance in mosquitoes [48].

Pyrethroid resistance in both species has been documented in multiple countries and regions of East Africa [45,49,50], southern Africa [51-54] and West Africa [55-57]. Further detailed studies are urgently required to establish current vector control operational impacts associated with this level of resistance. These findings suggest an increased contribution of these vectors to malaria transmission and hence great threat to the future use of LLINs in controlling these vectors.

The other probable cause for the observed increase in *An. funestus* population in this study area, which requires further investigation, might be a shift of *An. funestus* to outdoor and early evening and daytime biting behaviours, which increase their chances to survive and reproduce by feeding on unprotected humans, as recently documented *An. funestus* behaviours in Benin [58] and Senegal [59], West Africa.

In this study, both *An. funestus* and *An. gambiae s.l.* vector abundance varied with season. Increases in *An. gambiae s.l.* densities are facilitated by a wide range of ephemeral, sunlit, breeding habitats, such as hoof prints, rice puddles and ground depressions created during the rainy season [18,60]. The temporary nature of these habitats tends to reduce predation rate but also allows quick development of the juvenile stages, which results in *An. gambiae s.l.* dominating during the rainy season [18]. On the contrary, *An. funestus* prefer vegetated semi-permanent and permanent breeding habitats, such

as swamps and large ponds [18]. *Anopheles funestus* remained at a reasonable and detectable density across the rainy and dry seasons in the study areas and were significantly more abundant than *An. gambiae s.l.* in the dry season, probably due to their breeding habitat stability against desiccation [61].

Irrespective of seasonal variation in vector abundance, *An. funestus s.s.*, *An. gambiae s.s.* and *An. arabiensis* were all-important malaria vectors in the study area [12]. Despite high abundance of *An. arabiensis* and a higher EIR between 2008 and 2010, *An. funestus* contributed a relatively higher or equal EIR in 2011 and 2012. Historically, *An. funestus* has displayed high sporozoite prevalence [62] similar to that observed in this study and in a recent study conducted in neighbouring villages within the valley (Kaindoa *et al.,* unpublished). This trend of increase in abundance and high sporozoite prevalence of *An. funestus* has been also observed in Asembo district, western Kenya [45] and so appears to represent a trend across several regions of East Africa.

The huge increase in potential malaria transmission in 2012 (EIR = 226) coincided with an increase in abundance and sporozoite rates in *An. funestus* as it did in a neighbouring village in the valley (EIR = 467) (Kaindoa *et al.*, unpublished). The substantial increase in *An. funestus* and its reduced susceptibility to pyrethroids poses a serious threat that needs attention from vector control stakeholders. A separate study in West Africa also reported a rebound in malaria transmission partly being caused by resistance development in *An. gambiae* to pyrethroids [63].

A previous study has shown that despite high coverage and usage of LLINs, a high proportion of mosquitoes still enter houses [64]. Therefore, the increase in *An. funestus*, particularly in the dry season, is likely to exacerbate the problem. Therefore, new strategies to address resistance and outdoor biting behaviour in the early part of the evening as displayed by *An. funestus* and *An. arabiensis* are required. This can be achieved through improving the LLINs; for instance, recent development of nets which can target multiple resistant mosquitoes, Olyset® Plus [65], and by targeting vectors while outdoors using non-resistant compounds, either through larval source management in the dry season via autodissemination of insect juvenile hormone, e.g., pyriproxyfen [66,67], or by mosquito sterilization with pyriproxyfen [68], and killing them with toxic sugar-baited traps [69], non-chemical electric grid [70] and odour-baited traps [71].

Conclusion

This study shows that *An. funestus* and *An. arabiensis* are important malaria vectors sustaining malaria transmission, with a substantial increase in *An. funestus* and drastic reduction in *An. gambiae s.s.* in the year 2012. Malaria transmission significantly declined from 2008 to 2011 and rebounded in 2012 coinciding with an increased role of *An. arabiensis* and *An. funestus* in malaria transmission. Although fully susceptible to deltamethrin, *An. arabiensis* and *An. funestus* were found to be resistant and with reduced susceptibility to permethrin pyrethroid used for LLINs, respectively. These findings call for complementary vector control tools, robust vector surveillance systems and an insecticide resistance management plan to complement and preserve the efficacy of LLINs.

Competing interests

The authors have declared no competing interests.

Authors' contributions

DWL, CH, SD, SM, PJM, and GJD proposed the study hypothesis and experimental design. DWL and SSK performed statistical analysis and wrote the first draft of the manuscript. DWL supervised the surveillance and data collection. DWL, SM and PJM wrote the manuscript. All authors read and approved the final manuscript.

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Effective autodissemination of pyriproxyfen to breeding sites by the exophilic malaria vector Anopheles arabiensis in semi-field settings in Tanzania

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Abstract

Background: Malaria vector control strategies that target adult female mosquitoes are challenged by the emergence of insecticide resistance and behavioural resilience. Conventional larviciding is restricted by high operational costs and inadequate knowledge of mosquito-breeding habitats in rural settings that might be overcome by the juvenile hormone analogue, Pyriproxyfen (PPF). This study assessed the potential for *Anopheles arabiensis* to pick up and transfer lethal doses of PPF from contamination sites to their breeding habitats (*i.e.* autodissemination of PPF).

Methods: A semi-field system (SFS) with four identical separate chambers was used to evaluate PPF-treated clay pots for delivering PPF to resting adult female mosquitoes for subsequent autodissemination to artificial breeding habitats within the chambers. In each chamber, a tethered cow provided blood meals to laboratory-reared, unfed female *An. arabiensis* released in the SFS. In PPF-treated chambers, clay pot linings were dusted with 0.2 – 0.3 g AI PPF per pot. Pupae were removed from the artificial habitats daily, and emergence rates calculated. Impact of PPF on emergence was determined by comparing treatment with an appropriate control group.

Results: Mean (95% CI) adult emergence rates were (0.21 ± 0.299) and (0.95 ± 0.39) from PPF-treated and controls respectively ($p < 0.0001$). Laboratory bioassay of water samples from artificial habitats in these experiments resulted in significantly lower emergence rates in treated chambers (0.16 ± 0.23) compared to controls 0.97 ± 0.05) ($p < 0.0001$). In experiments where no mosquitoes introduced, there were no significant differences between control and treatment, indicating that transfer of PPF to breeding sites only occurred when mosquitoes were present; *i.e.* that autodissemination had occurred. Treatment of a single clay pot reduced adult emergence in six habitats to (0.34 ± 0.13) compared to (0.98 ± 0.02) in the controls ($p < 0.0001$), showing a high level of habitats coverage amplification of the autodissemination event.

Conclusion: The study provides proof of principle for the autodissemination of PPF to breeding habitats by malaria vectors. These findings highlight the potential for this technique for outdoor control of malaria vectors and call for the testing of this technique in field trials.

