Push-pull tactics to disrupt the host-seeking behaviour of malaria mosquitoes

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Push-pull tactics to disrupt the host-seeking behaviour of malaria mosquitoes

David J. Menger

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Abstract

Malaria remains a major health burden, especially in sub-Saharan Africa. The efficacy of the main vector control tools, insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS), is compromised by the development of physiological and behavioural resistance in the target mosquito species and by changes in the species composition of vector populations. These developments underline the need to develop novel vector control approaches which are complementary to insecticide-based methods. In this thesis, the potential of push-pull tactics as a tool to reduce malaria transmission is explored. It is described how the push-pull concept, originally designed for agricultural pest control, may be translated in a system that targets Anopheles mosquitoes. Several novel repellents are identified in the laboratory and a prototype push-pull system is tested in a semi-field setup. The system is improved and evaluated in a malaria endemic field setting and the push-pull approach is compared and combined with the existing practise of eave screening. Based on the experimental results it is concluded that (1) it is possible to reduce house entry of malaria and other mosquitoes using (spatial) repellents and/or attractant-baited traps; (2) the effect of repellents on house entry is larger and more consistent than the effect of attractant-baited traps; (3) the main function of the attractant-baited traps is to deplete mosquito populations through removal trapping; (4) the attractive and repellent components of the push-pull system complement each other and there is no or very little interaction between them; (5) a push-pull system based on repellent and attractive volatiles can be expected to reduce malaria transmission through a strong decrease of the entomological inoculation rate; (6) eave screening is a highly efficient method to reduce house entry of malaria and other mosquitoes and increases outdoor trap catches, while there is little added value in impregnating screening material with a repellent. In the last chapter, the issue of selection for insensitivity to the used compounds is discussed, as well as methods how to manage it. Furthermore, it is described how the principles of behavioural disruption on which push-pull tactics are based make the technique potentially suitable to target a wider selection of arthropod vectors of disease than malaria mosquitoes alone. It is concluded that future vector control strategies will probably consist of the integration of many different approaches, of which push-pull tactics may be one. By integrating different approaches, it will be possible to mitigate the development of resistance while targeting vectors in different life stages, uncompromised by changing behavioural patterns and changes in the composition of vector populations. This would require an integrated view on vector control, knowledge on the ecology of vectors and the political will to invest in programmes that focus on long term sustainable control.
Chapter 1

General Introduction

David J. Menger
Malaria and mosquitoes

Amongst all infectious diseases that affect human beings, malaria is one of the most deadly and it is the most important vector-borne disease. An estimated 198 million cases, causing 584,000 deaths, occurred worldwide in 2013 (WHO 2014). Malaria occurs throughout the tropics, with the majority of cases in sub-Saharan Africa, affecting mostly children under 5 years of age (WHO 2014).

Five species of *Plasmodium* parasites cause human malaria: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (White et al. 2013). They are transmitted by mosquitoes of the genus *Anopheles* (Diptera: Culicidae). Although there are over 450 anopheline species described, only about 70 are capable of transmitting human malaria, of which approximately 40 species can transmit the disease at a level of major concern to public health (Sinka et al. 2012). Some of the most effective vectors are extremely specialized in targeting humans as a blood host (anthropophily) and display a preference to rest inside human dwellings (endophily) (Besansky et al. 2004).

A malaria transmission cycle starts when an infected female mosquito injects sporozoites while blood-feeding on a human host. These sporozoites travel to the liver where they produce merozoites which, in turn, infect red blood cells. Inside the red blood cells the parasite reproduces asexually until the cells burst, causing fevers and other symptoms of malaria. Eventually, some parasites develop into gametocytes, that may be taken up by a next mosquito that takes a blood meal. Inside the mosquito’s midgut, the parasite reproduces sexually, producing sporozoites which migrate to the salivary glands, thereby closing the cycle (White et al. 2013). Diagnostic tools to identify malaria infection include microscopic analysis of blood films and rapid diagnostic tests (RDTs) that are based on antibody detection. There are different medications and formulations available to treat malaria. The WHO recommends an artemisinin-based combination treatment (ACT) (White et al. 2013). An effective vaccine is not yet available (Heppner 2013).

**Vector control**

Besides rapid diagnosis and treatment with ACT as curative measures, vector control remains the principal preventive strategy to combat malaria (WHO 2014). Vector
control aims to break the transmission cycle by reducing mosquito populations and preventing human-mosquito contact. The most widely used, and WHO-recommended, vector control tools are insecticide treated bed nets (ITNs) and indoor residual spraying (IRS) with persistent insecticides. Bed nets form a physical barrier and when impregnated with a pyrethroid insecticide to kill mosquitoes after contact, they provide a degree of protection at the community level (Lengeler 2004, Hill et al. 2006). IRS with insecticides such as dichlorodiphenyltrichloroethane (DDT) kills mosquitoes that feed and rest inside human dwellings. IRS utilizes various insecticides including DDT, whereas ITNs relies on pyrethroids (Pluess et al. 2010). ITNs and IRS can be used as single interventions, but in many areas they are used together (Okumu and Moore 2011).

Both ITNs and IRS result in human exposure to the used chemicals. Although pyrethroids and DDT have a low mammalian toxicity, there are concerns about the health effects on humans who are exposed for prolonged periods of time (Aneck-Hahn et al. 2007, Koureas et al, 2012).

Resistance

Although ITNs and IRS have contributed to an impressive decline of malaria in the last decade (Murray et al. 2013), this progress is threatened by the development of physiological and behavioural resistance in the target species. Resistance against pyrethroids has increased dramatically in recent years and is now widespread in malaria vectors across Africa (Ranson et al. 2011). DDT resistance first emerged in 1947, a year after its introduction for mosquito control, and it was the main cause that undermined the malaria eradication programme of the WHO in the 1960’s and 70’s (Hemingway and Ranson 2000). The current use of not only DDT, but also of other insecticides that are used for IRS is likewise compromised by spreading resistance (Van den Berg 2009).

Different mechanisms are responsible for resistance against insecticides and these include target site insensitivity, metabolic resistance and cuticular resistance (Ranson et al. 2011). Besides, mosquitoes have developed altered host-seeking behaviour as a result of the strong selection pressure on feeding indoors and at night. A shift from indoor to outdoor feeding as well as changes in biting times have been linked to the implementation of ITNs and IRS (Reddy et al. 2011, Russell et al. 2013, Moiroux et al.
In some areas changes have been observed in the species composition of vector populations (Lwetoijera et al. 2014). This last development may lead to the dominance of species that have different ecological characteristics, which are harder to target with conventional approaches (Besansky et al. 2004).

**New strategies**

The challenges outlined above call for new vector-control strategies which are less prone to the development of physiological and behavioural resistance and resilient against changes in the composition of vector populations. Such strategies imply the integration of different approaches that target vectors in their different life stages, take into account the ecology of the species and target them indoors as well as outdoors at any time they are active.

This view fits well with the concept of integrated vector management (IVM), which has gained increasing support over the last decade (WHO 2004, Van den Berg and Takken 2009, Van den Berg et al. 2013). The WHO defines IVM as ‘a rational decision-making process for the optimal use of resources for vector control’ (WHO 2008). It looks at the deployment of other vector control tools in addition to ITNs and IRS to address the problem of vector resistance. Such tools could include methods that target mosquitoes at the larval stage, such as the use of chemical or biological larvicides or the draining of breeding sites (Keiser et al. 2005, Fillinger and Lindsay 2011), but also measures that reduce contact between humans and adult mosquitoes such as the use of repellents and removal trapping (Lupi et al. 2013, Hiscox et al. 2012). These and other tools are in various stages of development, with some ready to be used and other still in the (field) testing phase (Takken and Knols 2009). Key requirements for the application of these tools are a high cost-effectiveness and user acceptability and suitable characteristics to be integrated in existing malaria control programmes.

**This thesis**

In this thesis I will explore the potential of combining repellents with attractant-baited traps in a so called “push-pull” system. Chapter 2 describes how the push-pull concept, originally designed for agricultural pest control, may be translated in a
system that targets Anopheles mosquitoes. The chapter provides an overview of existing tools such as repellents, attractive odour blends and traps, and suggests how these may potentially be combined in a tool directed at malaria vectors. In chapter 3, the repellency of nine selected compounds is determined in a newly developed bioassay that is based on a synthetic human odour blend. Two lactones, δ-decalactone and δ-undecalactone are identified as especially promising repellents. Chapter 4 deals with the testing of a prototype push-pull system in a semi-field setup. The effects of the repellent and attractive components are quantified in terms of mosquito house entry reduction and attractant-baited trap catches. Recommendations are provided for the practical implementation of the system in the field. In chapter 5, the push-pull system is modified and taken to the field, where the effect on the house entry of wild mosquitoes is determined. By adjusting an existing mathematical model, the impact of adding the push-pull system to existing vector-control tools on malaria transmission is predicted. Chapter 6 addresses the combination of push-pull tactics with eave screening to enhance the systems’ efficacy. In two field experiments, the effects of eave screening and the release of repellents, either in combination with attractant-baited traps or alone, on house entry and outdoor trap catches, are determined. In the final chapter, the outcomes of this thesis are discussed in the perspective of future vector-control strategies.

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Chapter 2

Push-pull: a semiochemical strategy for the control of malaria mosquitoes

David J. Menger, Joop J.A. van Loon and Willem Takken
Abstract

Although vector control has greatly contributed to recent declines in malaria incidence, the efficacy of the main vector control tools is compromised by the development of physiological and behavioural resistance in the target mosquito species. In this paper we investigate the possibility to develop a push-pull system based on semiochemicals, which targets malaria mosquitoes and is complementary to existing vector control methods. We discuss the potential of integrating repellent stimuli such as topical or spatial insect repellents (push) with attractant-baited traps that can be used for removal trapping (pull). Although topical mosquito repellents can provide good personal protection, the sole use of a repellent is unlikely to effectively reduce malaria transmission. However, repellents may contribute to malaria prevention in combination with other protective measures. Within a push-pull system, a safe, effective repellent is needed that could provide protection at a spatial scale for a prolonged period of time. Developments like microencapsulation and the impregnation of fabrics with long-lasting formulations may yield repellent-based tools that can effectively be deployed in a push-pull system. So far, little theoretical work has been done on the degree of trapping that will be required for mosquito population control. It is expected that trapping female mosquitoes at the stage when they are host-seeking or gravid is the most effective approach towards population control. To effectively reduce mosquito populations, baited traps should be able to compete with the attractiveness of the mosquito’s natural hosts. Recently-developed odour blends, which exhibit similar or greater attractiveness than humans, are a great step forward. Increasing understanding of the host-seeking behaviour of malaria mosquitoes may lead to the development of more effective trapping devices in the future.
Chapter 2

Background

Malaria remains one of the deadliest infectious diseases and the most important disease that is transmitted by an arthropod vector: mosquitoes of the genus *Anopheles* (Diptera: Culicidae) (WHO 2014). The efficacy of the main vector control tools, insecticide treated bed nets (ITNs) and indoor residual spraying with insecticides (IRS), is compromised by the development of physiological and behavioural resistance in the target species (Ranson et al. 2011, Van den Berg 2009) and by changes in the species composition of vector populations (Lwetoijera et al. 2014). These developments underline the need to move away from interventions relying exclusively on insecticides. New strategies should be designed in such a way that they are complementary to existing methods, but less prone to the development of resistance. Additionally, such interventions should also target mosquitoes feeding outdoors (Reddy et al. 2011, Russell et al. 2013). In this review we investigate the possibilities to develop a so-called “push-pull” system directed at malaria mosquitoes.

Push-pull

Push-pull is a term that was first coined in the context of integrated pest management (IPM) in agriculture (Rice 1986, Miller and Cowles 1990). It implies a behavioural manipulation of pest insects by the simultaneous use of repellent and attractive cues (Cook et al. 2007). The best known example is the use of Napier grass (*Pennisetum purpureum*) and Desmodium (*Desmodium* spp.) to protect cereal crops from stemborer moths (Kahn et al. 2011). Stemborers are strongly attracted to Napier grass (pull), which is planted as a border crop around a field with e.g. maize or sorghum and produces higher levels of attractive green leaf volatiles than these cereal crops. One of several species of *Desmodium* is intercropped with the main crop and produces volatiles that are repellent to the moths (push). This system is adopted by tens of thousands of farmers throughout East Africa, increasing crop yields and improving food security in the region (Kahn et al. 2011).

The mechanisms by which a push-pull approach functions typically involves semiochemical and/or visual cues. As insects use such cues to guide various behavioural patterns throughout their life-history (e.g. mating, feeding and oviposition behaviour), that behaviour may be manipulated by the deliberate and skilful release of specifically selected cues. The efficacy of the repellent and attractive
components may be enhanced by complementary or synergetic effects when they are released simultaneously in the same environment (Cook et al. 2007).

In recent years, research has provided more insight in how external cues govern different behavioural activities in malaria mosquitoes. Especially semiochemical cues are considered to be of major importance, but also visual and physical cues play a role in the search for mates, nectar, blood meals and a suitable oviposition site (Takken and Knols 1999). During the complex behavioural host seeking sequence, host-exhaled CO\textsubscript{2} and species-specific skin emanations attract mated females in search of a blood meal (Gillies 1980, Takken and Verhulst 2013). Visual cues, vertical targets and ground patterns may also help them in the host-seeking process (Snow 1987, Gibson 1995). Over a shorter distance, physical cues such as heat and moisture induce landing and biting behaviour (Takken et al. 1997, Spitzen et al. 2013). Male as well as female individuals take nectar meals and discriminate between plant species, possibly using kairomonal cues (Impoinvil et al. 2004, Manda et al. 2007, Nyasembe et al. 2014). In mosquito species other than malaria mosquitoes, sex pheromones have been shown to mediate the interaction between males and females, while an oviposition pheromone indicates a favourable site to deposit eggs to conspecific females (Nijhout and Craig 1971, Laurence and Pickett 1982, Mendki et al. 2000).

In the context of malaria control, cues that may be considered for inclusion in a push-pull system should affect mosquito behaviour in such a way that contact with a potential human blood host is avoided. As the stimuli in a push-pull system are generally non-toxic (Cook et al. 2007), it makes sense to integrate these stimuli with methods that reduce mosquito populations. One may think about protecting individual humans, households or communities against biting by host-seeking mosquitoes through the integration of repellent stimuli such as topical or spatial insect repellents (push) with attractant-baited traps for removal trapping (pull).

Visually and physically attractive traps may be augmented with attractive volatiles to lure mosquitoes over a larger distance. Depending on the chosen cues, such a trapping system could target individuals at different physiological conditions in their life cycle, e.g. those seeking for a blood-host, nectar or an oviposition site. The push component on the other hand, may employ volatile compounds that mask attractive cues emitted by potential human hosts or compounds that actively repel or deter host-seeking mosquitoes. Such compounds could be integrated with measures that
physically prevent mosquito-host contact, such as screens or bed nets.

In the next sections an overview is provided of existing techniques and identified behaviour-modifying cues that could potentially be included in a push-pull system to target malaria vectors.

**Push**

*Repellents*

Mosquito repellents, in the broadest sense of the concept, have been used by man since antiquity. Burning leaves or hanging bruised plants in houses are some of the oldest methods to protect against mosquito bites (Maia and Moore 2011). Their modern equivalents include mosquito coils and repellent emanators, which provide a certain degree of spatial protection (Ogoma et al. 2012a). The most widely used synthetic insect repellent is the compound DEET (N,N-diethyl-meta-toluamide), which is applied topically and offers personal protection for up to several hours depending on concentration and mosquito species (Rutledge et al. 1978, Walker et al. 1996, Costantini et al. 2004). An increasingly popular repellent is PMD (para-menthane-3,8-diol), which is derived from the essential oil of *Corymbia citriodora* or the lemon eucalyptus and shows an efficacy similar to that of DEET (Carroll and Loye 2006). Although registered topical repellents are generally considered safe when used as indicated, the intense use of repellents such as coils and sprays in confined spaces has been linked with serious adverse health effects (Koren et al. 2003, Osimitz et al. 2010, Waleed et al. 2013). Repellents from natural origin are often perceived as safer than synthetic compounds, although this is not necessarily the case (Trumble 2002).