Keywords: Autodissemination, Pyriproxyfen, *Anopheles arabiensis*, Malaria, Africa, Vector control, Semi-field system, Clay pots

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Background

Malaria remains one of mankind's leading public health challenges and a major economic burden for the developing nations where it is endemic. Disproportionately, 80% of all malaria cases and 90% deaths occur in Africa [1]. The World Health Organization (WHO) continues to recommend a range of combined strategies for malaria prevention with vector control, primarily through the use of insecticide-treated bed nets (LLINs) and indoor residual insecticide spraying (IRS), a key component of those strategies [2-4]. Despite great progress in reducing malaria transmission in Africa over the past decade, the future use of both of these interventions, and indeed any approach that relies on chemical insecticides, is seriously threatened by the emergence and ongoing spread of insecticide resistance [5-8]. Moreover, LLINs and IRS target only vectors that are active indoors, and even in areas where this has been successful, malaria transmission by outdoor biting and outdoor resting vector populations of *Anopheles arabiensis* and *Anopheles funestus* remains a serious public health challenge [9,10]. Effective sustainable tools or approaches with proven impact on outdoor biting and resting vector populations have yet to be developed.

Targeting the aquatic larval stages of the vector with conventional insecticides (larviciding), as a complement to LLINs and IRS, can be an effective method to suppress vector density [11], but it is limited by the difficult task, and high cost, of identifying and treating sufficient mosquito breeding habitats to impact the vector population [12,13]. WHO recommendations limit the use of larviciding to settings where larval habitats are few, findable, and easy to map and treat; typically this restricts larviciding to urban settings [14]. In rural settings where breeding habitats are abundant in number and character, this is a far greater challenge for which novel approaches are urgently needed.

Pyriproxyfen (PPF) is a juvenile hormone analogue (JHA) that interrupts normal development and metamorphosis of targeted mosquitoes [15]. Highly potent in terms of activity and specificity, it has low toxicity and a high margin of safety to non-target organisms [16] and to date, there has been no evidence of PPF resistance in any mosquito [17]. For effective mosquito control, WHO recommends a PPF dosage limit of 50 ppb, an extremely low level considering the maximum permissible level in drinking water is 300 ppb [18]. PPF can be delivered in formulations that persist in treated aquatic habitats for up to six months under field conditions [19,20]. PPF also has an additional unique benefit, termed autodissemination, which is defined as the ability of adult mosquitoes to pick up PPF from treated solid surfaces, retain and transfer it to breeding habitats in sufficient quantities to contaminate those habitats, rendering them unproductive either by killing larvae or preventing pupae from emerging to adults [21].

The few studies demonstrating the potential of autodissemination of PPF in vector control have been limited to the *Aedes* vectors of dengue and chikungunya viruses [21,22]. Small field trials in urban settings in Peru and Italy, against *Aedes aegypti* [21] and *Aedes albopictus* [22] respectively, resulted in significant adult emergence inhibition in treated areas. Many aspects of the biology of these *Aedes* species, such as their aggressive feeding, skip-oviposition (distributing portions of each egg batch in multiple habitats) and preference for relatively small volume man-made containers as breeding habitats, undoubtedly contribute to the prospect for exploiting autodissemination in urban control programs for dengue and chikungunya [19,21,22] and fabrication of efficient PPF contamination sites/stations [22,23]. The outdoor-active *Anopheles spp.* that transmit malaria in rural Africa breed in a wide variety of breeding habitats, ranging in size and character and across much larger areas [24] and are a much greater challenge for this approach.

This study reports on the first experiments undertaken in a large semi-field system in Tanzania, evaluating the potential of PPF autodissemination for control of *An. arabiensis* and probably other African malaria vectors. Here, the results of controlled experiments quantifying the efficacy of clay pots, a simple inexpensive PPF contamination station, for delivering PPF to resting adult female *Anopheles arabiensis* at levels that prevent emergence at untreated breeding habitats are presented, demonstrating for the first time that, in principle autodissemination of PPF can occur at operationally effective rates in an *Anopheles arabiensis*, an efficient African malaria vector.

Methods

Study site

This study was carried out at Kining'ina village (8.11417 S, 36.67484 E), in rural southern Tanzania, between May 2012 and October 2013 inside a semi-field system (SFS). Details of the design and use of this SFS have been provided previously [25,26]. Briefly, the SFS is an outdoor construction with mesh walls 4.53 m high, measuring 552.96 m^2 in total area but partitioned into six separate chambers each measuring 9.6 × 9.6 m. The concrete floors of the chambers were filled to a depth of 40 cm with local soil, and vegetation was allowed to grow naturally from the seeds therein. Although the SFS had six chambers, only four chambers were used for the experiments. A simple mud hut (1.75 m × 1.5 m, 2 m high) was built within each chamber to provide a shelter for a tethered cow bait, and possible resting location for mosquitoes. The simple mud hut was built to mimic the shelters used by communities to keep cows and not to represent an indoor set up.

Mosquitoes

All sets of experiments were performed using insectary-reared unfed mated *An. arabiensis* females aged 3 – 9 days post eclosion. It was assumed that mosquitoes at this age would have mated [27]. The *An. arabiensis* colony was established in March 2010, originating from individuals collected in Lupiro village within the Kilombero valley. It is reared routinely inside a semi-field system (SFS) under natural temperature and 12: 12 h light: dark photoperiod of that area. Larvae were fed Tetramin® fish food and adults maintained on human blood and 6% glucose solution. Mosquitoes were starved of sugar and water six hours prior to release in the experiments.

Experimental procedures and study design

Five experiments were conducted between May 2012 and September 2013: first, to investigate the existence of PPF autodissemination from PPF-treated clay pots to the breeding habitats by contaminated mosquitoes; second, to confirm that the observed PPF contamination at the experimental breeding habitats was mosquito-borne; third, to investigate mosquito resting site preferences inside the SFS; fourth, to measure the proportion of mosquitoes resting inside the clay pots that were subsequently able to contaminate oviposition sites; fifth, to measure amplification of autodissemination from limited numbers of treatment points to a greater number of breeding sites.

Experiment 1: Evaluation of PPF-treated clay pots for the delivery of pyriproxyfen to resting adult female mosquitoes for subsequent autodissemination

In every replicate of this experiment, 1500 – 5000 adult female *An. arabiensis* were released inside an SFS chamber, where a cow was provided for blood feeding, clay pots as resting sites during egg development, and water containers as oviposition sites. Clay pots have been used for sampling wild *An. arabiensis*, as adult females of this and other species will rest within these and similar vessels [28,29].