Mosquito repellents can interfere with the host-seeking behaviour of female mosquitoes on different levels. Often, the term repellency is used to refer to a range of behaviours that result in a reduction in the probability of human-vector contact, including movement away from a repellent source, interference with host detection and irritancy upon contact (WHO 2013). Studies on the molecular effects of repellents show that they act through multiple molecular mechanisms (Bohbot et al. 2011). Repellent compounds may interact with specific odorants at the binding site of an odorant receptor (OR) to inhibit or reduce odorant-evoked signals or they may independently elicit signals in the absence of odorants (Bohbot et al. 2011).
Malaria control

Many studies have shown that the topical application of a repellent greatly reduces the number of bites by malaria vectors that humans receive, and thereby the entomological inoculation rate (e.g. Le Goff et al. 1994, Govere et al. 2000, Lupi et al. 2013). The large-scale use of repellents has been suggested as a malaria intervention tool to reduce biting rates during episodes of high mosquito densities (Durrheim and Govere 2002) and bed nets treated with repellents have been proposed as an alternative to insecticide-treated nets in areas with widespread resistance (N’Guessan et al. 2008). Whether repellents prevent malaria, however, even when they significantly reduce biting rates, depends on the absolute number of bites a repellent-using individual still receives and on the infection rate of the mosquito population. Although there are reports of repellents providing good protection against malaria (Rowland et al. 2004), other studies found no or no significant reduction in the number of cases (Deressa et al. 2014, Sangoro et al. 2014), sometimes even when reductions in the biting rate were high (Dadzie et al. 2013). A recent review and meta-analysis concluded that “topical repellents are unlikely to provide effective protection against malaria”. However, the authors noted that there was “substantial heterogeneity between studies” and that additional well-designed studies on the effect of topical as well as spatial repellents were required (Wilson et al. 2014).

The main issue with repellents is that while they reduce the probability of human-vector contact, they lack the ability to reduce mosquito populations and cause the mass protective effect which makes insecticide-based methods so successful (Lengeler 2004, Hill et al. 2006). Therefore, a repellent needs to be integrated with another component, such as an attractant-baited trap, which could lure and kill the repelled mosquitoes, in order to deplete mosquito populations through removal trapping (Kline 2006, Okumu et al. 2010a).

Spatial repellents

A repellent for inclusion in a push-pull system would ideally have a spatial effect. Although the topical application of repellents can provide good personal protection for one individual at a time, the spatial dispersal of repellents may provide protection at the level of households or areas where people gather. Topical and/or spatial repellents may especially become important tools in the prevention of outdoor malaria transmission, which becomes increasingly important as existing measures mainly target indoor transmission (Killeen and Moore 2012).
It must be noted that the terms topical and spatial repellent do not reflect an absolute feature of a given compound. Rather, it says something about the range over which a compound is effective under the given circumstances and has to do with the compound’s volatility and the method of application or dispersal. The range at which a given concentration of compound induces a behavioural effect is a scale (centimetres, meters) and not a binary (topical or spatial) variable.

**Novel compounds**

There is no shortage of compounds, both from natural and synthetic origin, that have been suggested as (potential) repellents. Maia and Moore (2011) comprehensively reviewed many plant extracts and essential oils that have been tested for their repellent properties. *Cymbopogon* spp., or lemon grass (Poaceae), are the source of the popular citronella oil, but although citronella has a high initial efficacy, it rapidly evaporates, which drastically limits its value as a repellent (Trongtokit et al. 2005, Kongkaew et al. 2011). Fragrant members of the Verbenaceae and Lamiaceae have also been studied extensively for their repellent action. Some of these studies used whole potted plants, a practise which would fit extremely well in a push-pull strategy. Unfortunately, the measured effects have so far been moderate, with only 25 – 40% protection against malaria vectors (Seyoum et al. 2002, 2003). Periodic burning or thermal expulsion of leaves was more effective (20 up to 80% protection against various species of *Anopheles*), but of course also much more labour intensive. Burning or thermal expulsion of the leaves of *Corymbia citriodora*, the source of the previously mentioned PMD, provided 50 – 80% protection against various *Anopheles* spp. (Hassanali and Knols 2002, Dugassa et al. 2009).

The development of novel synthetic repellents has focussed mainly on finding alternatives for DEET, to be applied topically. Two compounds which have made it into commercial products are IR3535 and KBR 3023 (also known as picaridin) (Costantini et al. 2004). The identification of these molecules as mosquito repellents was the result of structure-activity modelling: by analysing the chemical structure of known repellents, the molecular structure of compounds having a similar biological effect could be predicted (Bohbot et al. 2014). This is only one out of several novel methods to identify new repellents. Others are based on a deeper understanding of the molecular structure of OR proteins or focus on automated high-throughput screening of thousands of compounds against a single OR (Tauxe et al. 2013, Bohbot et al. 2014). Such approaches may lead to the identification of novel repellents in the
future.

Enhanced longevity
Many candidate repellents, such as essential oils, are very volatile, implying that they rapidly evaporate, which limits the duration of their effect. Several techniques can reduce the evaporation rate of such a repellent. One is to add a another molecule to the blend; e.g. vanillin, which has been shown to prolong the protection time of various active compounds (Tawatsin et al. 2001). Another technique is microencapsulation. Microcapsules can be used to impregnate different kinds of textiles, and may preserve the effect of the repellent for weeks (N'Guessan et al. 2008, Miró Specos et al. 2010, Campos et al. 2013). This creates the possibility to manufacture repellent screens, curtains or garments.

There is, however, a trade-off between a compound’s spatial efficacy and the period for which the repellent effect lasts. In order to induce a behavioural effect at a distance from the source, a compound has to be volatile enough to be picked up in an adequate concentration by the mosquito’s olfactory system located in the antennae and maxillary palps. The higher the volatility, the higher the initial concentration of active compound in the air and the stronger and further ranging the effect. However, assuming a set quantity of compound, a higher volatility will lead to a faster depletion of the available amount of compound and thus to a shorter duration of the effect.

Volatile pyrethroids
Finally, exposure to sub-lethal concentrations of insecticides may also have a repellent effect. Volatile, or vaporized, pyrethroids such as transfluthrin and metofluthrin have been shown to be highly effective repellents, with a spatial effect (Kawada et al. 2008, Ogoma et al. 2012b). The drawback of such compounds, however, is that they are from the same chemical family as the compounds applied on bed nets, against which widespread resistance has emerged (Ranson et al. 2011). On top of that, prolonged exposure to these chemicals is associated with adverse health effects (Koureas et al. 2012).

Ultrasound devices
Besides chemical formulations, products that emit ultrasonic waves have been marketed as mosquito repellents. Although malaria mosquitoes are, in principle, sensitive to auditory cues (Pennetier et al. 2010), there is no evidence that ultrasound
would have any effect on the biting behaviour of mosquitoes (reviewed by Enayati et al. 2007).

**Pull**

*Trapping mosquitoes*
There are several examples of successful removal trapping programmes directed at nuisance insects or disease vectors (Day and Sjogren 1994). Most notable are the control programmes targeted at tsetse flies (*Glossina* spp.) that have reduced populations of these flies throughout Africa. Tsetse flies, which are responsible for the transmission of sleeping sickness (trypanosomiasis), are attracted to baited traps or targets, after which they are killed with an insecticide or through heat or starvation once trapped (Vreysen et al. 2013). By using odour baits, the efficacy of these traps or targets can be greatly enhanced (Vale 1993). Provided these devices are applied at the right density, they may suppress tsetse fly populations within a few months (Takken et al. 1986).

Historically, trapping of malaria mosquitoes has been a part of control programmes for the purpose of sampling and monitoring of populations (Mboera et al. 2000a). The interest in mass-trapping of adult mosquitoes as a control measure emerged in the 1990’s and was boosted by several successful experiments in the USA (Kline 2006). Traps, however, are relatively costly and most types require electricity, carbon dioxide and/or other natural or synthetic baits. The deployment of such traps in resource-poor areas where the disease burden of malaria is highest is therefore only relevant when their impact on the transmission of malaria is high. Ultimately the potential of odour-baited traps as a vector control tool will thus depend on their trapping efficacy, i.e. their attractiveness compared to the mosquito’s actual hosts. Indeed, a mathematical model by Okumu et al. (2010a) predicted that attractant-baited traps could play an instrumental role in the reduction of malaria transmission, provided they are more attractive than humans and used to complement (rather than replace) existing methods such as ITNs.

Trapping mosquitoes has a dual function. By removing the insects from the environment, immediate protection is provided to the hosts that would otherwise be attacked by those individuals. This direct protection can be enhanced by strategic placement of the trap, such that it is encountered before the host, and/or by
equipping the trap with an odour bait that is more attractive than the host. The second, indirect, method by which trapping provides protection is by its effect on the mosquito population. The constant removal of a proportion of the mosquito population may, over time, lead to a strongly reduced, or collapsed, population, which would drastically reduce transmission. Whether this collapse or reduction will actually happen depends on the efficacy of the traps and on the population ecology of the species (Kline 2006).

**Carbon dioxide**

Carbon dioxide is considered an important activator and attractant for host-seeking mosquitoes of many species (Gillies 1980, Mboera and Takken 1997). When malaria mosquitoes are exposed to a plume of CO\(_2\), they fly upwind in the direction of the source (Dekker et al. 2001). Once at close range, they rely on additional cues for the final stage of host-location. This is illustrated by windtunnel experiments in which mosquitoes fly towards a trap, but not into it, when exposed to CO\(_2\) alone, whereas the combination of CO\(_2\) + skin emanations leads to significantly higher trap entries than either of the two cues alone (Dekker et al. 2001, Spitzen et al. 2008). In the field, CO\(_2\) has been shown to increase trap catches when added to human scent or a synthetic host-odour mixture (Qiu et al. 2007, Jawara et al. 2009). Mosquitoes respond to elevated CO\(_2\) levels of a few promille above background levels and field studies suggest that they can detect a CO\(_2\) plume up to tens of meters downwind from a source, depending on the emission rate and mosquito species (Gillies 1980, Marinković et al. 2014).

Carbon dioxide can be obtained from different sources, such as propane, solid CO\(_2\) (‘dry ice’), or pressurized in steel cylinders (Kline 2002, Qiu et al. 2007). However, these methods have the drawback that they are expensive and difficult to obtain in regions like sub-Saharan Africa. Alternative methods have been developed over the last years, which are based on the fermentation of sugar or molasses by yeast (Smallegange et al. 2010a, Mweresa et al. 2014a). These methods have the advantage of being much cheaper and easier to apply in resource-poor areas, which may bring mass application into reach.

Besides these developments, a recent study by Turner et al. (2011) identified a ketone, 2-butanone, as ‘a dose-dependent activator of the cpA neuron’ (a CO\(_2\) detecting neuron, located on the maxillary palp) in several mosquito species,
including malaria mosquitoes, in an electrophysiological assay. This may offer new possibilities for methods to mimic a CO$_2$ source in order to attract host-seeking mosquitoes.

**Human and synthetic odour blends**

Research conducted over the last 25 years has elucidated the role of skin emanations as mosquito attractants in addition to CO$_2$. Takken and Knols (1999) comprehensively reviewed the work on the role of human and synthetic odours in host-seeking behaviour of malaria mosquitoes up to then. Studies by De Jong and Knols (1995) and Dekker et al. (1998) had shown that, once in close vicinity of a human, mosquitoes of different species were attracted to different body parts of human volunteers and that this preference was odour-mediated. Several studies also suggested compounds that could be responsible for this attraction, such as carboxylic acids, ammonia and lactic acid (Knols et al. 1997, Braks et al. 2001).

In the years thereafter it was shown that the products of skin bacteria are important attractants for malaria mosquitoes (Braks et al. 2000, Verhulst et al. 2009, 2010). Healy and Copland (2000) and Healy et al. (2002) showed that 2-oxopentanoic acid, in combination with a source of heat comparable to human skin temperature, elicited a landing response from *An. gambiae*. Smallegange et al. (2005) found that a synergism between ammonia, lactic acid and carboxylic acids is responsible for much of the attractiveness of human beings. A later study by Smallegange et al. (2009) revealed the specific contributions of individual aliphatic carboxylic acids and identified tetradecanoic acid (C14) as a key compound that mediates attraction.

In the meantime, field experiments showed that it was possible to catch high numbers of malaria mosquitoes using traps baited with human odour in combination with CO$_2$ (Njiru et al. 2006, Jawara et al. 2009) and explored the potential of synthetic blends as odour baits (Qiu et al. 2007). A breakthrough in the development of an attractive synthetic mixture came when Okumu et al. (2010b) developed a synthetic odour blend comprised of CO$_2$, ammonia and carboxylic acids that was more attractive than humans. Although in windtunnel experiments such blends remained inferior to human odour (Smallegange et al. 2010b), Mukabana et al. (2012a) produced a new synthetic blend that was more attractive than humans in a field setting, by adding the alcohol 3-methyl-1-butanol to a standard blend of CO$_2$, ammonia, lactic acid, and tetradecanoic acid (the additional effect of the latter had
been confirmed in a field study by Jawara et al. 2011). Currently, a blend of ammonia, lactic acid, tetradecanoic acid, 3-methyl-1-butanol and butan-1-amine, augmented with CO$_2$, appears the most attractive bait for An. gambiae s.l. and An. funestus (Van Loon et al. In Press). A recent contribution of Nyasembe et al. (2014) highlights the potential of plant-based synthetic odour baits to catch malaria mosquitoes.

**Physical cues**
Several physical cues play a role during the host-seeking behaviour of malaria mosquitoes. For monitoring purposes, light traps placed next to a human sleeping under a bed net have been used as an alternative to human landing catches (Mbogo et al. 1993, Davis et al. 1995, Ndiath et al. 2011). Light increases trap catches when used next to a human bait indoors, but not outdoors (Costantini et al. 1998). This may indicate that light is a more effective lure in confined spaces, possibly because it promotes flight in the presence of host odours. Mweresa et al. (2014b) showed that the combination of light + human was a stronger attractant than light, a human or a synthetic odour blend alone. However, since light is an unspecific lure, it also attracts other insects than mosquitoes. Besides, a light source indoors at night time may be experienced as inconvenient by the inhabitants.

An experiment by Olanga et al. (2010) addressed the role of warmth and moisture in the orientation towards a human or synthetic odour source. The authors showed that a rise in temperature of a few degrees above background level, at around 1 m from the human or synthetic odour source, and an increase in the relative humidity of the air (to 75-85%) only play a minor role in the host-seeking process. However, Spitzen et al. (2013) showed that when it comes to a landing response, heat is an essential cue to induce this behaviour once a mosquito is at close range of its blood-host.