Eight 10 L clay pots were placed on the ground: 5 around the perimeter of the SFS chamber and the other 3 around the walls of the mud hut. Each pot was lined with black cotton that had been dampened with water and dusted with PPF powder (0.2 – 0.3 g AI per clay pot; Sumilarv®, Sumitomo Chemical Co. Ltd., Japan). Dusting was done by unevenly sprinkling 2 – 3 g of 10% AI PPF powder over all surface of dampen cotton cloth using a makeup/painting blush. The cotton cloth was treated with PPF after being attached inside around the circumference of clay pot using 3 mm aluminium wire to ensure maximum containment of PPF powder (Figure 1C). Pots were allowed to dry for 24 hours, facilitating the PPF powder to attach lightly to the fabric while not hindering its pickup by mosquitoes that contacted it. Two identical artificial breeding habitats (2.5 L plastic basins, 21 cm in diameter; filled with 250 g of soil and 2 L of water; water levels were replenished as required) were buried with the rim at ground level, 5 m apart and between 1 and 8 m from clay pots (Figure 1). At the start of each experiment, 1,500 – 5,000 unfed female mosquitoes (aged between three and nine days post eclosion and caged with males until used) were released at 18.00 hrs. A cow, tethered inside the mud hut, was available for the first three days to permit blood feeding.

The experiment was allowed to run for 25 days following release of the mosquitoes, to allow 10 days until the first pupae developed and a further 15 days to harvest all pupae from the artificial aquatic habitats that successively developed from eggs laid by released mosquitoes. The breeding habitats were visually examined daily for the presence of eggs and larvae to confirm if mosquitoes visited the habitats. Each day, pupae developed from deposited eggs were removed, counted and transferred to an insectary where they were maintained under the cage in cups containing water from the habitats until they emerged as adults or died.

Control experiments were run simultaneously in a separate chamber using an identical protocol but without any PPF application to the cotton lining of the clay pots. A total of six replicates of both treatment and control experiments were run, over a period of 6 months. Treatment and control chambers were separated by a distance of 3.2 m and, to avoid PPF contamination of the control chamber, the same SFS chambers were used in all replicates for treatment and control. Of importance, control and treatment were not rotated but fixed between chambers, when one replicate was on-going in a pair of control and treatment chambers; the other pair of control and treatment chambers was put into uses. Where control and treatment chambers were adjacent to each other, a panel of white cloth was mounted on one side of partition net to prevent movement of PPF particles between chambers. A break of at least seven days between replicates minimized the chance of any mosquitoes surviving from the previous replicate. PPF contamination between replicates was minimized by spraying the chamber structure, the hut and vegetation with water, new plastic basins were used and cow were thoroughly cleaned by washing with only water without soap before each replicate. Successful contamination and dissemination was evaluated by comparing the differences in pupal mortality and emergence inhibition from the basins between treated experiments and controls.

PPF contamination of water in the experimental breeding habitats was investigated further by two methods. First, immediately after recording first stage larvae in the breeding habitats (typically 5–8 days after mosquito release), three 150 ml water sub-samples were collected

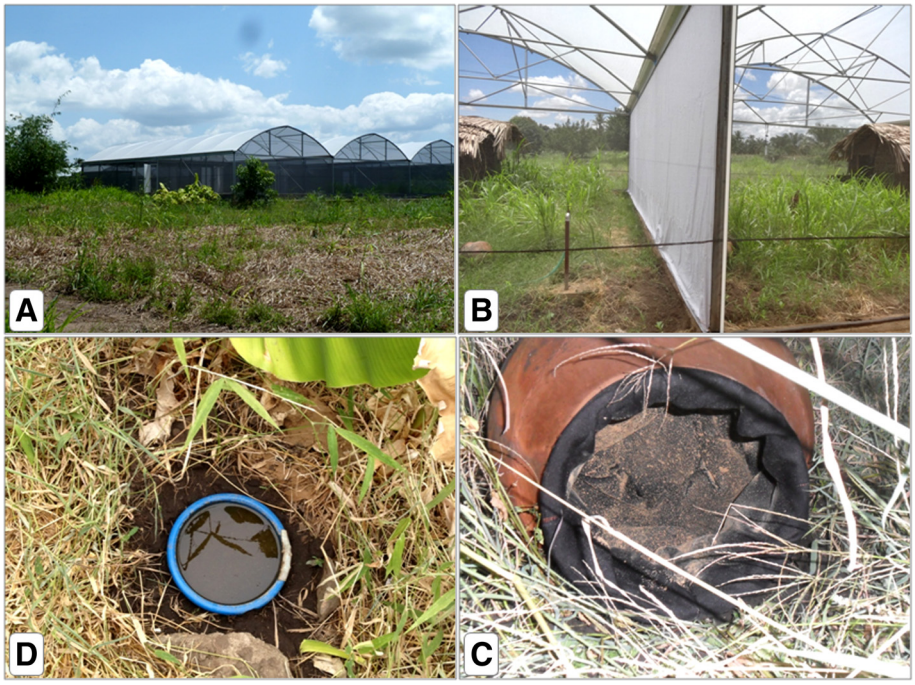

Figure 1 (A) The semi field system used in experiments; (B) adjoining chambers with huts for housing bait cows visible in each; (C), Pyriproxyfen (PPF)-treated cloth interior of a clay resting pot placed on the ground within a chamber; (D) plastic basin sunk in the ground within a chamber to provide the artificial habitat for egg laying.

from each habitat and transferred to separate 250 ml glass beakers. Twenty 2nd or 3rd instar *An. arabiensis* larvae taken from the laboratory colony (*i.e.* fresh uncontaminated individuals) were placed in each beaker and daily mortality and emergence rates were recorded until all were dead or had emerged as adults. The procedures were repeated twice, i.e. only in two consecutive experiments of the six experimental replicates.

In a second bioassay, at the termination of each experimental replicates (*i.e.* day 25 following initial introduction of adult females) and after pupation of all larvae and removal of all pupae, 250 second or third instar *An. arabiensis* larvae taken from the laboratory colony (*i.e.* fresh uncontaminated individuals) were introduced in each breeding habitat (assumed to be contaminated with PPF from previously released adults) and daily mortality and emergence was recorded until all were dead or had emerged as adults. The procedures were repeated twice, i.e. only in two consecutive experiments of the six experimental replicates.

Experiment 2: Confirmation that pyriproxyfen contamination of breeding habitats was mosquito-borne

To examine whether the PPF impact on adult emergence from the breeding habitats observed in the previous experiment might have resulted from the passive carriage by wind currents, or by other organisms (*e.g.* other invertebrates, amphibians, rodents, etc.), two tests were conducted using the setup of experiment 1.

In the first test, 250 second or third instar *An. arabiensis* larvae taken from the laboratory colony were introduced in the two breeding habitats with fresh water and soil in both treatment and control SFS chambers, which had been prepared exactly as described for Experiment 1. No adult mosquitoes were released in either chamber. The daily pupation, mortality and emergence rates were recorded until all pupae were dead or had emerged as adults. The experiment was allowed to run until all had pupated.

In the second test, the chambers used for treatment and control were reversed, *i.e.* the control was run in the chamber previously used for treatment and *vice versa*. A total of 5,000 adult female mosquitoes were released in each chamber and two replicates of the second test were conducted and breeding habitats productivity were monitored as described in experiment 1.