**Oviposition cues**
Besides trapping techniques which are directed at host-seeking mosquitoes, methods that target other stages of their life cycle could be part of a push-pull system. If it would be possible to effectively trap female mosquitoes at the stage where they are about to reproduce, this would be an effective approach for population control (Depinay et al. 2004, Herrera-Varela et al. 2014). The selection of an oviposition site by a gravid mosquito determines to a large extent the survival of offspring and the species’ distribution (Refsnider and Janzen 2010, Morris 2003).
To assess the suitability of potential larval habitats, female mosquitoes use olfactory cues along with other chemical and physical cues (Rejmánková et al. 2005, Bentley and Day 1989). Despite a growing number of studies on the oviposition behaviour of Anopheles mosquitoes, their habitat preferences are much less well understood than those of other mosquitoes, notably Culex spp., for which an oviposition pheromone has been identified (Ferguson et al. 2010, Laurence and Pickett 1982, Beehler et al. 1994). Nevertheless, it is clear that physical characteristics (Huang et al. 2005, 2006, Balestrino et al. 2010) and water vapour (Okal et al. 2013) play a role in the selection of a suitable aquatic habitat, while semiochemicals of microbial origin (Lindh et al. 2008, Sumba et al. 2004) and other volatiles have also been suggested as potential kairomonal cues (Blackwell and Johnson 2000, Rinker et al. 2013).

**Trap types**

Different trap types have been developed for sampling and/or removal trapping of malaria and other mosquitoes. The ones that are used most often include various traps developed by the Center for Disease Control of the United States of America (CDC traps) and several types of counterflow traps such as the Mosquito Magnet (MM) series by the American Biophysics Corporation (North Kingstown, USA) and the BG Sentinel trap by Biogents (Regensburg, Germany).

Early mass trapping experiments in the USA in the 1990’s deployed CDC traps baited with CO$_2$ + 1-octen-3-ol. In these experiments, populations of the black salt-marsh mosquito Ochlerotatus taeniorhynchus, Culex nigripalpus and Anopheles atropos mosquitoes were successfully controlled using a protective barrier of traps between the source (i.e. breeding sites) of the mosquitoes and the target area (in this case a resort area) (Kline 2006). Another experiment that used Mosquito Magnet (MM) type traps placed along a nature trail in the same area was also successful in reducing population size of O. taeniorhynchus, although a protective barrier of the same traps around a residential area had much less impact (Kline 2006).

In Tanzania, Mboera et al. (2000a) evaluated the efficacy of CO$_2$-baited CDC traps, MM traps and electric nets for trapping wild mosquitoes and concluded that MM traps and electric nets were superior to CDC traps for sampling outdoor flying An. gambiae and Cx. quinquefasciatus. Similar results were reported by Xue et al. (2008) who showed that a recent version of the MM trap, the MM-X trap, baited with various compounds outcompeted CDC traps in the field. Schmied et al. (2008)
compared the MM-X trap with the BG Sentinel trap for catching *An. gambiae* s.s. with food odour and/or CO$_2$ as baits. They concluded that the BG Sentinel trap “showed a consistently higher catching efficiency” when it was placed into a pit to lower the opening to just above ground level.

MM-X traps were also used by Jawara et al. (2009) to determine the optimal placement of attractant-baited traps in and around human dwellings in The Gambia for collecting host-seeking mosquitoes during the malaria season. It was concluded that traps placed immediately outdoors, under the roof, with the outlet opening 15 cm above the ground were the best compromise between efficacy and convenience. Although the traps caught high numbers of mosquitoes, this had no effect on house-entry rates. However, later studies with improved blends showed significant reductions in mosquito house entry (Hiscox et al. 2014, Menger et al. 2014). Mathematical models that assume area-wide coverage of such devices also predict reductions in house entry (Okumu et al. 2010a).

An ongoing field trial that deploys odour-baited traps at a large-scale is the SolarMal project on Rusinga Island in western Kenya (Hiscox et al. 2012). It has the aim “to demonstrate proof of principle for the elimination of malaria using the nation-wide adopted strategy of LLINs and case management, augmented by mass trapping of mosquito vectors”. The Suna Trap, a novel type of counterflow trap was especially developed for this purpose (Hiscox et al. 2014). During a series of laboratory and (semi-) field experiments it caught higher or equal numbers of *An. gambiae* s.l. compared to CDC or MM-X traps (Hiscox et al. 2014). The Suna Trap is intended to be baited with (synthetic) human odour and CO$_2$.

A different type of attractant-baited trap is the Ifakara Odour-Baited Device and modifications thereof (Okumu et al. 2010c). These are large (several cubic meters) canvas boxes that can be baited with attractive (synthetic) odour and fitted with insecticide-treated panels. Two different varieties that have recently been developed are the Ifakara Tent Trap (ITT) and the Ifakara Odour Baited Station (IOBS). The IOBS proved to be more efficient than the ITT in catching several mosquito species and when compared to MM-X traps it was equally effective in catching *An. arabiensis*, but less effective for *Culex* or *Mansonia* spp. Another novel device that is still being tested is the Mosquito Landing Box (MLB) (Matowo et al. 2013). It can be baited with odours and treated with insecticides to kill visiting mosquitoes. So far, only a prototype was
evaluated, which was effective in catching *An. arabiensis*, *An. funestus*, *Culex* spp. and *Mansonina* spp.

A tent trap which differs from the ones described above is presented by Krajacich et al. (2014). It consists of a regular modern dome-shaped tent, which is modified to trap mosquitoes using an ingenious suction system. In villages in rural Senegal it caught *An. gambiae s.l.* and *Culex* spp. In direct comparison with human landing catches it was equally effective for *Cx. quinquefasciatus* but less so for *Aedes aegypti*.

Another interesting variation on the theme of odour-baited devices is the Lehmann’s funnel entry trap (Diabaté et al. 2013). Most easily described as a combination of eave screening and trapping, it uses the attraction of people sleeping indoors to trap host-seeking mosquitoes, without the need for an artificial bait and without insecticides. During a field test in Burkina Faso, it reduced house entry by 70 to 80%.

Oviposition traps resemble a suitable larval habitat in order to lure gravid female mosquitoes that are ready to oviposit. For control purposes such traps may be enhanced with a larvicide or entomopathogenic fungus. They are available for *Culex* spp. and *Aedes* spp. (Mboera et al. 2000b, Perich et al. 2003, Snetselaar et al. 2014) and may be deployed for the control of malaria mosquitoes in the future.

Other tools that have been suggested for sampling, and possibly for control, of malaria vectors are resting pots or boxes (Odiere et al. 2007). These artificial resting sites target especially semi-gravid and gravid females (Mahande et al. 2010). Experiments in Tanzania have demonstrated that cow urine acts as a bait that enhances the pots’ attractiveness (Kweka et al. 2009, 2010).

**Conclusion**

The principal goal of vector control strategies is to prevent malaria transmission. Whether a combination of protective repellents and removal trapping will lead to a large enough reduction in the entomological inoculation rate to achieve this goal will depend on the efficacy of the tools and on several other factors such as the density of vectors and their infection rate, their feeding preferences and behavioural and ecological characteristics.
Topical mosquito repellents can provide good personal protection against nuisance biting and disease transmitting mosquito species. Although the sole use of a repellent is unlikely to provide effective protection against malaria, repellents may contribute to malaria prevention in combination with other protective measures. Except for the use of mosquito coils and emanators of repellent insecticides, which have been linked to health risks, there are few examples of effective repellents with a spatial effect. Within a push-pull system, a safe, effective repellent is required that could provide protection at a spatial scale (e.g. the house level) for a prolonged period of time (e.g. days or weeks). Although there are fundamental limits to the longevity and the spatial range in which a repellent can be effective, developments like microencapsulation and the impregnation of textiles with long-lasting formulations may yield repellent-based tools that can effectively be deployed in a push-pull system. The use of novel techniques such as structure-activity modelling or automated high-throughput screening may result in the identification of new classes of repellents in the future.

To effectively reduce a mosquito population, the attractiveness of the traps compared to the mosquito’s actual hosts is essential to the success of the intervention. The development of odour blends which exhibit similar or greater attractiveness than humans creates the opportunity to develop odour-baited traps that may not only catch large numbers of mosquitoes, but that could also deflect mosquitoes away from potential human blood-hosts. A vast number of studies on the host-seeking behaviour of malaria mosquitoes has led to a more accurate understanding of this complex behavioural process. The appreciation of host seeking as a series of interconnected steps in which CO$_2$, host odours and body heat all play important roles, may lead to the development of more effective trapping devices. Such traps would require odour blends with a long-lasting effect and which are inexpensive and safe to use. Several studies have already addressed the longevity of odour-impregnated materials and further experiments are ongoing (Mukabana et al. 2012b, Mweresa et al. 2015). So far, little theoretical work has been done on the degree of trapping that is required to reduce malaria transmission (Weidhaas and Haile 1978, Kline 2006). Whereas for population control of tsetse flies an additional daily mortality of 2 to 3% to the female population is considered sufficient (Vreysen et al. 2013), this fraction would probably be much higher for anopheline mosquitoes (> 13% according to Weidhaas and Haile 1978). Considering the mosquito life cycle, it can be expected that trapping female mosquitoes at the stage when they are host-seeking or gravid and looking for an oviposition site is the most effective approach towards population control.
Chapter 2

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Chapter 3

Assessing the efficacy of candidate mosquito repellents against the background of an attractive source that mimics a human host

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Abstract

Mosquito repellents are used around the globe to protect against nuisance biting and disease-transmitting mosquitoes. Recently, there has been renewed interest in the development of repellents as tools to control the transmission of mosquito-borne diseases. We present a new bioassay for the accurate assessment of candidate repellent compounds, using a synthetic odour that mimics the odour blend released by human skin. Using DEET (N,N-diethyl-meta-toluamide) and PMD (p-menthane-3,8-diol) as reference compounds, nine candidate repellents were tested, of which five showed significant repellency to the malaria mosquito *Anopheles gambiae sensu stricto* (Diptera: Culicidae). These included: 2-nonanone, 6-methyl-5-hepten-2-one, linalool, δ-decalactone and δ-undecalactone. The lactones were also tested on the yellow fever mosquito *Aedes aegypti* (*Stegomyia aegypti*) (Diptera: Culicidae), against which they showed similar degrees of repellency. We conclude that the lactones are highly promising repellents, particularly because these compounds are pleasant-smelling, natural products that are also present in human food sources.
Chapter 3

Introduction

Mosquito repellents are used around the globe as measures of protection against nuisance biting and disease-transmitting mosquitoes. Recent studies bring have indicated that, in combination with other strategies, both topical and spatial repellents may help to control mosquito-borne diseases (Achee et al. 2012, Killeen and Moore 2012, Debboun and Strickman 2013). However, this potential is compromised by the need to develop more effective compounds.

A common first step in the identification of promising repellents is the laboratory testing of candidate compounds (WHO 2009, 2013). Compounds with effects that are equal to or stronger than that of DEET (N,N-diethyl-meta-toluamide), the current standard amongst mosquito repellents, are interesting candidates for further studies. The term ‘repellent’ is used here to refer to any compound that has an effect on the behaviour of mosquitoes, which results in a reduction in human-vector contact and therefore provides personal protection. This definition thus includes ‘movement away from the source’ (repellency in the strict sense) as well as ‘inhibition of attraction’ (interference with host detection and/or feeding response) (after: WHO 2013).

Much laboratory testing of repellents makes use of human subjects as sources of attraction from which mosquitoes need to be repelled. Examples include the widely used arm-in-cage tests (e.g. Barnard and Xue 2004, Amer and Mehlhorn 2006) as well as various olfactometer bioassays (Feinsod and Spielman 1979, Dogan and Rossignol 1999). Although testing with human subjects is a necessary final step, this method has various drawbacks. Recent studies have shown that individuals differ significantly in their attractiveness as hosts (Verhulst et al. 2010, 2011) and thus, in a scientifically sound design, compounds should be tested repeatedly in a reasonably large group of individuals. This method is labour-intensive and generally perceived as inconvenient, especially when it concerns the screening of large numbers of compounds.

Several authors have addressed the need for a standardized bioassay to test repellents (Dogan et al. 1999, Klun et al. 2005, Kröber et al. 2010). Although these alternatives tackle most of the problems described above, the use of a single attractive compound such as lactic acid or of a warm object in combination with carbon dioxide (CO₂) may only partially represent the attraction of a human being
who emits a blend of attractive odorants from a warm and moist skin surface (Curran et al. 2007, Gallagher et al. 2008) and in addition exhales CO$_2$, which activates the mosquito’s host-seeking behaviour and makes it more sensitive to attractants (Takken 1991, Dekker et al. 2005).

Over the last years, experimental progress has led to the development of artificial odour baits of similar or even greater attractiveness than human-produced odours (Okumu et al. 2010a, Mukabana et al. 2012a). In this paper we describe a landing bioassay that makes use of such an odour bait, in combination with pulses of CO$_2$, to elicit mosquitoes to land on and probe into a warm and moist surface. We determined the effect of nine candidate repellents on the number of landings made by a group of mosquitoes, using DEET and PMD ($p$-menthane-3,8-diol) as reference compounds for the purpose of comparing their efficacy.

In two subsequent experiments, we first used *Anopheles gambiae* Giles s.s., one of the most important vectors of malaria in sub-Saharan Africa, to screen all candidate compounds. This experiment is followed by tests with two of the best candidate repellents that have our particular interest against *Aedes aegypti* (*Stegomyia aegypti*), a vector of yellow fever, dengue and other diseases.

**Material and methods**

*Mosquitoes*

The mosquitoes used in the experiments were reared in climate chambers at the Laboratory of Entomology of Wageningen University, The Netherlands. The original population of *An. gambiae* s.s. was collected in Suakoko, Liberia. A colony of *Aedes aegypti* was established with mosquitoes obtained from the Swedish University of Agricultural Sciences (SLU).

Mosquitoes were kept under photo : scotophase conditions of LD 12 : 12 hours at a mean ± standard deviation (SD) temperature of 27 ± 1°C and relative humidity of 80 ± 5%. Adults were kept in 30 x 30 x 30 cm gauze wire cages and had access to human blood on a Parafilm® membrane every other day. Blood was obtained from a blood bank (Sanquin Blood Supply Foundation, Nijmegen, The Netherlands). A 6% glucose solution in water was available *ad libitum*. Eggs were laid on wet filter paper and then placed in a plastic tray with tap water for emergence. Larvae were fed on Liquifry® No
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1 (Interpet, Dorking, U.K.) for the first three days and then with TetraMin® baby fish food (Tetra GmbH, Melle, Germany) until they reached the pupal stadium. Pupae were collected from the trays using a vacuum system and placed into a plastic cup filled with tap water for emergence.

The mosquitoes to be used in the experiments were placed in separate cages as pupae (An. gambiae s.s.) or upon emergence as adults (Aedes aegypti); they were given access to a 6% glucose solution but did not receive blood meals. The day preceding the experiment, five to eight day old female mosquitoes were placed in release cages with access to tap water in cotton wool until the experiment. Both experiments took place during the last four hours of the scotophase, a period during which An. gambiae s.s. females are highly responsive to host odours (Maxwell et al. 1998). Although Aedes aegypti is primarily a day-feeding mosquito, our colony displays aggressive biting behaviour during the last hours scotophase, which conveniently allowed us to test both species during the same period of the day.

Description of the bioassay
The bioassay was set up in a climate-controlled room of constant air temperature and relative humidity (RH). Climate parameters were adjusted to mimic tropical (dawn) conditions. Temperature was maintained at 24 ± 1°C and RH was kept between 60 and 75%. During the experiments these parameters were continuously monitored using a Tinyview® data logger with display (Gemini Data Loggers (UK) Ltd, Chichester, U.K.).

Because repellents are usually highly volatile compounds and are often tested at relatively high concentrations, there is a risk that the set-up may become contaminated when these substances are tested. Therefore, the bioassay used replaceable 30 x 30 x 30 cm Bugdorm® cages as flight chambers (Mega-View Science Co. Ltd, Taichung, Taiwan).

Mosquitoes were attracted to a landing surface: a heated circular plateau (Ø 15 cm) that was positioned underneath the gauze bottom of the Bugdorm® cage. A layer of ten stacked moist filter papers (Ø 8 cm) was placed on top of the heating plateau. Stainless steel gauze was placed over the papers on which the strips releasing the odour blend were laid (see below). A transparent plastic cylinder was placed around the plateau to concentrate the warm, humid air within the area above the plateau.
The temperature in the centre of the landing stage was kept at 34 ± 2°C, comparable to the temperature of human skin.

The five-compound odour bait, which simulates the smell of a human foot, provided baseline attraction against which repellency could be measured. This bait consists of ammonia, L-(+)-lactic acid, tetradecanoic acid, 3-methyl-1-butanol and butan-1-amine. The individual compounds were released from nylon strips (cut from panty hoses: 90% polyamide, 10% spandex; Marie Claire SA, Borriol, Spain) (Okumu et al. 2010b). Concentrations were optimized for this release method: ammonia (25%), L-(+)-lactic acid (88–92%), tetradecanoic acid (16% in ethanol), 3-methyl-1-butanol (0.01% in paraffin oil) and butan-1-amine (0.001% in paraffin oil). Strips measuring 26.5 cm x 1 cm were impregnated with the attractive compounds by submerging them into an Eppendorf tube containing 1 ml of solution. Subsequently, they were stored at room temperature for three to five hours. Hereafter the strips were hung for half an hour under a fume hood to allow excess fluid to drip off. Finally they were wrapped in aluminium foil and stored at 4°C in a refrigerator until use. A two-second pulse of CO₂ at 2.17 mL/min was released into the Bugdorm® cage at intervals of eight sec through a teflon tube on top of the cage. In preliminary studies, this combination of the artificial odour bait + CO₂ had shown a similar, or slightly higher, attraction as a human hand (Supplementary Figure 1).

A glass screen, placed 10 cm in front of the flight chamber, separated the behavioural observer from the experimental cage to minimise interference by human emanations with the mosquitoes under study. Figure 1 shows a schematic representation of the experimental setup. In the ceiling of the experimental room a fan generated suction to exhaust volatiles emitted by both the observer and the bioassay setup.

Measuring repellence
In the absence of a repellent compound, mosquitoes released into the cage were highly attracted to the heated landing surface and they alighted and inserted their proboscis through the gauze in search of a blood-host. Repellence was measured as the number of landings in the presence of a candidate repellent relative to the number of landings in the absence of a repellent compound during 8 min observation time.

A candidate repellent was released from a nylon strip that was prepared identically to
the method used for the attractive compounds, with the exception that the strips were not hung up under a fume hood but stored in Eppendorf tubes at 4°C directly after their preparation to prevent loss of active compound. The strips with the candidate repellents were taken out of their solution just before the start of the experiment and allowed to leak out on filter paper for 10 sec before they were placed in the experimental setup. Strips were laid directly on the landing stage, in a circle, within the area through which the attractant blend permeated.

**Figure 1. Schematic representation of the repellent bioassay.** It shows the flight chamber containing the assay cage and the position of the circular landing platform (arrow) emitting a 5-component attractant blend and moisture, on which the repellent-impregnated nylon strip was applied. The vertical rectangles are glass screens; that in front of the assay cage serves to separate the observer from the mosquitoes’ environment.

After one minute of acclimatization time, landings within the circular area delineated by the treated strip were counted for a period of 8 min. A landing was defined as the total period for which a mosquito maintained contact with the landing stage. Walking/hopping around on the landing stage as well as short (< 1 sec) take offs immediately followed by landing again were included in one landing. A new landing was recorded when a mosquito had left the stage for more than 1 sec before landing again. Landings shorter than 1 sec during which no probing took place were ignored.
Candidate compounds and experimental design

The repellent effect of nine candidate compounds was tested and compared with the effect of DEET. Candidate compounds included 1-dodecanol (1DOD), 2-nonanone (2NON), 6-methyl-5-hepten-2-one (6MHO), 2,3-heptanediol (23HD), 2-phenylethanol (2PHE), eugenol (EUG), δ-decalactone (dDL), δ-undecalactone (dUDL) and linalool (LNL). These compounds were selected from a large list of potentially behaviour-disrupting organic compounds (BDOCs) that was in turn based on studies of the olfactory receptors of An. gambiae s.s. in ex vivo heterologous olfactory receptor expression assays (Wang et al. 2010) and in vivo electrophysiological studies (Qiu et al. 2006, Carey et al. 2010, Suer, 2011). Nine BDOCs that had shown to inhibit attraction or reduce the overall response in dual-choice olfactometer bioassays (part of this work was published by Smallegange et al. (2012)) were now tested for repellency.

Furthermore, PMD was included as a comparator because it is a relatively new repellent that is now commercially available in Europe as a natural alternative to DEET. The compound is derived from the essential oil of Eucalyptus citriodora and PMD-based repellents have previously been shown to be effective against mosquitoes of several genera, including vectors of human disease (Carroll and Loye 2006 and references therein).

In a preliminary experiment we measured the effect of concentration on repellency, using DEET, PMD, catnip oil and oleic acid as repellents. From a concentration range of 0.1, 1 and 10%, significant repellent effects were found for both 1 and 10%, but not with 0.1%. As 10% DEET or PMD completely inhibited landing behaviour for the 8-min observation period, one would not be able to identify stronger repellents at this concentration. Therefore, in the current experiment, all compounds were tested at a 1% concentration. All compounds were dissolved in ethanol. An ethanol treated strip (ETH) served as the negative control and an untreated nylon strip (NTR) was used to determine the effect of the solvent.

A new, unique assay cage was assigned to each compound. Each compound was tested eight times (n = 8). All replicates of a certain compound were carried out in the same cage. For each testing day, the order in which compounds were tested was randomized.
For each individual test, ten naive *An. gambiae* s.s. females were released into the experimental cage. After one minute acclimatization time, their behaviour was observed for 8 min as described under the heading ‘measuring repellence’. Normality of the data and homogeneity of variances were determined for the number of landings as a function of treatment using the Shapiro-Wilk test and Levene’s test respectively. The $\alpha$-value of pair-wise comparisons was adjusted for the number of comparisons, using Bonferroni correction.

**Results**

**Experiment 1: An. gambiae s.s.**
A General Linear Model (GLM) confirmed that candidate repellents affected the number of landings ($P < 0.001$), whereas there was no significant effect of temperature and relative humidity of the room. Multiple t-tests showed that seven compounds significantly reduced the number of landings compared to the solvent-only treatment. The order of increasing efficacy was (reduction percentage): 2-nonanone (61%); 6-methyl-5-hepten-2-one (66%); linalool (70%); $\delta$-decalactone (75%); DEET (84%); PMD (89%) and $\delta$-undecalactone (91%; Figure 2). No significant differences were found among the effective compounds (Tukey’s post-hoc test). No knockdown effects were observed following exposure to any of the compounds.

**Experiment 2: Aedes aegypti**
The number of landings was affected by the candidate repellents (GLM, $P < 0.001$) and not by testing day, temperature and relative humidity of the room. Multiple t-tests showed that all selected compounds significantly reduced the number of landings compared to the control. The order of increasing efficacy was (reduction percentage): PMD (47%); $\delta$-undecalactone (57%); DEET (58%) and $\delta$-decalactone (66%; Figure 3). Between the selected compounds, there were no significant differences (Tukey’s post-hoc test). No knockdown effects were observed following exposure to any of the compounds.

**Discussion**

**Candidate repellents**
We successfully used the bioassay to quantify the repellent effect of nine candidate repellents. In addition to the commercially available repellents DEET and PMD, five
other compounds proved to have significant repellent effects. Linalool, a terpene alcohol produced by many plant species, has previously been suggested to have a repellent effect on mosquitoes (Hwang et al. 1985, Park et al. 2005). Similarly, 2-nonanone is produced by some plant species and has been suggested as an insect mimetic attractant (Borg-Karlson and Groth 1986). As part of a larger experiment, it was screened for repellency by Innocent et al. (2008), who found 53% repellency against An. gambiae s.s. at a 1% concentration, which is not very different from the

Figure 2. Effects of the candidate repellents on An. gambiae s.s. Bars show the mean number of landings made by a group of 10 females during 8 min. Error bars indicate the standard error; n = 8 for all treatments. Asterisks indicate significance for P-values smaller than < 0.0042, the adjusted error rate based on Bonferroni’s correction. N/A: not applicable, see the materials and methods section for treatment abbreviations.
Another ketone, 6-methyl-5-hepten-2-one, has been identified as a human skin emanation with an inhibitory effect on flight and probing activity in *Aedes aegypti* (Logan et al. 2008). Interestingly, whereas Logan et al. (2008) identified this inhibitory effect at low concentrations, we have observed attraction at low concentrations and inhibition of attraction at high concentrations for *An. gambiae* s.s. in previous dual-choice olfactometer bioassays (data not shown). In the current experiment, linalool, 2-nonanone and 6-methyl-5-hepten-2-one showed repellent
effects comparable to DEET, at similar concentrations.

As for the two lactones, δ-decalactone and δ-undecalactone, to our knowledge the present study represents the first illustration of these compounds as having behavioural effects on host-seeking mosquitoes (patent pending). Pask et al. (2013) performed a structure-activity study on *An. gambiae s.s.* using a heterologous expression system of olfactory receptor (OR) proteins and demonstrated that AgOR48 has highest binding affinity to δ-lactone, δ-undecalactone and δ-dodecalactone among a range of lactones differing in ring size and the length of the linear carbon chain.

Both δ-decalactone and δ-undecalactone are natural products present in food sources such as edible fruits and dairy products (Lin and Wilkens 1970, Mahajan et al. 2004). Their odour is generally described as fruity, coconut-like and pleasant. These characteristics make them excellent candidate repellents to test for further applications. Studies to explore the potential of these compounds in an odour based push-pull system (Cook et al. 2007) are currently ongoing.

**Repellent assay**

The set-up in which these experiments were conducted was especially designed to rapidly establish repellent effects of a range of candidate compounds. Most repellent assays use a vertebrate host as source of attraction (see Debboun et al. 2006). Vertebrates emit a wide range of odorant cues (Penn et al. 2007, Gallagher et al. 2008) of which some are attractive to mosquitoes (Takken and Knols 1999). The heat produced by the vertebrate body is a further cue that induces landing responses (Healy et al. 2002, Spitzen et al. 2013). The use of live hosts however, is cumbersome, expensive and causes variation in results because of the daily variation in attractiveness of an individual host and wide variation between hosts (Verhulst et al. 2010, 2011). In the current assay we overcame these variable effects by using a synthetic olfactory cue, which has the advantage of a constant level of attractiveness to the mosquito (Okumu et al. 2010a, Mukabana et al. 2012a). This allowed us to observe the behavioural responses to a compound on different testing days without the confounding effect of host variation. The landing surface emanating a kairomone blend is a cheap and reproducible method of odour dispensing and can rapidly be refreshed in between experiments. Thus, this assay can be employed as a high-throughput system for evaluation of candidate repellent products.
The 8-min observation time was chosen as a suitable period for the initial assessment of candidate repellents. It facilitated rapid screening and at the same time allowed for enough landings to determine significant differences between the control and the treatments. To observe the effect of a compound over time, the assay can be used unaltered as the attractive blend remains active for very long times (up to several months in a field situation, Mukabana et al. 2012b).

Existing bioassays that use a synthetic source of attraction use either a single attractive compound or heat (e.g. Dogan et al. 1999, Grieco et al. 2005, Kröber et al. 2010). The bioassay presented here mimics a natural host by dispensing skin odorants, heat and moisture in the presence of CO$_2$, after which a candidate repellent is applied, just as may occur in a natural setting where skin is treated with repellent compounds. The bioassay therefore approximates the stimuli emanating from a live host, but without the inter- and intra-individual variation expressed by humans. Therefore, it can be used to rapidly and reliably test the efficacy of candidate repellents.

This study has identified the strong repellent effect of four compounds, two of which, δ-decalactone and δ-undecalactone, had not been identified previously as mosquito repellents. Their application as spatial or topical repellents, in vector-control programmes or otherwise, should be further explored as they may provide a safe and effective alternative for, or addition to, existing methods.

In conclusion, our repellent assay rapidly identifies inhibitory behavioural effects of candidate repellents in the absence of a live host. A new class of repellents, δ-lactones, the efficacy of which is similar to or greater than that of DEET, has been added to the repertoire of chemical mosquito repellents.

**Acknowledgements**

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the malaria mosquito *Anopheles gambiae sensu stricto* to an active kairomone blend: laboratory and semi-field assays. *Physiological Entomology* 37: 60-71


Supplementary Figure 1. Mean number of landings per mosquito recorded during preliminary experiments. A human hand (HH) or the 5-component attractant blend (AB) was used as an odour source. Data are shown for control (no repellent) treatments only. Error bars show the standard error of the mean (SEM). For a total of 20 experiments (12 HH and 8 AB), the attractant blend was slightly more attractive (overall mean of 2.91 landings per mosquito, versus 2.42 for the human hand) and less variable (average SEM of 0.17 versus 0.33 for the human hand). N = 8 for all experiments, except HH10 (n = 14), AB6 (n = 10) and AB8 (n = 10). Number of landings is expressed per mosquito to allow comparison of experiments in which different group sizes (five or ten mosquitoes per replicate) were used.
Chapter 4

A push-pull system to reduce house entry of malaria mosquitoes

David J. Menger, Bruno Otieno, Marjolein de Rijk, W. Richard Mukabana, Joop J.A. van Loon and Willem Takken

Abstract

Mosquitoes are the dominant vectors of pathogens that cause infectious diseases such as malaria, dengue, yellow fever and filariasis. Current vector control strategies often rely on the use of pyrethroids against which mosquitoes are increasingly developing resistance. Here, a push-pull system is presented, that operates by the simultaneous use of repellent and attractive volatile odorants. Experiments were carried out in a semi-field set-up: a traditional house which was constructed inside a screenhouse. The release of different repellent compounds, para-menthane-3,8-diol (PMD), catnip oil and delta-undecalactone, from the four corners of the house resulted in significant reductions of 45% to 81.5% in house entry of host-seeking malaria mosquitoes. The highest reductions in house entry (up to 95.5%), were achieved by simultaneously repelling mosquitoes from the house (push) and removing them from the experimental set-up using attractant-baited traps (pull). The outcome of this study suggests that a push-pull system based on attractive and repellent volatiles may successfully be employed to target mosquito vectors of human disease. Reductions in house entry of malaria vectors, of the magnitude that was achieved in these experiments, would likely affect malaria transmission. The repellents used are non-toxic and can be used safely in a human environment. Delta-undecalactone is a novel repellent that showed higher effectiveness than the established repellent PMD. These results encourage further development of the system for practical implementation in the field.
Chapter 4

Background

Mosquitoes are the dominant vectors of pathogens that cause infectious diseases such as malaria, dengue, yellow fever and filariasis (Gratz 1999, WHO 2013). Vector control strategies are aimed at disrupting transmission cycles and are an important tool in the prevention of these diseases. Current vector control strategies often rely on the use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS) (Van den Berg and Takken 2008, Thomas et al. 2012). However, the rapidly increasing resistance of mosquitoes to the active chemicals on which these strategies depend implies a serious limitation of their efficacy (Ranson et al. 2011, Kanza et al. 2012, Mawejje et al. 2012, Ochomo et al. 2012).

The literature provides examples of various alternative vector control tools that could be employed as supplements to, or possibly even as replacements of, ITNs and IRS (reviewed by Takken and Knols 2009). A tool which has previously proven its value in the context of agricultural pest management is the so called ‘push-pull system’ (Cook et al. 2007). A push-pull system manipulates the behaviour and/or distribution of pest insects by the simultaneous use of repellent and attractive stimuli. In this paper, a push-pull system is introduced, that is directed at the major African malaria vector Anopheles gambiae sensu stricto (s.s.). The system is based on removal trapping and the release of spatial repellents.