Experiment 3: Mosquito resting site preference inside the semi field systems

To determine the proportions of released mosquitoes that rested inside the clay pots in the experimental setup, adult female mosquitoes were released inside treated and control SFS chambers, as described for experiment 1. On each morning over the following three days (an average period

for eggs development before mosquito visits the habitats to lay eggs), all mosquitoes found resting inside clay pots and walls and ceiling of the cattle hut were collected using mouth aspirators, counted and recorded as either fed or unfed. The experiment was repeated twice, first with 2,000 mosquitoes and then with 4,000 mosquitoes released in each chamber (released mosquitoes were increased in the second replicate to increase the proportion of mosquitoes to be recaptured).

Experiment 4: Determining contamination rates of the Anopheles arabiensis population resting inside clay pots

To estimate the proportion of *An. arabiensis* contaminated with PPF in this setup, 5,000 unfed adult female mosquitoes were released inside both treated and control SFS chambers, where only clay pots were treated with PPF as described in experiment 1. On each of the three mornings after release, a maximum of 60 mosquitoes (30 from each of the resting sites) were collected inside all clay pots and mud huts (walls and ceiling) and assessed for their feeding status. Following resting behaviour in mosquito after acquiring a blood meal, mosquitoes were collected 36 hrs after release to ensure that high proportion was blood-fed. Individual mosquitoes were collected with separate mouth aspirators and held in a plastic cup (approximately 30 – 60 minutes) to avoid cross-contamination until use. Mosquitos were killed by refrigeration and each mosquito was suspended in 50 ml of water containing 10 third stage larvae of laboratory-reared *An. arabiensis* to monitor larval mortality and pupa emergence inhibition, over 12 days. In addition, the plastic collection aspirators were rinsed with water to remove any possible PPF particles and clean water added to a total volume of 50 ml in which 10 third-stage larvae were suspended, and followed up as just described. The experiment was repeated twice.

To calculate the proportion contaminated, a maximum mortality threshold above an upper 95% CI from a control section was set. Thus an observed larval or pupal mortality in a bioassay cup above the set threshold in the treatment arm, implied that the suspended mosquito was contaminated. The estimated contamination in the treatment section was corrected using Abbot's formula [30], where the control larval mortality was greater than 5%. Corrected contamination = [% Contamination –% mortality in control)/(100 –% mortality in control)] × 100.

Experiment 5: Determination of autodissemination efficiency with fewer treatment points and more breeding habitats

The impact of few treated clay pots (1–2) with PPF to deliver PPF contamination to resting mosquitoes was determined in two tests. In the first test, only two of the eight pots were treated with PPF and compared to a control section where all eight pots remained untreated. A batch of 5,000 unfed female *An. arabiensis* were released once in a control and treatment chambers.

In the second test, only one pot was treated with PPF in treatment section, and 5,000 unfed female *An. arabiensis* were released in a control and treatment chambers, in three consecutive batches of 2,000, then 2,000 and lastly 1,000, with an interval of one day between releases. The rationale of releasing different mosquito batches was to facilitate multiple visiting events of mosquitoes to the habitats, which were likely to occur mosquitoes are released in different batches rather than single batch. This also mimic what is likely to happen in nature where different mosquitoes are likely to transit in the same clay pots over time.

In both tests, six breeding habitats were provided, and pupae collected from individual habitat were monitored as described until all were dead or had emerged as adults.

Data analysis

All data were analysed using R v2.12.2 [31] and the lme4 package [32] for generalized linear mixed effects models. The differences in the total number of pupae collected and proportion emerged between control and treatment SFS chambers were determined with Poisson and binomial distribution respectively using a best-fit generalized linear mixed effect model. While treatment groups (with/without PPF) were classified as fixed effect in the model, experimental replicates, numbers of mosquito released, numbers of larvae, total numbers of pupae collected per control and treatment chambers, and numbers of breeding habitats per control and treatment chambers were assigned as random effects for the autodissemination of PPF and larval bioassay data.

Results

Experiment 1: Evaluation of PPF-treated clay pots for the delivery of pyriproxyfen to resting adult female mosquitoes for subsequent autodissemination

The results of the experiments measuring the impact of PPF-treated resting pots on emergence from nearby breeding habitats are summarized in Figure 2. In the six replicates carried out, an average proportion (95% CI) of adult emerged per experimental replicate was 0.95 ± 0.39) in the control group compared to 0.21 ± 2.99) in the PPF treatments ($p < 0.0001$) (Figure 2C). There was no difference in the mean number (95% CI) of pupae collected from the treatment group (717 ± 622.8) compared with the control group (590 ± 220.9) ($p = 0.579$) (Figure 2A), suggesting that oviposition behaviour of mosquitoes after PPF treatment was not affected by the treatment. However, mean (95% CI) proportion of adult emerged from collected pupae were significantly high in the control group (558 ± 201.9) compared with the treatment group (130.5 ± 155.6) ($p < 0.0001$) (Figure 2B). Low

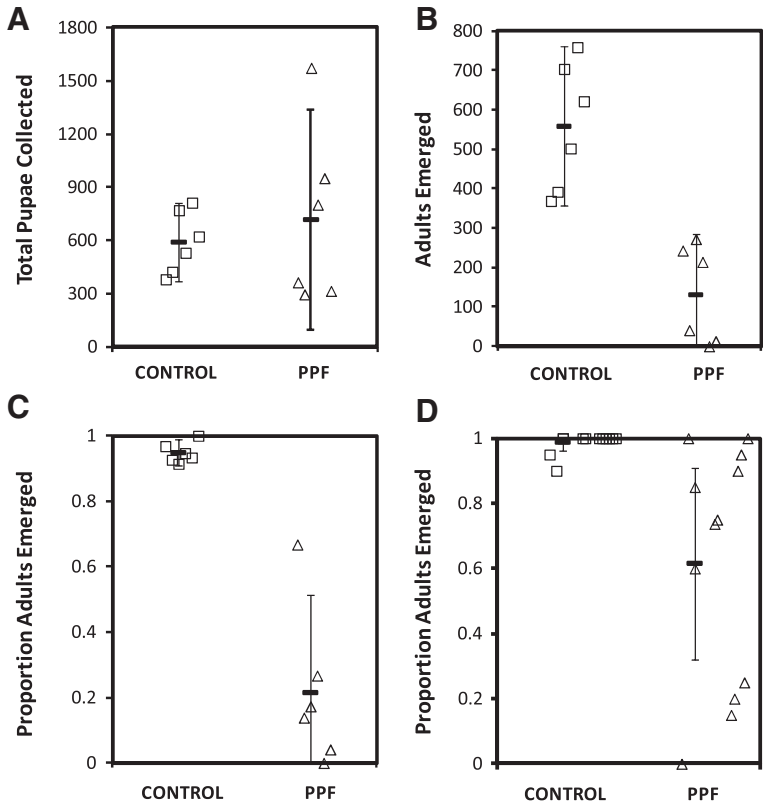

Figure 2 Number of pupae produced (A), adults emerged (B), proportion of adult emerged (C) in the breeding habitats and proportion of adult emerged from larval bioassay on water samples from control and PPF - treated sections (D).

adult emergence rate observed in the treatment chambers strongly suggest the occurrence of PPF autodissemination events mediated by gravid female mosquitoes attempting to oviposit.