Removal trapping is a strategy that aims at reducing the target insect population with attractive traps placed in strategic locations. This strategy is effective against tsetse flies (Glossina spp.), which transmit trypanosomiasis (sleeping sickness), and against other disease vectors (Day and Sjogren 1994). Recent laboratory and field experiments have led to the development of odour blends based on ammonia, L-lactic acid and carboxylic acids which, in combination with carbon dioxide (CO₂), can be used as baits to effectively trap tropical mosquitoes, including malaria vectors (Braks et al. 2001, Smallegange et al. 2005, 2009, Okumu et al. 2010a, Jawara et al. 2011, Verhulst et al. 2011, Mukabana et al. 2012a).

Repellents can be applied topically for personal protection, e.g. the widely used insect repellent DEET (N,N-diethyl-meta-toluamide), but can also be dispersed spatially to protect a space, e.g. the burning of repellent-impregnated coils, candles that contain certain essential oils or leaves of specific tree species (Lindsay et al. 1996, Seyoum et
al. 2002, Alten et al. 2003, Dugassa et al. 2009). Repellents that exhibit a spatial effect may be considered for inclusion in a push-pull system.

The use of push-pull tactics fits within the emerging view that vector control strategies should be expanded beyond insecticide-dependent methods (Thomas et al. 2012). Combining the mechanisms of attraction and repellency has the potential to result in a synergistic effect (Cook et al. 2007). By ‘pushing’ mosquitoes away from certain places using repellents, one could stimulate their movement towards other places where they are ‘pulled’ into traps baited with attractive cues. Now that highly attractive synthetic odour blends that mimic human scent are at the disposal of the scientific community, the remaining challenge lies in the development or selection of effective spatial repellents directed at the target group.

In this paper, two experiments are presented in which it is demonstrated how (1) a push-pull system was employed in a semi-field situation where it successfully reduced house entry of the predominant malaria vector in sub-Saharan Africa, *An. gambiae* s.s. and (2) this push-pull system was improved with the introduction of a novel mosquito repellent that displays a superior spatial effect.

**Methods**

*Mosquitoes*

The mosquitoes (*An. gambiae* s.s., Mbita strain; henceforth termed *An. gambiae*) were reared under ambient atmospheric conditions in screenhouses (larvae) and indoors (adults) at the Thomas Odhiambo Campus (TOC) of the International Centre of Insect Physiology and Ecology (*icipe*) located near Mbita Point township in western Kenya. Mosquito eggs were placed in plastic trays containing filtered water from Lake Victoria. All larval instars were fed on Tetramin® baby fish food which was supplied thrice per day. Pupae were collected daily and placed in mesh-covered cages (30 × 30 × 30 cm) prior to adult emergence. Adult mosquitoes were fed on 6% glucose solution through wicks made from adsorbent tissue paper.

Female mosquitoes of 3 – 6 days old since eclosion that had no prior access to blood were used for the semi-field experiments. The mosquitoes were collected from the colony at 12:00 h each day and stored for 8 h in the colony room with access to water on cotton wool. Within 15 min before the start of the experiment the cups with the
mosquitoes were transported to the experimental set-up.

Description of the set-up
The experiments were conducted at the Mbita Point Research & Training Centre of icipe in Kenya. Experiments took place in the MalariaSphere (Figure 1), a screenhouse into which a traditional house was built surrounded by natural vegetation (Knols et al. 2002). The traditional house possesses an eave, through which mosquitoes that are released into the screenhouse may enter, as they would do in a natural situation when an attractive host is present inside (Snow 1987). The MalariaSphere was set up as described by Knols et al. (2002), with the only modification that no breeding sites were present.

Figure 1. The MalariaSphere; a screenhouse with a traditional house constructed inside (image copied from Knols et al. 2002).

Experimental design
Both experiments explored the effects of attractant-baited traps and the dispersal of repellents around the traditional house. Four different set-ups were tested during experiment 1 and eight different set-ups were tested during experiment 2. During all tests, one attractant-baited trap (see below) was placed inside the experimental house to represent a human being. The house entry of the mosquitoes was measured by the number of mosquitoes caught by the trap inside the house.
Each night at 20:00 h, 200 female mosquitoes were released into the MalariaSphere. At 6:30 h the next morning the experiment was terminated by closing and switching off the ventilators of all traps. The traps were then placed in a freezer for several minutes to inactivate the mosquitoes, after which the numbers of trapped mosquitoes were determined.

**Experiment 1**
The four set-ups that were tested during experiment 1 included: (1) a control set-up in which only the attractive trap inside the house was present, (2) a push-only situation in which a repellent was released from the four corners of the house, (3) a pull-only situation in which four attractant baited-traps were positioned around the house and (4) a situation in which the total push-pull system was set up with both the repellent and the attractant components in place. See Table 1 for the presence/absence of the specific traps during the treatments and Figure 2 for an overview of their positions. Each set-up was tested during eight different nights, thus a total of 32 tests was carried out during the same number of nights. The order of the tests was not fully randomized in order to minimize the risk of contamination of the MalariaSphere with the used odours. The repellent compound selected for this experiment was para-menthane-3,8-diol (PMD) (Carroll and Loye 2006). Nylon strips were impregnated with a 40% solution of commercially available Citriodiol™ (containing > 64% PMD) as described below. At the start of each test, the mosquitoes were released from four different spots around the house (50 mosquitoes per spot), see Figure 2.

<table>
<thead>
<tr>
<th>Table 1. Placement of attractants and repellents in experiment 1 (Yes/No).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

See also Figure 2.

**Experiment 2**
During experiment 2, eight different set-ups were tested. This study compared the effect of three different repellents in push-only situations as well as in situations in...
which both a repellent and the attractive blend were released; see Table 2 and Figure 3 for a comprehensive overview of which repellent compound was used during the different tests, the presence/absence of the repellent and attractive components and their positions. Each set-up was tested during six different nights, thus a total of 48 tests was carried out, during the same number of nights. The order of the tests was not fully randomized in order to minimize the risk of contamination of the MalariaSphere with the used odours. PMD (see experiment 1), catnip essential oil (e.o.) (Bernier et al. 2005, Birkett et al. 2011) and delta-undecalactone (dUDL; patent pending) (Menger et al. 2014) were used as repellents. Strips were impregnated with 40% solutions (catnip e.o. and dUDL were dissolved in paraffin oil) as described below. During experiment 2, all 200 mosquitoes were released from one central point between the entrance of the screenhouse and the experimental hut (see Figure 3).

**Attractant-baited traps**
Mosquito Magnet® X (MM-X) traps (Njiru et al. 2006, Qiu et al. 2007) were baited with CO₂ and a five-compound odour blend, which simulates the smell of a human
Table 2. Placement of attractants and repellents in experiment 2 (Yes/No).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Attractant inside</th>
<th>Attractant outside</th>
<th>Repellent outside</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>Y</td>
<td>N</td>
<td>Y (PMD)</td>
</tr>
<tr>
<td>3</td>
<td>Y</td>
<td>N</td>
<td>Y (Catnip)</td>
</tr>
<tr>
<td>4</td>
<td>Y</td>
<td>N</td>
<td>Y (dUDL)</td>
</tr>
<tr>
<td>5</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>6</td>
<td>Y</td>
<td>Y</td>
<td>Y (PMD)</td>
</tr>
<tr>
<td>7</td>
<td>Y</td>
<td>Y</td>
<td>Y (Catnip)</td>
</tr>
<tr>
<td>8</td>
<td>Y</td>
<td>Y</td>
<td>Y (dUDL)</td>
</tr>
</tbody>
</table>

See also Figure 3.

Figure 3. Experimental set-up of experiment 2. Green represents an MMX trap baited with attractant, red represents an MMX trap dispersing the repellent. The asterisk indicates the mosquito release points. Numbers indicate the treatments at which the trap or dispenser was present (see also Table 2).

foot (Mukabana et al. 2012a, Menger et al. 2014). The individual compounds of the attractive blend were released from nylon strips (cut from panty hoses: 90% polyamide, 10% spandex, Marie Claire®) (Okumu et al. 2010b). Concentrations were optimised for this set-up and release method: ammonia (2.5% in water), L-(+)-lactic-
acid (85%), tetradecanoic acid (0.00025 g/l in ethanol), 3-methyl-1-butanol (0.000001% in water) and butan-1-amine (0.001% in paraffin oil) (see Table 3). Nylon strips (26.5 cm x 1 cm) were impregnated with the attractive compounds by dipping three strips in 3.0 ml of compound in a 4 ml screw top vial (experiment 1) or by dipping individual strips into an Eppendorf tube containing 1 ml of solution (experiment 2). Before use, strips were dried for 9–10 h at room temperature. During experiment 1 for every experimental night a set of freshly impregnated strips was used. During experiment 2 strips were used for a maximum of 12 consecutive nights. During daytime, the strips were packed in aluminium foil and stored at 4°C in a refrigerator. The five strips were held together with a safety pin and hung in the outflow opening of the MM-X trap using a plastic covered clip. CO₂ was produced by mixing 17.5 g yeast with 250 g sugar and 2.5 L water (Smallegange et al. 2010) and released from the MM-X trap together with the odours. MM-X traps equipped with the attractive blend were positioned with the outflow opening at the optimal height of 15–20 cm above the floor surface (Jawara et al. 2009).

Table 3. Composition of the attractive blend.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>2.5% (v/v)</td>
<td>Water</td>
</tr>
<tr>
<td>L-(+)-lactic acid</td>
<td>88-92% (w/w)</td>
<td>Water</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>0.00025 g/l</td>
<td>Ethanol</td>
</tr>
<tr>
<td>3-Methyl-1-butanol</td>
<td>0.000001% (v/v)</td>
<td>Water</td>
</tr>
<tr>
<td>Butan-1-amine</td>
<td>0.001% (v/v)</td>
<td>Paraffin oil</td>
</tr>
</tbody>
</table>

Dispersal of the repellents

To disperse the repellents, MM-X traps were used of which the suction mechanism was disabled; leaving only the outflow mechanism functional (Okumu et al. 2010a). The repellent compounds were applied to nylon strips identically to the attractants. However, because of their volatility the strips with repellent were dried for only 1 h (experiment 1) or 10 min (experiment 2). One repellent strip was used per MM-X trap. Freshly prepared strips were used each night. The MM-X traps that dispersed the repellent were hung from the lowest part of the roof of the traditional house, with the outflow opening about 1 m above the floor, to intercept mosquitoes that would enter through the eaves of the experimental hut.
Statistical analysis

For both experiments, the trap catches inside and (when applicable) outside the experimental house were compared between all treatments. The Shapiro-Wilk test was used to test the normality of the data and Levene’s test was used to test for equality of variances. Subsequently, the differences between trap catches inside the house in experiment 1 were analysed using analysis of variance (ANOVA) followed by Bonferroni post-hoc tests. Trap catches outside were compared using an independent-samples t-test. Differences between trap catches inside the house in experiment 2 were analysed using ANOVA followed by Games-Howell post-hoc tests. Trap catches outside the house were compared using ANOVA followed by Bonferroni post-hoc tests.

Results

Experiment 1

During the control tests, the attractant-baited trap inside the house caught on average 62.0 (SEM 8.7) or 31.0% of the released mosquitoes. The release of PMD (push only), removal trapping (pull only) and the combination of both strategies (push-pull) all significantly reduced the house entry of *An. gambiae* compared to the control situation (ANOVA: F = 21.53, df = 3, p < 0.001; Bonferroni post-hoc tests at α = 0.05, see Figure 4).

When PMD was released from the four corners of the house, the number of trapped mosquitoes dropped to 31.1 (8.2); a reduction of nearly 50%. With four attractant-baited traps placed around the house, even fewer mosquitoes entered the house, with the trap indoors catching only 21.3 (2.1) mosquitoes on average. The four traps outdoors caught 107.3 (15.4) mosquitoes or 53.7% of the total number released. With both the push and the pull components in place, the number of mosquitoes trapped indoors was lowest, with only 14.4 (4.0) mosquitoes on average, or 7.2% of the total number released. This implies a reduction of more than 75% compared to the control treatment. The traps outdoors caught an average of 115.4 (16.3) mosquitoes in the push-pull scenario.

Experiment 2

In the absence of repellent dispensers or removal trapping, the attractant-baited trap inside the house caught 82.0 (4.0) mosquitoes on average; 41.0% of the total number


released. As in the previous experiment, all treatments significantly reduced the number of mosquitoes trapped in the experimental house (ANOVA: $F = 70.08$, df = 7, $p < 0.001$; Games-Howell post-hoc tests at $\alpha = 0.05$, see Figure 5).

The push-only treatment in which delta-undecalactone was dispensed caused a significantly stronger reduction (81.5%) than the treatments with PMD or catnip e.o (45.7% and 56.5% resp.), of which catnip e.o. performed slightly (ns) better. Removal trapping (pull only) led to a 82.3% reduction, with the trap inside the house catching only 14.5 (2.0) mosquitoes on average. The push-pull treatment employing delta-

Figure 4. Mean number of mosquitoes trapped inside and, when applicable, outside the experimental house. For all treatments $n = 8$, error bars indicate the standard error of the mean. Bars not sharing the same character are significantly different at $\alpha = 0.05$ with Bonferroni post-hoc tests.
undecalactone as a repellent provided the strongest reduction, 95.5%; only 3.7 (0.7) mosquitoes were caught inside the house on average; 1.9% of the total number released. The total number of mosquitoes trapped outdoors did not differ significantly between the treatments that included removal trapping.

**Discussion**

*Efficacy of the push-pull system*

An attractant-baited trap placed inside a traditional house caught 31% (experiment 1) to 41% (experiment 2) of the mosquitoes released in the screenhouse. Therefore, host-seeking female mosquitoes must have entered the house attracted by the combination of odour + CO₂ that was deployed to mimic a potential host. This confirms that the odour blend + CO₂ functions analogous to a human host in terms of

![Figure 5. Mean number of mosquitoes trapped inside and, when applicable, outside the experimental house.](image)
inducing house entry as a component of host-seeking behaviour (Okumu et al. 2010a, Mukabana et al. 2012a).

The release of PMD from the four corners of the house resulted in a significant reduction of over 45% in house entry of host-seeking mosquitoes. Therefore, anyone being indoors would have received fewer mosquito bites under this treatment. Experiment 2 showed that this effect improved significantly (to 81.5%) when PMD was replaced by delta-undecalactone. The placement of attractant-baited traps around the house significantly reduced the number of mosquitoes trapped inside the house, in both experiments. Instead of entering the house, a high percentage (53.7% and 44.1% resp.) was lured into the traps placed outdoors.

These examples show that it is feasible to trap or repel host-seeking mosquitoes before house entry, thereby rendering protection to the house occupants. The highest reductions in house entry (up to 95.5%), and thus the highest degrees of protection, were achieved by simultaneously repelling mosquitoes from the house (push) and removing them from the experimental set-up by trapping (pull). Although outdoor trap catches were slightly elevated when both push and pull were present, compared to pull only, there was no statistical indication that a greater push led to a greater pull or vice versa. Rather than a synergistic interaction between both components, the attractant and repellent seem to have independent effects that, by their different modes of action, complement each other.

**Spatial repellency**

The results also show that PMD, catnip e.o. and delta-undecalactone, had an effect on the mosquitoes over a large distance, as the places from where the repellents were dispensed were approx. 3 m apart. Released in an appropriate way, in the present experiments by active dispersion from nylon fabric, these compounds thus act as spatial repellents.

PMD has previously been shown to be an effective repellent against mosquitoes of several genera, including vectors of human disease (Carroll and Loye 2006 and references therein). Catnip e.o. has also been reported as an insect repellent, with proven effect on mosquito species of several genera including *Aedes*, *Anopheles* and *Culex* (Bernier et al. 2005, Birkett et al. 2011, Zhu et al. 2006, Polsomboon et al. 2008).
Delta-undecalactone was first identified in studies of the olfactory receptors of *An. gambiae* using *ex vivo* heterologous olfactory receptor expression assays (Wang et al. 2010) and *in vivo* electrophysiological studies on antennal sensilla (Qiu et al. 2006, Carey et al. 2010, Suer 2011). Subsequently, it was selected for tests in a repellent bioassay, where it showed an equal or higher level of repellency than DEET [28]. The superior spatial repellent effect it displayed in this experiment underlines its potential as a new repellent that may be used for the control of mosquito vectors of disease. Because delta-undecalactone is a natural product present in edible fruits and dairy products (Lin and Wilkens 1970, Mahajan et al. 2004), regulatory issues concerning its use as a repellent are expected to be limited making it a suitable compound for inclusion in vector-control programmes.

**Field implementation**

The outcome of this study suggests that a push-pull system based on odorant volatiles may successfully be employed to target mosquito vectors of human disease. Reductions in house-entry of the magnitude observed in this study, would likely affect malaria transmission, especially in areas where mosquito densities are low and malaria risk is directly related to the entomological inoculation risk (Smith et al. 2007). So far, house entry reductions of this magnitude are only known for pyrethroid insecticides (e.g. Kawada et al. 2008, Ogoma et al. 2012). The results presented here justify the decision to keep working on a field-proof push-pull system based on a combination of non-pyrethroid repellents and attractants.

The usefulness of push-pull systems for control of mosquito-borne diseases will not only depend on their efficacy in repelling and trapping mosquitoes, but also on their applicability and cost-effectiveness (Okumu et al. 2010c). For malaria control, vector control measures should be affordable and usable in rural African settings. In its current shape, employing up to nine electrically-powered MM-X traps, the push-pull system presented here does not meet these requirements. Therefore, follow-up experiments are planned to further optimize this system and explore the practical implementation of an odour-based push-pull system that is less dependent on electric power.

Attractant odour baits have been reported that can be formulated to last for several months (Mukabana et al. 2012b). Odour-baited traps can be operated and maintained by house owners, preferably through a community approach, improving the
sustainability of this vector control method. Studies on repellent formulation and passive distribution mechanisms are still required.

Finally, this system may also be considered in areas where most malaria transmission occurs outdoors (Reddy et al. 2011, Russell et al. 2013), where it is expected to increase the efficacy of existing methods such as ITNs and IRS that do not target host-seeking mosquitoes outside the house.

Conclusion

This study shows a strong spatial effect of PMD, catnip oil and delta-undecalactone, when dispensed around a house in a semi-field set-up. Combined with an attractant in a push-pull strategy, the volatile repellents caused highly significant reductions in house entry of the major African malaria vector \textit{An. gambiae}. These results encourage further development of the system for practical implementation in the field.

Acknowledgements

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References


Chapter 4


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Chapter 5

Field evaluation of a push-pull system to reduce malaria transmission


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Abstract

Malaria continues to place a disease burden on millions of people throughout the tropics, especially in sub-Saharan Africa. Although efforts to control mosquito populations and reduce human-vector contact, such as long-lasting insecticidal nets and indoor residual spraying, have led to significant decreases in malaria incidence, further progress is now threatened by the widespread development of physiological and behavioural insecticide-resistance as well as changes in the composition of vector populations. A mosquito-directed push-pull system based on the simultaneous use of attractive and repellent volatiles offers a complementary tool to existing vector-control methods. In this study, the combination of a trap baited with a five-compound attractant and a strip of net-fabric impregnated with micro-encapsulated repellent and placed in the eaves of houses, was tested in a malaria-endemic village in western Kenya. Using the repellent delta-undecalactone, mosquito house entry was reduced by more than 50%, while the traps caught high numbers of outdoor flying mosquitoes. Model simulations predict that, assuming area-wide coverage, the addition of such a push-pull system to existing prevention efforts will result in up to 20-fold reductions in the entomological inoculation rate. Reductions of such magnitude are also predicted when mosquitoes exhibit a high resistance against insecticides. We conclude that a push-pull system based on non-toxic volatiles provides an important addition to existing strategies for malaria prevention.
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Introduction

Malaria continues to place a substantial burden on people throughout the tropics and especially in sub-Saharan Africa. Current prevention efforts focus on long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) to control mosquito populations (WHO 2013). Although these measures have led to significant decreases in the number of malaria cases, progress is threatened by the development and rapid spread of insecticide-resistance (Ranson et al. 2011, White et al. 2013). An additional threat is the shift from indoor to outdoor feeding as well as changes in biting times that have been observed following the implementation of ITNs and IRS (Reddy et al. 2011, Moiroux et al. 2012, Russell et al. 2013, Sougoufara et al. 2014). Furthermore, changes in the composition of vector populations may lead to the dominance of species with a different ecology, which are harder to target with conventional approaches (Dabiré et al. 2012, Lwetoijera et al. 2014).

A mosquito-directed push-pull system, which operates by the simultaneous use of attractant and repellent cues, offers a possible alternative or addition to current vector control methods (Cook et al. 2007, Kahn et al. 2011). Previous experiments have shown that a push-pull system employing attractant-baited traps and spatial repellents can be effective at lowering the house entry of malaria mosquitoes by as much as 95% in an experimental setup (Menger et al. 2014a). Residents would receive considerable protection by such a reduction in mosquito exposure.

Thus far, however, all research concerning this type of push-pull system for malaria mosquitoes has taken place under semi-field conditions. For practical implementation of the system in rural Africa it is important that the system be low-tech and not dependent on electric power. This would involve limiting the number of attractant-baited traps and finding an alternative for electric power-dependent systems for the dispersal of repellents. Moreover, the system should be designed in such a way that it can run independently for a prolonged period of time.

Recent large-scale field studies are exploring the potential of mass-trapping of mosquitoes by employing a single attractant-baited trap per household that can run on solar power (Hiscox et al. 2012). Supplementing this with a passive (i.e. not requiring energy input) repellent release mechanism would provide a push-pull system that is both user-friendly and practical for real-world implementation.
Impregnated textile fabrics can be employed as suitable materials for passive dispersion of repellents (Mweresa et al. 2014a). Durable textiles can also be used for eave-screening, providing a combination of two efficient mechanisms by creating a physical as well as a chemical mosquito barrier. A prolonged passive release of repellent compound can be achieved by using a microencapsulation technique (Campos et al. 2013). Microcapsules can be impregnated into many different kinds of fabric and offer a novel method to control the release of active compounds. This technique makes it possible to obtain a longer lasting repellent effect than when the active compound is directly applied to the textile (N'Guessan et al. 2008, Miró Specos et al. 2010).

In the present study we first determined the longevity of the repellent effect of a fabric that was impregnated with porous microcapsules containing delta-undecalactone, a compound which has recently been shown to have strong repellent properties against several mosquito vectors of disease (Menger et al. 2014a,b). Subsequently we deployed a push-pull system that uses a trap baited with a five-compound attractive blend + CO₂ (Menger et al. 2014a) in combination with this repellent-impregnated fabric in a malaria-endemic village in western Kenya. We explored the possible effects of large-scale application of the described push-pull intervention on human-mosquito contact and malaria transmission by adapting an existing mathematical model.

Results

*Laboratory experiment*

Experiments were conducted in the set up described by Menger et al. (2014b), in which mosquitoes (*Anopheles coluzzii*, formerly *An. gambiae s.s. form M*) were given the opportunity to land on an artificial bait. At all tested times, t = 0, t = 1 month, t = 3 months and t = 6 months, a significant repellent effect was found for fabric impregnated with microencapsulated delta-undecalactone (Independent Samples t-test, p < 0.001 for all comparisons; Figure 1). The reduction in the number of landings was similar (ranging from 47 to 61%) at all tested time points.

*Field experiment*

Four treatments were tested in Kigoche village in Kisumu county, western Kenya: (i) the control treatment, in which a house received neither repellent-impregnated fabric
nor an attractant-baited trap. (ii) a push-only treatment in which only the repellent-impregnated fabric was installed, (iii) a pull-only treatment in which an attractant-baited MM-X trap was installed outside the house and (iv) a push-pull treatment in which both the repellent-impregnated fabric and the attractant-baited trap were in place. For the duration of the experiment, houses were occupied by one male volunteer only, who slept under an untreated bed net. The house entry rate of mosquitoes was determined by CDC light trap catches (Lines et al. 1991, Costantini et al. 1998). Preceding the experiment, a baseline study was carried out in order to be able to correct for randomization bias, as treatments were not to be rotated between houses because of possible residual effects.

During the entire experiment, 1,791 mosquitoes were caught inside the houses
(96.9% female, 3.1% male) of which 1,724 (96.3%) were anophelines and 67 (3.7%) culicines. The anopheline population consisted of 80.2% *An. funestus* s.l. and 19.8% *An. gambiae* s.l. A sub-sample of 188 individuals of *An. funestus* was molecularly studied for sub-species composition (Koekemoer et al. 2002, Cohuet et al. 2003). The 177 samples that were successfully amplified were all *An. funestus* s.s. Out of 184 *An. gambiae* individuals that were analysed molecularly (Scott et al. 1993), 171 were successfully amplified and all were *An. arabiensis*.

Statistical analyses were done for the overall CDC trap catches and for the anopheline sub-group, other sub-groups were considered too small to carry out reliable statistics, but their values are reported below and more details can be found in Tables S1 and S2 in the supplementary information.

The four houses that were selected for the intervention from the baseline study were the ones that were most similar in terms of mean trap catches and variation over the subsequent nights (Table 1). Within the five-week intervention phase, there was no increase or decrease in trap catches as a function of time (GLM with overall CDC trap catches as dependent variable, ‘intervention’ as a fixed factor and ‘week’ as a covariate, full-factorial: p = 0.001 for intervention, p = 0.629 for week and p = 0.711

**Table 1. Mean number (+SD) of mosquitoes caught during the baseline phase.**

<table>
<thead>
<tr>
<th>House</th>
<th>Baseline Mean</th>
<th>Baseline SD</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.75</td>
<td>6.944</td>
<td>Push-pull</td>
</tr>
<tr>
<td>2</td>
<td>6.63</td>
<td>3.739</td>
<td>not selected</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>5.228</td>
<td>Pull</td>
</tr>
<tr>
<td>4</td>
<td>15.75</td>
<td>4.301</td>
<td>Control</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>7.091</td>
<td>Push</td>
</tr>
<tr>
<td>6</td>
<td>6.25</td>
<td>6.819</td>
<td>not selected</td>
</tr>
<tr>
<td>7</td>
<td>21.63</td>
<td>14.262</td>
<td>not selected</td>
</tr>
<tr>
<td>8</td>
<td>7.88</td>
<td>3.871</td>
<td>not selected</td>
</tr>
</tbody>
</table>

For all houses n = 8, except for house 3 (n = 7). Four houses were selected for the different interventions.
for intervention*week). Therefore the samples over the whole intervention period were pooled, resulting in 25 replicate measurements for each group.

Significant reductions in house entry of mosquitoes were found for all interventions (Figure 2). The push-only intervention reduced mosquito house entry by 52.8% compared to the control. The pull-only intervention reduced mosquito house entry by 43.4% and the push-pull intervention reduced mosquito house entry by 51.6% (Table 2).

![Figure 2. Mean number of mosquitoes caught inside the houses.](image)

**Figure 2. Mean number of mosquitoes caught inside the houses.** Error bars indicate standard error of the mean (SEM), n = 8 for the baseline data (n = 7 for house 3) and n = 25 for the intervention data. Asterisks indicate a significant difference-in-differences between the control and the intervention: * p < 0.05; ** p < 0.01; *** p < 0.001.

Considering anopheline mosquitoes only, the results were fairly similar, with all interventions resulting in significant reductions in house entry (Figure 3). The impact of the different interventions was 55.1% for the push-only, 44.4% for the pull-only and 51.1% for the push-pull intervention (Table 3). For *An. funestus*, house entry reductions were 59.5, 47.4 and 48.9% for the push-only, pull-only and push-pull interventions, respectively (Table S1 for more details). House entry reductions for *An. gambiae* s.l. were 32.9, 29.3 and 39.0% respectively (Table S2). No further calculations
were done for the *Culex* and *Mansonia* subgroups, as the low numbers of caught individuals (58 and 9 in total, respectively) would not allow us to draw reliable conclusions.

**Table 2. Mean overall CDC trap mosquito catches for the different interventions.**

<table>
<thead>
<tr>
<th>Intervention</th>
<th>House</th>
<th>Baseline</th>
<th>Intervention</th>
<th>Difference</th>
<th>Difference (%)</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>15.75</td>
<td>15.60</td>
<td>-0.15</td>
<td>-1.0%</td>
<td>n/a</td>
</tr>
<tr>
<td>Push</td>
<td>5</td>
<td>14.00</td>
<td>6.48</td>
<td><strong>-7.52</strong></td>
<td>-53.7%</td>
<td>-52.8%</td>
</tr>
<tr>
<td>Pull</td>
<td>3</td>
<td>11.00</td>
<td>6.12</td>
<td>*-4.88</td>
<td>-44.4%</td>
<td>-43.4%</td>
</tr>
<tr>
<td>Push-pull</td>
<td>1</td>
<td>21.75</td>
<td>10.32</td>
<td>***-11.43</td>
<td>-52.6%</td>
<td>-51.6%</td>
</tr>
</tbody>
</table>

For the baseline data n = 8 (n = 7 for house 3) and n = 25 for the intervention data. Asterisks indicate a significant difference-in-differences between the control and the intervention: * p < 0.05; ** p < 0.01; *** p < 0.001.

**Figure 3. Mean number of anopheline mosquitoes caught inside the houses.** Error bars indicate standard error of the mean (SEM), n = 8 for the baseline data (n = 7 for house 3) and n = 25 for the intervention data. Asterisks indicate a significant difference-in-differences between the control and the intervention: * p < 0.05; ** p < 0.01; *** p < 0.001.
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The MM-X traps placed outdoors in the pull-only and push-pull treatments caught 1,356 mosquitoes (95.6% female, 4.4% male) in total, of which 616 (45.4%) were anophelines and 740 (54.6%) culicines. The anophelines were 52.1% *An. funestus*, 43.8% *An. gambiae* s.l. and 4.1% other anopheline spp. The mean number of mosquitoes caught outside in the push-pull treatment (29.16, SEM 4.32) was not significantly different from the mean number caught in the pull-only treatment (25.08, SEM 2.54).

### Malaria transmission model

To simulate the effect of implementation of the push-pull strategy on a large scale, we adjusted an existing mathematical model by Okumu et al. (2010a). We used the default settings of the model, with exceptions for: bed net use (Ch), which was set at 67%; human availability (ah), which was translated to relative human availability (rah) to model the effect of house entry reduction (expressed as push efficacy: ps) and; attractiveness of the attractant-baited traps (λt). The effects of possible push-pull interventions in a situation in which pyrethroid resistance is widespread (reducing excess mosquito mortality (θm)) was explored in a second scenario.

Model simulations predict the impact of a large-scale push-pull intervention on the EIR (Figures 4 and 5). Under the given assumptions, either repellent barriers or odour-baited traps alone result in evident reductions of the EIR. However, the strongest reductions are obtained when combining push and pull.