In the laboratory bioassay measuring the effect of breeding habitat water on development of larvae, an average proportion (95% CI) of 0.987 ± 0.02 emerged to adults in water from the controls, while only 0.62 ± 0.29 emerged from the treatment group ($p = 0.0003$), (Figure 2D).

In the second larval bioassay, laboratory-reared larvae placed in the breeding habitats after the clay pot experiment ended, had significantly lower average (95% CI) emergence proportion in the treatment chamber (0.16 ± 0.23) compared to the control chamber (0.97 ± 0.05) ($p < 0.0001$), which confirm auto dissemination of PPF to the breeding sites. Attrition of introduced larvae due to predation and other natural causes were similar in both groups (315/500 and 359/500 larvae accounted for in control and treated groups respectively) and there was no evidence of any increase in larval mortality due to PPF ($p = 0.773$). All introduced larvae emerged successfully or died within 20 days of the start of the experiment.

Experiment 2: Confirmation that pyriproxyfen contamination of breeding habitats was mosquito-borne

In the first test of experiment 2 carried out, laboratory-reared larvae were placed in the breeding habitats of control and treatment chambers, prepared as described for experiment 1, except that here, no mosquitoes were released. The result of the single replicate showed that there was no difference in average (95% CI) proportion adult emergence per day between treatment (0.63 ± 0.24) and control sections (0.69 ± 0.32), ($p < 0.0001$). The total number of pupae collected from breeding habitats in the control (n = 379) and treatment (n = 392) chambers were not different ($p > 0.05$).

In the second test of experiment 2, the design of experiment 1 was repeated by releasing 5,000 adult female mosquitoes in each experimental chamber except here, the control was run in an SFS chamber previously used for PPF treatment, and *vice versa* for the treatment. Average (95% CI) adult mosquito proportion emergence were significantly higher in the control group, both before (0.95 ± 0.39) and after (0.72 ± 0.34) the locations were switched compared to the treatment (0.21 ± 2.99) and (0.05 ± 0.07) ($p < 0.0001$). The results of both experiments demonstrated that reductions in emergence rates

in the breeding habitats occurred only when adult mosquitoes were present in the PPF-treated chamber.

Experiment 3: Mosquito resting site preference inside the semi field systems

All recaptured mosquitoes from different resting sites were blood fed. A mean (95% CI) recapture rate of (0.385 ± 0.02) was achieved in all of the replicates carried out, with no difference seen between control (0.38 ± 0.005) and treatment groups (0.39 ± 0.021). ($p = 0.266$). Although, total number of mosquitoes recaptured increased when the number of mosquitoes released was greater ($p = 0.006$), the proportion of mosquito recaptured remains similar between replicates ($p = 0.543$). As Figure 3 shows, the majority of mosquitoes were collected from the ceiling and walls within the hut with 17% found within the resting pots.

Experiment 4: Determining contamination rates of Anopheles arabiensis population resting inside clay pots

As determined by their ability to inhibit adult emergence in a laboratory bioassay, 100% of all mosquitoes collected inside treated clay pots were found to be PPF-contaminated, while approximately 72% of those found resting in the hut within the treated chamber, were contaminated. Mosquitoes from PPF treated clay pots and huts caused (0.005 ± 0.007) and (0.52 ± 0.06) average adult emergence proportion from exposed larvae respectively in larval bioassay. In the control chamber, an average (95% CI) of (0.925 ± 0.08) of all larvae successful emerged to adults during larval bioassay using mosquitoes collected from clay pots and cattle shed in the control chamber.

Experiment 5: Determination of autodissemination efficiency with fewer treatment points and more breeding habitats

In both tests, impacts of PPF on pupal emergence were observed in all habitats in the treated chambers. When two clay pots were tested, the mean (95% CI) pupae collected from all breeding habitats were similar between control (52.57 ± 26.98) and treatment (62.92 ± 34.15) chambers, ($p = 0.522$). Similarly, the mean number of pupae collected was not different between control (100.34 ± 19.65) and treatment (104.88 ± 23.66) chambers when one clay pot was tested ($p = 0.883$). The mean proportion (95% CI) of emerged adults was significantly reduced in the treated chambers when two (0.33 ± 0.18) or only one (0.34 ± 0.13) clay pots were treated compared with the respective controls (0.82 ± 0.12); (0.98 ± 0.02); $p < 0.0001$)).

Discussion

Previous field studies have demonstrated the potential for the autodissemination technique when applied to free flying population *Aedes* mosquito species under field settings [21,22]. In this study, we also proved the occurrence of PPF autodissemination using captive populations of malaria vector *An. arabiensis* under semi-field settings. Overall, autodissemination of PPF by *An. arabiensis* inhibited 82% of adult emergence, which is compatible with the control level of 80% recommended by WHOPES for controlling malaria vector juvenile stages [33] under semi-field conditions. In some cases, for example experiment 1, Figure 2C, total emergence inhibition in PPF-treated sections was achieved with no single adult mosquito emerging from these habitats. Larval bioassays showed a significantly lower adult emergence rate in the treatment sections compared to the control further

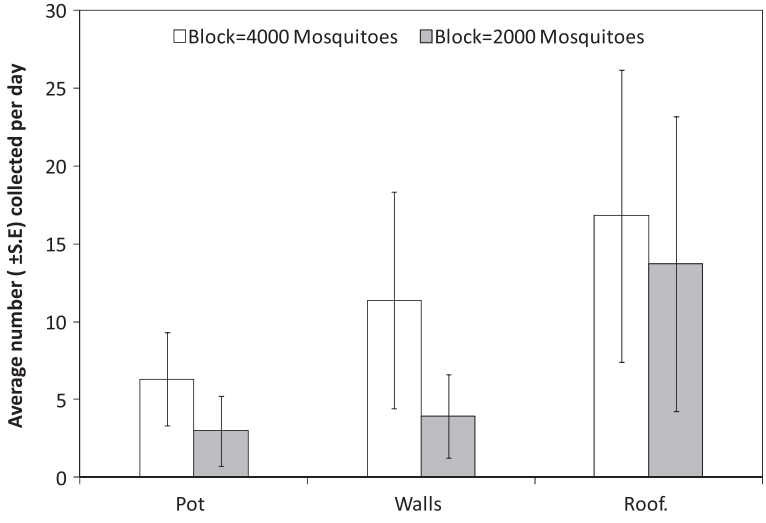

Figure 3 Average number of mosquitoes collected at different resting sites inside the Semi Field Systems.

confirming the delivery of PPF to the breeding habitats in all experiments. More importantly, by introducing insectary larvae directly in the habitats, an even lower emergence rate was observed compared to the control sections. This could be due to the presence of organic matter in the breeding habitats that would allow PPF adsorption and could prolong its persistence in aquatic habitats [34].

Though not clearly elucidated by the data presented here, it remains as a limitation of current study, that wide range and many number of mosquito released (1,500 – 5,000) in relation to number and size of breeding habitats might have affected the productivity of the habitats provided (pupae as a proxy indicator) by causing high larval mortality in the habitats due to overcrowding factors [35], and result in relative small number of pupae collected. However, the reason for a wide range was due to shortage of mosquitoes with a same age whereas many mosquitoes were released to make sure that our experiments were not confounded by shortage of mosquitoes following natural mortality and scavenging. Surprisingly, variations in the numbers of mosquitoes released did not affect the proportions of adults that ultimately emerged from the pupae in the contaminated breeding habitats, the inclusion of the numbers of mosquitoes released resulted in the best model. Since the numbers of mosquitoes visiting contamination stations would have differed between experiments and replicates, variation in mosquito numbers released and pupae collected from the were qualified as random rather than fixed factor.

Importantly, similar emergence rate recorded in the absence of mosquitoes between control and treatment chambers in first test of experiment 2 indicate that passive transfer of PPF (which might have confounded or potentially artificially enhanced any observed impact) did not occur at any stage in these studies. In addition, similar impact of PPF on adult emergence observed in the second test of experiment 2 as the results of released mosquitoes before and after switching locations of control and treatment chambers confirmed that dissemination by ovipositing mosquitoes alone was responsible for transfer of the effective dosages of PPF to the breeding habitats.

In assessing potential mosquito resting sites for targeting with PPF inside SFS, similar number of mosquitoes recaptured between control and treatment groups indicated that PPF does not repel resting mosquitoes. Overall, the proportions of recaptured adult female mosquitoes were few; this might have been caused by restricted collections from few designated places, and missed those resting in the vegetation grown inside the experimental chambers. High resting preference of mosquitoes to the wall and ceiling of the mud hut compared to the clay pots, highlight the potential of targeting these sites with PPF.

The results of experiment 5 are of particular importance because they demonstrated that only one treated resting pot competing with alternative untreated resting sites including seven clay pots and resting sites within the mud hut was sufficient to inhibit > 65% adult emergence in six breeding habitats via ovipositing mosquitoes alone. These findings are very promising and highlight the potential that autodissemination offers for amplification of limited numbers of treatment points to significant levels of effective breeding habitat treatment coverage. Clearly, field-based experiments and mathematical modelling should now be designed to investigate this further and establish the relationship between contamination stations and habitats coverage.

The mechanism of PPF delivery to mosquitoes is crucial for the overall success of the autodissemination technique [21-23]. In this study, the use of clay pots as a point source for PPF application effectively delivered PPF to the mosquitoes resting within and at rates sufficient to enable autodissemination. The attractiveness and usefulness of clay pots as an outdoor and indoor sampling tool for malaria and other disease vectors as well as a delivery tool for entomopathogenic fungi has been described elsewhere [28,36,37]. Although absolute numbers of mosquitoes resting inside clay pots are relatively low, these tools are considered to be efficient for sampling blood fed mosquitoes compared to many other sampling techniques [29]. The results presented here indicate that this simple and affordable method has additional potential in vector control.

When aquatic habitats are limited, the minority of mosquitoes that are contaminated in clay pots and then carry lethal doses of PPF to their aquatic habitats also affect the offspring of uncontaminated mosquitoes. Thus, contaminated adults amplify the impact of their own contamination by affecting the offspring of all mosquitoes that share the contaminated mosquito's breeding site [38,39]. Although not investigated in this study, field deployment of autodissemination approach is predicted to be affected by number of mosquitoes visiting the habitats, size of the breeding habitats and distance of the habitats from PPF contamination stations. Moreover, targeting only the clay pots with PPF resulted in the effective contamination of mosquitoes that were ultimately collected from the huts, suggesting that blood-fed mosquitoes move between resting sites during that phase of their gonotrophic cycle. This is clearly an advantage in terms of optimizing the effect of PPF through further "coverage amplification of the habitats" whereby PPF is likely to be delivered to many breeding habitats by PPF-contaminated mosquitoes using few habitats, and potentially might act to reduce the number and costs of contamination stations required [40]. Clay pots, by providing shelter from rain and sunlight, might also prolong the lifespan of single PPF treatments, an

important consideration in any 'insecticide'-based program. However, it should be noted that this experimental design provides only estimates, rather than actual numbers, of mosquitoes that rest or pass through clay pots and of whether they are contaminated or not.

The impact of PPF varies at different stages of the mosquito's life cycle. Previous work has shown that mosquitoes that are contaminated within 24 hrs of a blood meal become sterilized and do not lay eggs [41,42] but this sterilization effect does not occur when exposure to PPF occurs beyond 24 hrs after the blood meal. However, in the experiments reported here, the test mosquitoes produced large numbers of developing offspring in the artificial habitats provided, suggesting that the clay pots set outside the cattle sheds, were not visited by blood-fed mosquitoes until sometime after completion of feeding when egg-maturation was underway. If so, then it was while resting outdoors after the blood meal that these mosquitoes were contaminated, and targeting this stage of the gonotrophic cycle (i.e. >24 hours after blood feeding) may maximize delivery of PPF to the breeding habitats [23]. Alternatively, if PPF-contamination occurred immediately after or within 24 hours of blood feeding, then it suggests that these PPF-sterilized mosquitoes, despite not being gravid, went on to visit the breeding habitats where they prevented emergence of the next generation of mosquitoes from the eggs laid by uncontaminated adults.

Although a key necessity for its success is the development of efficient contamination stations, a role performed very well by the clay pots in the experiments reported here, the autodissemination technique potentially can target both indoor and outdoor biting mosquitoes, susceptible and pyrethroid resistant mosquito strains at their larval habitat, with impacts on adult mosquito density and malaria transmission [14,40,43]. The integration of this method of control with current vector control measures (LLINs and IRS) could help in the control of outdoor biting vectors such as *An. arabiensis* as well as providing an approach to managing insecticide resistance [44]. The autodissemination of insecticides by adult mosquitoes for the control of malaria is likely to work better in the dry season when the breeding habitats are few and stable with reduced water flushing [38,40]. With recent development of highly potent formulation up to 10% AI PPF dust, which is effective at ultra-low dose, it might be possible to effectively contaminate greater volumes than current possible using malaria vectors and other mosquitoes that share the habitats with Anopheles mosquitoes.

This is the first study to investigate the potential for using PPF autodissemination for the control of *An. arabiensis,* one of the efficient African malaria vectors. The results are very promising and indicate that this approach offers an opportunity to be considered amongst future malaria control strategies in Africa. Before its full potential can be assessed, further vector studies will be required in key areas: 1) the effectiveness seen in these semi-field experiments must be demonstrated under full field conditions; 2) quantitative studies on 'amplification' are required to determine the numbers and densities of treatment points required to deliver effective control at breeding sites; 3) investigations of impacts on other species sharing the breeding sites, including other vectors, nuisance mosquitoes and non-target species.

Competing interests
The authors have declared no competing interests.