### Table 3. Mean CDC trap catches of anopheline mosquitoes for the different interventions.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>House</th>
<th>Baseline</th>
<th>Intervention</th>
<th>Difference</th>
<th>Difference (%)</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>15.63</td>
<td>15.12</td>
<td>-0.51</td>
<td>-3.3%</td>
<td>n/a</td>
</tr>
<tr>
<td>Push</td>
<td>5</td>
<td>13.63</td>
<td>5.68</td>
<td>-7.95</td>
<td>-58.3%</td>
<td>-55.1%</td>
</tr>
<tr>
<td>Pull</td>
<td>3</td>
<td>10.86</td>
<td>5.68</td>
<td>-5.18</td>
<td>-47.7%</td>
<td>-44.4%</td>
</tr>
<tr>
<td>Push-pull</td>
<td>1</td>
<td>21.75</td>
<td>9.92</td>
<td>-11.83</td>
<td>-54.4%</td>
<td>-51.1%</td>
</tr>
</tbody>
</table>

For the baseline data n = 8 (n = 7 for house 3) and n = 25 for the intervention data. Asterisks indicate a significant difference-in-differences between the control and the intervention: * p < 0.05; ** p < 0.01; *** p < 0.001.
In the first scenario, assuming 67% ITN coverage and susceptible mosquitoes, the initial EIR is estimated at 18.5 infectious bites per year (Figure 4, see also Figure S1). Combining a repellent barrier with a push efficacy of 55% reduction in house entry (as was found in this study and is indicated with a solid arrow in Figure 4) with an odour-baited trap that has the same attractiveness as a human being (Okumu et al. 2010b, Mukabana et al. 2012a) (the green line / triangles in Figure 4) would reduce the EIR to 2.9 infectious bites per year. If the push efficacy can be improved to 80% (which is deemed feasible by screening the eave entirely with repellent material and is indicated with a dotted arrow in Figure 4), the combination with a trap reduces the EIR to 1.0 in our model (a nearly 20-fold reduction). The repellent barrier only reduces the EIR to 13.5 or 6.9 when the push efficacy is 55 or 80%, respectively. Attractant-baited traps alone are estimated to reduce the EIR to 6.5, if the attractiveness of the traps is the same as that of a human being.

Figure 4. Model simulations showing the entomological inoculation rate (EIR) as a function of different levels of push efficacy. Push efficacy is expressed as the percentage of house entry reduction and pull efficacy is expressed as the relative attractiveness of the trap, compared to a human being. In this scenario mosquitoes are fully susceptible to insecticides.
In the second scenario, mosquitoes are assumed to have high resistance against the insecticides used on ITNs. The EIR is calculated to be much higher, with an initial value of 49.7 infectious bites per year (Figure 5, see also Figure S2). In this case a repellent barrier with a push efficacy of 55% reduction in house entry (solid arrow) that is combined with an odour-baited trap that has the same attractiveness as a human being (green line / triangles) reduces the EIR to 6.3 infectious bites per year. In case the push efficacy can be improved to 80%, the combination with a trap is predicted to reduce the EIR to 1.9 (a more than 20-fold reduction). The repellent barrier in the absence of a trap reduces the EIR to 27.8 or 11.0 when the push efficacy is 55 or 80%, respectively. Attractant-baited traps alone, having the same attractiveness as a human being, are predicted to reduce the EIR to 17.3.

Figure 5. Model simulations of a scenario in which mosquitoes are highly resistant against insecticides. Shown is the entomological inoculation rate (EIR) as a function of different levels of push efficacy. Push efficacy is expressed as the percentage of house entry reduction and pull efficacy is expressed as the relative attractiveness of the trap, compared to a human being.
Discussion

Interpretation of results
This study showed how repellent-treated fabrics, whether or not in combination with an attractant-baited trap, reduced mosquito house entry by approximately 50% in a field setting. Model simulations predict that when a push-pull intervention is applied on a large scale, up to 20-fold reductions in the EIR may be obtained by combining the repellent fabric and attractant-baited traps.

Behavioural tests in the repellent bioassay showed a consistent repellent effect of the treated fabric, which was maintained for a period of at least six months. Because samples were stored in plastic bags in a refrigerator in between the tests, evaporation of volatiles from the fabric was presumably much lower than under field circumstances. The fabric intended for use in the field study was prepared identically and stored for two months in the same way, thus we expect it to have been similarly efficient by the time it was applied in the field.

During the field experiment, we found that even a 10 cm wide strip of this repellent-treated fabric reduced mosquito house entry by 52.8%. This must have resulted from a ‘barrier’ of repellent, as the fabric did not physically close off the eave, leaving ample space for mosquitoes to fly over as they did in the control treatment with untreated fabric. Mnyone et al. (2012) similarly closed off the eaves partially with baffles, and demonstrated that such imperfect barriers do not affect house entry of An. gambiae s.l. and An. funestus. Snow (1987) reported that endophilic host-seeking mosquitoes fly towards a human-occupied house (presumably following a CO₂ gradient (Spitzen et al. 2008, Jawara et al. 2009)) where, upon reaching a vertical wall, they fly upwards until entering through the eave. For this reason, we chose to apply the fabric to the lower part of the eave, closing off the bottom 10 cm rather than the middle or upper section, to make sure that mosquitoes would encounter the fabric before entering the house.

The employment of an attractant-baited trap outside the experimental house reduced mosquito house entry by 43.4%. This suggests that mosquitoes were lured into the trap before they could enter the house. This is an unexpected result, as previous observations indicated that outdoor traps do not directly influence mosquito house entry (Jawara et al. 2009). However, the positioning of the trap, relative to the
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location of mosquito breeding sites or resting places, may potentially influence the trap’s efficacy in luring mosquitoes away from a house before entering (Day and Sjogren 1994). Moreover, the outdoor trap caught 25 mosquitoes per night on average, which is a considerably higher number than the 6 individuals caught by the CDC trap indoors during the pull-only intervention or the 16 individuals that were caught on average in the control house. Although these catches cannot be compared directly since different trapping methods were used indoors and outdoors, it confirms findings from previous studies showing that attractant-baited traps are a very potent tool to remove large numbers of mosquitoes (Okumu et al. 2010b, Mukabana et al. 2012a).

When the repellent-treated fabric and the attractant-baited trap were combined, mosquito house entry was reduced by 51.6%. This reduction is a bit higher than the reduction achieved by the attractant-baited trap alone, but rather similar to the reduction achieved by the repellent alone. This result may seem surprising, but is actually in line with our earlier conclusion that ‘rather than a synergetic interaction, both components (i.e. the push and the pull) seem to have independent effects’ (Menger et al. 2014a). It could thus be concluded that there is no additive effect of the attractant-baited trap and that the repellent-treated fabric, which has the higher impact and is also much cheaper, should be the recommended intervention.

Model simulations however, show that when all households are covered by the intervention, malaria transmission is reduced most effectively by augmenting the existing prevention efforts with the complete push-pull system. Whereas both the repellent barrier and the attractant-baited trap reduce the EIR independently, it is their combination that causes the strongest (up to 20-fold) reductions. This shows that the short term effects of a limited number of traps, as measured during the field experiment, may greatly differ from the effect of large-scale deployment of traps over a longer period of time, as simulated in the model. In the push-pull intervention of our experiment, an average of 29 mosquitoes per night were caught in the outdoor trap, compared to 10 mosquitoes by the trap indoors. Large-scale deployment of traps that catch such high numbers of mosquitoes is expected to affect malaria transmission by reducing the mosquito’s lifespan and by depleting mosquito populations (Hiscox et al. 2012, Kline 2006). It is because of this indirect effect, that simultaneous deployment of the attractant and repellent may still lead to a greater
impact on the EIR, not through a synergism, but rather through complementary functions. Especially in the high insecticide-resistance scenario, it is the combination of the repellent barrier and attractant-baited traps that is able to bring the EIR down to values that would drastically reduce malaria transmission.

The required efficacy of the push and the pull components lies within the range of what has experimentally been shown to be feasible. For example, a repellent barrier with an efficacy of 55% has been found in this study for the house entry of anopheline mosquitoes. This efficacy could most likely be improved by closing off much more of the eave, instead of leaving most of it open (as was done here for experimental purposes). In a previous study in a semi-field setup, house entry was reduced by 80% using only a repellent (Menger et al. 2014a). Odour baits with an attractiveness similar to that of humans have already been identified (Okumu et al. 2010b, Mukabana et al. 2012a) and are currently being deployed in a large field trial (Hiscox et al. 2012).

The dominant malaria vector trapped indoors was *An. funestus* (80% versus 20% *An. arabiensis*). This is in line with the acknowledgement of *An. funestus* as an anthropophilic and endophilic vector, whereas *An. arabiensis* is a much more opportunistic feeder that may attack cattle as well as humans, indoors or outdoors (Besansky et al. 2004). Indeed, in the outdoor traps, the proportion of *An. arabiensis* was much higher (44%) and closer to the proportion of *An. funestus* (52%). Whereas the repellent barrier is expected to affect mainly indoor transmission, outdoor traps may have an impact on indoor as well as outdoor transmission, because they target vector species with diverse host-seeking behaviours. Further studies should elucidate the functioning of the respective push and pull components in more detail (e.g. the spatial range of the repellent barrier and the optimal placement of the attractant-baited trap relative to it).

Push-pull as a vector-control tool

In our study, fabrics treated with delta-undecalactone reduced mosquito house entry. When implemented as a vector-control tool, one would not use narrow strips of fabric that leave open most of the eave for mosquitoes to enter, as was done in this study for experimental purposes. Rather, one would close off all openings as much as possible, to install a physical barrier, in addition to the semiochemical one. This of course, brings to mind the practise of screening eaves and/or ceilings, which has
already proven to be an effective measure against mosquito house entry (Lindsay et al. 2003, Kirby et al. 2009, Kampango et al. 2013). However, house screening is difficult in the typical mud-walled houses that make up the majority of houses in the village in which this study was conducted, or indeed in many other traditional hand-built houses that are commonly found in the African countryside. The many cracks and uneven edges hinder the complete closure of the eave, or other openings, with gauze or netting. However, eave screens which are impregnated with a long lasting spatial repellent would not need to close off each little hole and crack as they would serve as a semiochemical barrier as well. Furthermore, net fabric made of cotton is cheap, readily available and allows some degree of air circulation, the main purpose of eaves.

Field experiments employing a repellent to reduce house entry are many, but few report effects of the magnitude observed in this study for a prolonged period of time (i.e. more than a few hours) (Maia and Moore 2011). One category of repellents that do cause very significant reductions in house entry are the volatile pyrethroids (Kawada et al. 2008, Ogoma et al. 2012). Application of these volatile, or vaporized insecticides resulted in house entry reductions of over 90% in houses with open eaves or similar constructions. However, there are two main objections against the use of insecticides. The first is the development of physiological and behavioural resistance in the target species (Ranson et al. 2011, IR-mapper). Although to repel mosquitoes is not the same as to kill them, and thus may be less prone to the development of resistance, these chemicals are from the same class, the pyrethroids, as those used on bed nets (which are meant to kill) and structurally similar. The second, but no less important, argument against pyrethroid insecticides is the concern about the health effects on humans who are exposed to the chemical for prolonged periods of time (Koureas et al. 2012). A volatile insecticide, dispensed in or around human dwellings would be inhaled, increasing one’s exposure to potentially harmful chemicals. Delta-undecalactone is a natural product that is present in food sources such as edible fruits and dairy products and its odour is generally described as fruity, coconut-like and pleasant (Lin and Wilkens 1970, Mahajan et al. 2004).

In the system presented here, the push and the pull component appear to operate independently. In other words, mosquitoes that are pushed away from the house, do not have a greater chance of being pulled into the trap. This may actually be an advantage, as it would decrease the chance that mosquitoes develop insensitivity to
the repellent, which would be stimulated if mosquitoes that are pushed away would have a greater chance of dying in a trap. However, this observation would have to be confirmed in a larger field study, as in the current situation mosquitoes that were repelled may have been diverted to surrounding houses that did not receive the intervention (Maia et al. 2013). Therefore, a field study in which a majority of houses in the area receives the push-pull intervention, and all houses are monitored, is a recommended next step.

Based on model simulations, we expect that in a scenario in which coverage of the intervention is high, the greatest benefit can be gained by using both repellent barriers and odour-baited trapping devices to reduce malaria transmission. An advantage of using an odour-baited trap next to a repellent is that mosquitoes are not only repelled from a house, but also actively removed by the trap. As previously shown for trypanosomiasis (sleeping sickness) and other vector-borne infectious diseases, baited traps can be a very efficient tool to lower vector populations and reduce transmission (Day and Sjogren 1994, Kline 2006). As the odour-bait is a blend that consists of five different compounds, all of which are also present in human skin emanations, it is unlikely that mosquitoes would rapidly become insensitive to it.

In conclusion, the push-pull system based on attractive and repellent volatiles seems a promising addition to the repertoire of integrated vector management, as it may contribute strongly to malaria prevention. It is expected to add to the effect of existing methods such as ITNs and IRS, especially in areas where insecticide resistance is widespread and in situations where malaria transmission occurs outdoors. Its efficacy to reduce malaria transmission should be confirmed in larger-scale field experiments, preferably in combination with existing vector-control tools.

Materials and Methods

Components of the push-pull system

Attractant
A five-compound odour bait, which simulates human scent, was used as an attractant in both the laboratory and field experiments (Mukabana et al. 2012a, Hiscox et al. 2014, Menger et al. 2014b). In the laboratory experiment, it provided baseline attraction against which the activity of candidate repellents could be measured. In the
field experiment the odour bait was used in combination with CO\textsubscript{2} to bait the mosquito traps. The blend consists of ammonia, L-\((+)-lactic\)-acid, tetradecanoic acid, 3-methyl-1-butanol and butan-1-amine. Individual compounds were released from nylon strips in concentrations optimized for this release method (Okumu et al. 2010c, Mukabana et al. 2012b).

**Repellent**

The repellent used in this study was delta-undecalactone, a novel repellent which has been shown to be effective against *An. coluzzii*, *An. gambiae* and *Aedes aegypti* mosquitoes in laboratory and semi-field setups (Menger et al. 2014a,b). The repellent was released from microcapsules incorporated into cotton netting.

Microcapsules containing delta-undecalactone were produced by a solvent evaporation technique using an oil-in-water emulsion (Senhorini et al. 2012, Chaiyasat et al. 2013). We selected as shell material poly(lactic acid), a biodegradable polymer that is non-toxic, environmentally friendly and that has been thoroughly studied for its use in encapsulating hydrophobic drugs (Wischke and Schwendeman 2008). The core material was delta-undecalactone, which was slowly released by diffusion through the porous shell. The microcapsules consisted of 30\% wt. delta-undecalactone (determined by thermogravimetric analysis) and were applied onto 100\% cotton net fabric that was especially designed for this purpose (Leno structure, 65 g/m\textsuperscript{2}, provided by Utexbel, Belgium). The application on the substrate was performed by padding, thereby obtaining a wet pickup of 67\%, and the product was dried at 110°C. The result was a repellent-impregnated fabric containing 2.18 g dry microcapsules per m\textsuperscript{2}. Figure 6 shows a scanning electron microscope (SEM) image of this fabric, confirming the presence of the microcapsules.

**Laboratory experiment**

**Mosquitoes**

The mosquitoes (*An. coluzzii*, formerly *An. gambiae s.s.* form M) used in the laboratory experiment were reared in climate chambers at the Laboratory of Entomology of Wageningen University, The Netherlands. The original population was collected in Suakoko, Liberia, in 1987 (by courtesy of Prof M. Coluzzi).