Authors' contributions
DWL, CH, SD, SM, PJM and GJD proposed the study hypothesis and experimental design. DWL, SSK and CH performed statistical analysis and wrote the first draft of the manuscript. DWL supervised the experiments and data collection. DWL, SM and PJM wrote the manuscript. All authors read and approved the final manuscript.

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Short Report: Comprehensive Sterilization of Malaria Vectors Using Pyriproxyfen: A Step Closer to Malaria Elimination

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Abstract. One of the main challenges to malaria elimination is the resilience of vectors, such as *Anopheles arabiensis*, that evade lethal exposure to insecticidal control measures or express resistance to their active ingredients. This study investigated a novel technology for population control that sterilizes mosquitoes using pyriproxyfen, a juvenile hormone analogue. Females of *An. arabiensis* were released in a semifield system divided into four equal sections, and each section had a mud hut sheltering a tethered cow providing a blood source for mosquitoes. In all sections, the inner mud hut walls and roofs were lined with black cotton cloth. In one-half of the sections, the cloth was dusted with pyriproxyfen. An overwhelming 96% reduction in adult production was achieved in pyriproxyfen-treated sections compared with control sections. This unprecedented level of control can be exploited to design new vector control strategies that particularly target existing behaviorally resilient and insecticide-resistant populations.

INTRODUCTION

Current frontline malaria vector control interventions, such as long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS), have contributed greatly to the recent successes in malaria control.[1] However, these tools are more effective against vector species that primarily feed indoors on humans and rest indoors. They are less effective against outdoor feeding and resting mosquitoes. *Anopheles arabiensis*, currently mediating most of the residual malaria transmission in east Africa,[2,3] is not optimally controlled by LLINs and IRS, because it commonly feeds outdoors on humans or cattle, rests outdoors, and can enter but then rapidly exit houses containing these products without exposure to lethal doses of their active ingredients (AIs).[3,4]

Another challenge to malaria vector control is the development of resistance in malaria vectors against all classes of insecticides currently used for LLINs and IRS, particularly pyrethroids, the most widely used and the only class approved for use in bednets.[5]

Pyriproxyfen (PPF) is a juvenile hormone analogue that traditionally has been used in aquatic habitats to prevent mosquito larvae and pupae from developing into adults. However, it can also sterilize adult mosquitoes on contact.[6–8] This study builds from our previous work performed in laboratory conditions showing that *An. arabiensis* mosquitoes were particularly vulnerable to sterilization immediately after blood feeding.[8] Adult mosquitoes can also transfer PPF from resting sites to breeding sites to interfere with immature development.[9,10] Here, we show, for the first time, an operationally practicable means of controlling a free-flying captive population of *An. arabiensis* using PPF.

MATERIALS AND METHODS

This study was carried out in southern Tanzania inside a semifield system (SFS) with walls consisting of netting only, and therefore, the microclimate inside it closely resembled the natural environment outside of it.[11] The SFS was divided into four equal sections, with a space volume of approximately 360 m^3 each. In each section, a mud hut sheltering a tethered cow, eight clay pots, and four plastic basins with soil and water were designed to provide blood, resting, and oviposition sites for mosquitoes (Figure 1). In all sections, the inner mud hut walls and roofs were lined with black cotton cloth, and in one-half of the sections, the cloth was dusted with PPF powder (0.6–0.8 g AI/m^2). In total, 5,000 unfed 3 to 9-day-old insectary-reared *An. arabiensis* females, previously caged with equivalent numbers of males, were released per section, with a cow to provide blood for the first 3 days only. Mosquitoes used in the experiments were starved 6 hours before release. Therefore, they fed on the cow, and after 3 days, a solution of 6% glucose was set up at multiple locations inside the SFS for sugar feeding. These mosquitoes remained in the SFS to complete their gonotrophic cycle. All pupae that subsequently developed from the aquatic habitats were removed, counted, and reared in small cages to monitor the numbers of emerging adults and therefore, the impact of PPF exposure on the mosquitoes' ability to produce viable offspring. Seven days after larvae were observed in the habitats, 150 mL water were collected from every habitat using a glass beaker to determine whether PPF had been transferred to these habitats by contaminated mosquitoes during oviposition.[12] To assess the presence of PPF in each beaker, larval bioassays were conducted using second and third instar larvae from the insectary. Twenty *An. arabiensis* larvae were introduced in each beaker and monitored daily until all larvae and pupae had either died or developed and emerged to adults. Five replicates each of the control and treatment were completed in three separate experiments in the following setup. In the first experiment, two replicates (treatment and control) were run (5,000 × 4 = 20,000 mosquitoes); in the second experiment, two replicates (treatment and control) were run (5,000 × 4 = 20,000 mosquitoes), and in the third experiment, one replicate (treatment and control) was run (5,000 × 2 = 10,000 mosquitoes), making a total of 50,000 mosquitoes reared and released.

All statistical analyses were conducted in R v2.12.2[13] (R Development Core Team, University of Auckland,

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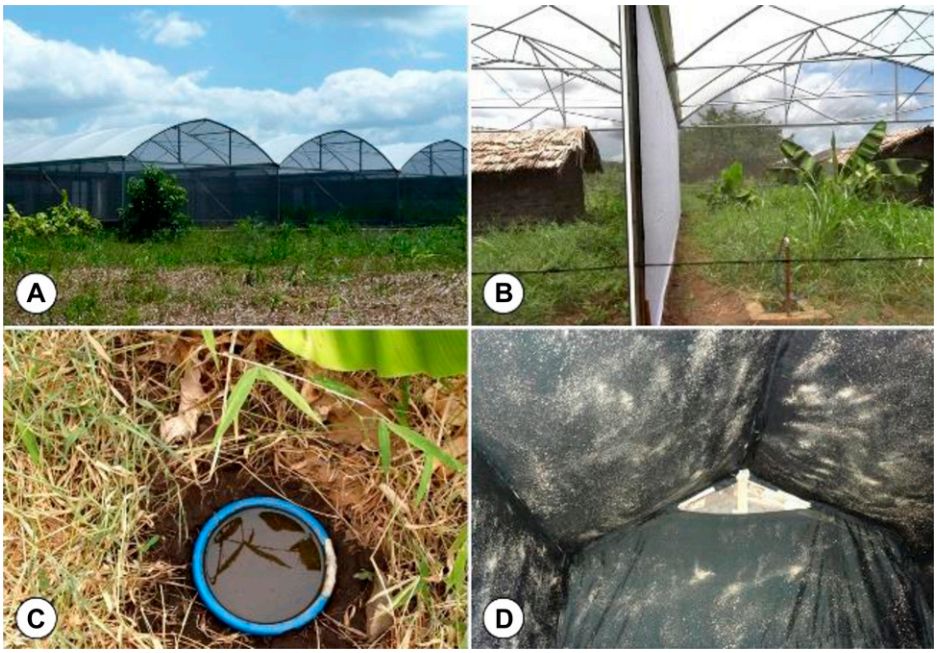

FIGURE 1. Semifield system (SFS) setup. (**A**) SFS with (**B**) mud huts built inside each section to shelter a cow and (**C**) breeding habitats. (**D**) Mud huts were lined with black cloth and dusted with PPF in treatment sections.

Auckland, New Zealand) using the lme4 package for generalized linear mixed effects models.[14] To determine any differences in the numbers of pupae or adults produced between treated and control sections, a generalized linear mixed effects model with a Poisson distribution and a log link function for count data was performed. The treatment group (control or PPF) was classified as a fixed effect, whereas SFS section nested within experiment was put in as a random effect as per the experimental design. A visual inspection of the plots of error versus fitted values distribution was used to determine the best model fit. The model was then tested with each nested parameter separately to determine the underlying variation. SFS section was found to count for a lot of variation and therefore, required the full nested model to be retained. The differences in pupal emergence rates in both SFS habitats and the bioassays experiments were compared by fitting a generalized linear mixed effects model with binomial error structure and logit link function for proportion data. The data were fitted to a model including treatment as a fixed effect and breeding habitat nested within SFS section nested within experiment as a random effect as per the experimental design. Visual inspection of the plots of error versus fitted values distribution was used to determine the best model fit. Model reduction was conducted by removing nested parameters one by one; however, the full nested model was retained.

RESULTS

Experiments lasted between 11 and 16 days from release of adult mosquitoes to collection of the last pupae in the breeding habitats. An overwhelming 95% reduction in pupal production and 96% reduction in adult production were achieved in PPF-treated sections compared with control sections (Figure 2A and B). In four of five replicates, exposure to this juvenile hormone analogue completely sterilized all mosquitoes; not a single pupa or new adult was seen. The few adults emerging from a PPF-treated section in the fifth replicate probably resulted from mosquitoes that had been contaminated with PPF but were not completely sterilized and managed to lay eggs. The pupae collected in the PPF-treated section showed a significantly lower emergence rate (82%; 164/201) compared with the control (95%; 4,132/4,349; χ^2 [1] = 65.6, $P < 0.001$) (Figure 2C). This result suggested possible PPF autodissemination to the breeding habitats by contaminated mosquitoes. However, bioassays with insectary larvae reared in water from the control and PPF-treated habitats showed similar emergence rates (Figure 2D). A similar pattern has been observed in recent studies (Lwetoijera DW and others, unpublished data), where PPF activity is more pronounced in breeding habitats with organic material than water samples kept in glass beakers.

DISCUSSION

The striking level of sterilization seen in this key malaria vector reveals an exciting new opportunity for malaria vector control. This technology is a practical, novel technology for population control that sterilizes mosquitoes rather than killing them. It offers the chance to develop new tools that are not compromised by existing resistance mechanisms. New paradigms in vector control are in great demand, especially for vectors such as *An. arabiensis*[4,15] and other anophelines[16] that exhibit flexibility in feeding and resting indoors and outdoors and minimize their contact with conventional adulticides applied indoors. The findings reported here have limitations given that the experiments were conducted within an enclosed environment on insectary-reared mosquitoes that had never been subjected to insecticide pressure. However, this technology can be readily adapted in natural conditions to assess its impact on wild populations of *An. arabiensis*.

Treating walls and roof linings with PPF comprehensively sterilizes captive populations of free-flying *An. arabiensis*,

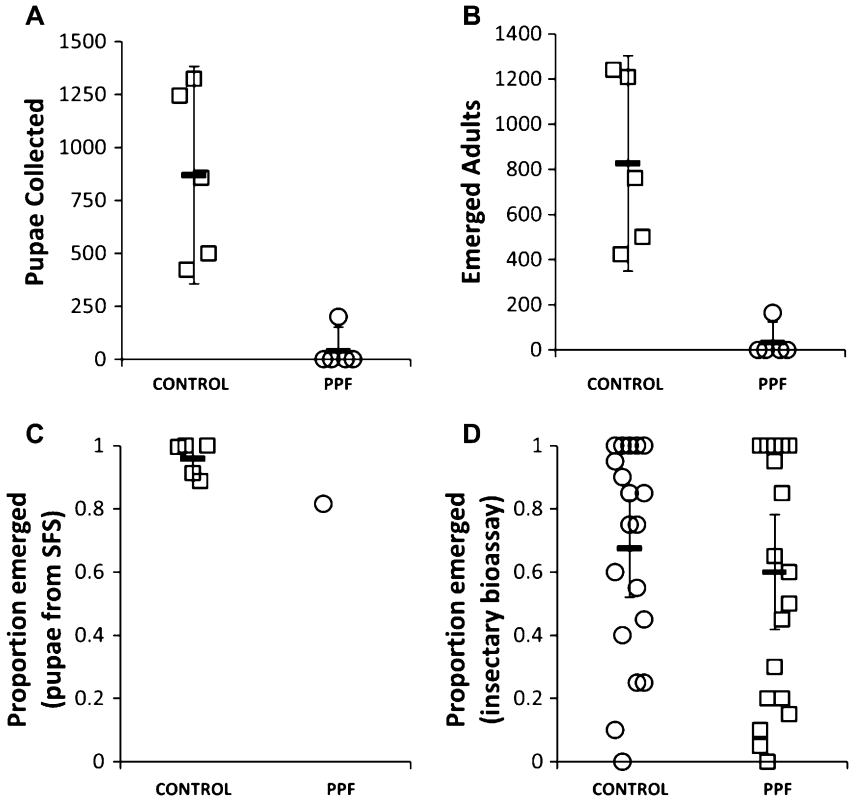

FIGURE 2. Impact of PPF on mosquito emergence. Number of (**A**) pupae produced and (**B**) adults emerging from control and treated sections and the proportion of adult emergence in (**C**) SFS and (**D**) insectary bioassays.

making it a powerful control tool and an easy complement to LLINs and IRS. PPF-treated materials could be deployed outdoors in areas where mosquitoes rest or transit, such as areas where people gather in the early hours of the evening and inside and outside of cattle sheds. These treated materials could also be specifically designed to attract resting mosquitoes. Similar substrates are already exploited for the delivery of conventional insecticides.[17] Our prototype uses a safe and registered insecticide class that has yet to be deployed against adult malaria vectors. Alternatives to conventional adulticides are desperately needed. The physiological resistance to pyrethroids, recently characterized in populations of *An. arabiensis* from Zanzibar, precipitated the substitution of pyrethroids for a less cost-effective carbamate compound with a history of resistance development in malaria vectors.[18,19] No resistance to PPF has been reported in mosquitoes (J. Invest and others, unpublished data), and no cross-resistance has been observed between PPF and other classes of insecticides of public health interest. PPF could be applied in combination, mosaics, or rotations with current insecticides to mitigate the emergence of resistance.[5] It is remarkably stable in the shade and available in a variety of commercial formulations that fit this new application.

The indication that the few mosquitoes that managed to lay eggs from the PPF-treated section also transferred PPF to their breeding habitats and significantly reduced subsequent mosquito emergence is a welcome development. The autodissemination of PPF by adult mosquitoes has been already observed in *Aedes* species,[9,10] and we are working to prove the same phenomenon in malaria vectors.

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