Mosquitoes were kept under 12:12 h photo:scotophase at a temperature of 27 ± 1°C
and relative humidity (RH) of 80 ± 5%. Adults were kept in 30 × 30 × 30 cm gauze wire cages and were given access to human blood through a Parafilm membrane every other day. Blood was obtained from a blood bank (Sanquin Blood Supply Foundation, Nijmegen, The Netherlands). A 6% glucose solution in water was available \textit{ad libitum}. Eggs were laid on wet filter paper and then placed in a plastic tray with tap water for emergence. Larvae were fed on Liquifry No 1 (Interpet, UK) for the first three days and then with TetraMin baby fish food (Tetra, Germany) until they reached the pupal stage. Pupae were collected from the trays using a vacuum system and placed into a plastic cup filled with tap water for emergence.

The mosquitoes intended for the experiments were placed in separate cages as pupae. They had access to a 6% glucose solution but did not receive blood meals. The day preceding the experiment, 5-8 day old female mosquitoes were placed in release cages with access to tap water in cotton wool until the experiment. Both experiments took place during the last four hours of the scotophase, a period during which \textit{An. gambiae} females are highly responsive to host odours (Maxwell et al. 1998).
Bioassay

The bioassay was set up in a climate-controlled room at constant air temperature (24 ± 1°C) and RH between 60 and 75%. During the experiments these parameters were monitored using a Tinyview data logger with display. Central to the bioassay was a landing stage to which mosquitoes were attracted. It consisted of a heated circular plateau (Ø 15 cm) that held the five-compound odour blend and was positioned underneath the gauze bottom of a flight chamber. The temperature at the centre of the landing stage was kept at 34 ± 2°C, comparable to the temperature of human skin, causing the mosquitoes to land and probe with their proboscis through the gauze in search of a blood-host.

Measuring repellence

A 15 cm x 15 cm cutting of the repellent-treated fabric was compared to an identical cutting of untreated fabric. The fabric was laid down on the bottom of the flight chamber, over the landing stage. Repellence was measured by releasing ten female mosquitoes into the flight chamber. After one min. of acclimatization time, the number of landings on the fabric covering the landing stage was counted during eight min. A landing was defined as the total period during which a mosquito maintained contact with the landing stage. Walking/hopping around on the landing stage as well as short (< 1 s) take offs immediately followed by landing again were included in one landing. A new landing was recorded when a mosquito had left the stage for more than 1 s before landing again. Landings shorter than 1 s during which no probing took place were ignored.

Design and data analysis

The treated and the control fabric were tested eight times, with four replicates per day of each, in random order, during two subsequent days. The tests were performed within a week after the treatment had taken place and were repeated after one, three and six months. In between tests, the fabric was stored at 4°C in a refrigerator. IBM SPSS Statistics 19 was used for data analysis. For the different moments in time, the number of landings on the treated fabric was compared to the control. A Shapiro-Wilk test was used to test for normality. T-tests were performed to determine significant reductions at α = 0.05.

Field experiment
Study site
Kigoche village is located in Kisumu county in western Kenya. It lies adjacent to the Ahero rice irrigation scheme (00°08′19″S, 34°55′50″E) at an altitude of 1,160 m above sea level (Mukabana et al. 2012a). Kigoche has an average annual rainfall of 1,000 - 1,800 mm and an average RH of 65%. Mean annual temperatures in the area vary between 17°C and 32°C. Rice cultivation is the main occupation of the inhabitants. Most houses in the village are mud-walled with open eaves, have corrugated iron-sheet roofs, no ceiling and are either single- or double- roomed. Eaves, about 20 cm wide, increase ventilation in the houses and form the predominant entry points for mosquitoes (Snow 1987, Lindsay and Snow 1988). Malaria caused by *Plasmodium falciparum* is endemic in the village. The area experiences a long rainy season between April and June and a short rainy season in October - November. During these periods, mosquito breeding sites proliferate, and mosquito populations rapidly increase in size. The domestic animal population comprises cattle, goats, sheep, chickens, ducks, dogs and cats, with cattle being most abundant. The main staple food is maize. Rice is primarily grown as a cash crop.

Houses
Eight traditional, mud-walled houses were selected for the baseline study (see below). The minimum distance between any two selected houses was 30 m, but other (unselected) houses were present around and in between. Based on the mosquito catches during the baseline experiment, four out of the eight houses were selected for the subsequent push-pull experiment.

Measuring house entry
Mosquitoes were attracted into a house by a volunteer sleeping under an untreated bed net. Eight male volunteers were recruited to sleep in the houses, one person per house. There were no other people sleeping in the house. The CDC light trap was installed at the foot end of the bed, with the top cover hanging approximately 15 cm above the matrass. The light of the trap was disabled, in order to collect only mosquitoes attracted by the volunteer. Power for the fan was supplied by a 6 V dry cell battery. Vaseline petroleum jelly was applied to the string from which the trap hung down, preventing ants from reaching the mosquitoes in the trap. The eight volunteers rotated amongst the houses. Each night the collection of mosquitoes started at 19:30 h and stopped at 6:30 h in the morning.
Chapter 5

Trapped mosquitoes were killed in a freezer and morphologically identified. Culicine mosquitoes were identified to genus level and anophelines were divided into *An. funestus* sensu lato (s.l.), *An. gambiae* s.l. and other *Anopheles* spp. Individual *An. funestus* s.l. and *An. gambiae* s.l. mosquitoes were placed into 2 ml Eppendorf tubes with silica gel and a piece of cotton wool to be further identified with a polymerase chain reaction (PCR) (Scott et al. 1993, Koekemoer et al. 2002, Cohuet et al. 2003). The abdominal status of female mosquitoes was categorized as unfed, blood-fed or gravid.

**Interventions**
The four treatments that were tested during the field experiment were: (i) the control treatment, in which a house received neither repellent-impregnated fabric nor an attractant-baited trap. (ii) a push-only treatment in which only the repellent-impregnated fabric was installed, (iii) a pull-only treatment in which an attractant-baited trap was installed outside the house and (iv) a push-pull treatment in which both the repellent-impregnated fabric and the attractant-baited trap were in place.

The repellent was released from a 10 cm wide strip of the fabric described above, which was applied inside the eave, around the full circumference of the house (Figure 7, A and B). The strip was stretched in the lower part of the eave, closing off only the bottom 10 cm but leaving ample space for mosquitoes to enter the house. The control and pull-only treatments received an untreated strip of fabric that was applied the same way as the treated fabric used in the push and push-pull treatments. Strips remained in place over the entire study. See Table 4 for a comprehensive overview of the presence/absence of the specific elements during the treatments.

**Table 4. Overview of which push and pull elements were present during the various interventions.**

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Fabric in eave</th>
<th>MMX trap outside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>untreated</td>
<td>No</td>
</tr>
<tr>
<td>Push only</td>
<td>treated</td>
<td>No</td>
</tr>
<tr>
<td>Pull only</td>
<td>untreated</td>
<td>Yes</td>
</tr>
<tr>
<td>Push-pull</td>
<td>treated</td>
<td>Yes</td>
</tr>
</tbody>
</table>
The attractant-baited traps were of the Mosquito Magnet X (MM-X) type (Njiru et al. 2006, Qiu et al. 2007), baited with the five-compound blend described above and CO₂ produced by the fermentation of molasses by yeast (Smallegange et al. 2010, Mweresa et al. 2014b). Traps were installed outside, with the odour outlet positioned at 15 cm above ground level (Figure 7C) (Jawara et al. 2009). A 12V battery provided power for the MM-X traps. Surgical gloves were worn when handling the traps, to avoid contamination with human odour.

![Image of the push-pull system](image)

**Figure 7. The components of the push-pull system.** Panels A and B: The 10 cm wide strip of fabric as it was applied inside the eave, around the full circumference of the house. Panel C: The attractant baited MM-X trap as it was installed outside the house.

### Study design

Data from a baseline study allowed us to correct for initial differences between the houses in terms of mosquito entry by using a difference-in-differences method rather than a simple cross-sectional comparison to estimate the impact of the interventions (Baker 2000). The baseline study was conducted during eight subsequent nights (a full rotation of all volunteers), to determine the house entry of mosquitoes for eight different houses. Hereafter, four houses were selected based on the mean number of mosquitoes caught and the variation between the different nights (see details in the results section). Treatments were randomly assigned to the selected houses.

Immediately following the baseline study, the push-pull experiment ran for five subsequent weeks. During the first two rounds of eight nights, sampling took place...
every night \(((n = 8) \times 2)\). For the last three weeks, sampling took place three nights a week \(((n = 3) \times 3)\). House entry was measured by CDC trap catches, as during the baseline study. The differences between the mean indoor catches were corrected for by subtracting the mean trap catches of the baseline study from the data obtained during the intervention phase. For a conservative estimate, we used the pooled variance of the intervention phase data, which was larger than the pooled variance of the baseline data, for further testing. The mean trap catches of the different interventions were compared with the control treatment using a General Linear Model (GLM) followed by Dunnet’s post-hoc test. Testing was one-sided (treatment < control) with overall \(\alpha = 0.05\). IBM SPSS Statistics 19 was used to generate GLMs and post-hoc tests.

**Ethics statement**

This study was part of a series of studies that were approved by the ethical review committee of the Kenya Medical Research Institute (KEMRI/RES/7/3/1). The purpose and procedures of the study were explained to local leaders, household heads and volunteers before seeking permission to carry out the study. Volunteers and house owners were informed about the nature of the study and consented after having read and understood the protocol of the study prior to signing two copies of the written consent form approved by the ethics committee of KEMRI. One of the copies was kept by the participant while the second one was retained for the project record. During the experiment there was daily communication with the volunteers, who had continuous access to artemisinin combination therapy (ACT) in case of infection with malaria. The individual in Figure 7C has provided specific permission for his picture to be used in this publication.

**Malaria transmission model**

**General description**

The deterministic and static model by Okumu et al. (2010a) describes and quantifies the most essential activities of malaria mosquitoes in the process of malaria transmission. Over 70 parameters describing these activities are included in the model, roughly captured in ecological parameters, intervention parameters and parameters that are derived from combinations of those. The model assumes that the population is homogeneously exposed to mosquitoes, no cumulative or time effects are considered and biting finds place exclusively indoors and during the night. See
Okumu et al. (2010a) for full details concerning the parameterization of all variables and literature references. Using the entomological inoculation rate (EIR, the average number of infectious bites received by a person in a year (Smith et al. 2007)) as a proxy, we determined the effect of a possible push-pull intervention on malaria transmission for a number of scenarios.

Model settings
We used the default settings of the model, with exceptions for the following parameters:
Bed net use (Ch) is set at 67%, i.e. 2/3 of the population is assumed to possess a bed net and sleep under it. The model acknowledges the dual efficacy of ITNs, using one parameter to express the excess diversion ($\theta_D$) and another parameter to express the excess mortality ($\theta_m$) that a mosquito experiences upon attacking a human being sleeping under an ITN. The latter parameter is adjusted in a second series of scenarios that explored the effect of pyrethroid resistance (see below).

In order to include the influence of repellent-induced house entry reduction (push efficacy) on the EIR, we interpreted this effect as a human being less available for a blood meal. Push efficacy is thus represented by reduced availability of all humans (those with and those without a bed net) for blood meals. Thus, when the efficacy of the push (ps) is defined as the fraction of mosquitoes that is prevented from entering the house by the repellent barrier, then the availability of humans (ah) decreases through $ah \times (1 - ps)$, which results in the relative availability of humans (rah). We used rah instead of ah in all scenarios, considering house entry reduction of 0 – 100% (Menger et al. 2014a, this paper). In the absence of the push-intervention ps = 0, thus rah = ah.

We used the relative attractiveness of the attractant-baited traps ($\lambda_t$) as a measure for the efficacy of the pull. The efficacy of the pull is the attractiveness of the trap compared to that of a human being, thus when $\lambda_t = 1$, the trap is as attractive as a human being. We considered values of 0, 0.5, 1 and 2 for $\lambda_t$ (Okumu et al. 2010b, Mukabana et al. 2012a). In the absence of the pull intervention $\lambda_t$ is set to 0. Availability of odour-baited traps, which in the original model is linked to human availability, was set to 0.0012, its default value, identical to that of a human being in the absence of the push intervention. Each household, assumed to consist of six people, is supposed to possess one odour-baited trap. Therefore, using the default
number of people (1000), the number of odour-baited traps is set to 167.

To explore the effects of possible push-pull interventions in a situation where pyrethroid resistance is widespread, the excess mortality that a mosquito experiences upon attacking a human being sleeping under a bed net was reduced in a second series of scenarios. A recent review by Strode et al. (2014) addressing the risk difference, in terms of mortality, for a mosquito attacking someone sleeping under a non-treated net versus someone sleeping under an ITN, allowed us to reliably estimate this parameter, which we set to 0.4 (from 0.7 in the default scenarios) to mimic a high resistance situation.

Acknowledgements

We wish to thank the volunteers in Kigoche village who made possible the field study, the rearing staff in Wageningen for supplying the mosquitoes used in the laboratory experiment and Saskia Burgers of Biometris for advice on the statistical analyses. Maxime Durka of Devan Chemicals is gratefully acknowledged for commenting on the sections about microencapsulation.

References


Chapter 5

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IR-Mapper (not dated) IR Mapper consolidates reports of insecticide resistance in malaria vectors onto filterable maps to inform vector control strategies. [http://www.irmapper.com/]


Chapter 5


Maia MF, Moore SJ (2011) Plant-based insect repellents: a review of their efficacy, development and testing Malaria Journal 10(S1): S11


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Chapter 5


Supplementary Figure 1. Model simulations showing the entomological inoculation rate (EIR) as a function of different levels of pull efficacy. Pull efficacy is expressed as the relative attractiveness of the trap, compared to a human being. Push efficacy is expressed as the percentage of house entry reduction. In this scenario mosquitoes are fully susceptible to insecticides.
Supplementary Figure 2. Model simulations of a scenario in which mosquitoes are highly resistant against insecticides. Shown is the entomological inoculation rate (EIR) as a function of different levels of pull efficacy. Pull efficacy is expressed as the relative attractiveness of the trap, compared to a human being. Push efficacy is expressed as the percentage of house entry reduction.
### Supplementary Table 1. Mean catches of *Anopheles funestus* mosquitoes for the different interventions.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>House</th>
<th>Baseline</th>
<th>Intervention</th>
<th>Difference</th>
<th>Difference (%)</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>12.75</td>
<td>13.12</td>
<td>0.37</td>
<td>2.9%</td>
<td>n/a</td>
</tr>
<tr>
<td>Push</td>
<td>5</td>
<td>10.13</td>
<td>4.40</td>
<td>-5.73</td>
<td>-56.6%</td>
<td>-59.5%</td>
</tr>
<tr>
<td>Pull</td>
<td>3</td>
<td>8.57</td>
<td>4.76</td>
<td>-3.81</td>
<td>-44.5%</td>
<td>-47.4%</td>
</tr>
<tr>
<td>Push-pull</td>
<td>1</td>
<td>14.00</td>
<td>7.56</td>
<td>-6.44</td>
<td>-46.0%</td>
<td>-48.9%</td>
</tr>
</tbody>
</table>

For the baseline data n = 8 (n = 7 for house 3) and for the intervention data n = 25.

### Supplementary Table 2. Mean catches of *Anopheles gambiae s.l.* mosquitoes for the different interventions.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>House</th>
<th>Baseline</th>
<th>Intervention</th>
<th>Difference</th>
<th>Difference (%)</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>2.88</td>
<td>2.00</td>
<td>-0.88</td>
<td>-30.6%</td>
<td>n/a</td>
</tr>
<tr>
<td>Push</td>
<td>5</td>
<td>3.50</td>
<td>1.28</td>
<td>-2.22</td>
<td>-63.4%</td>
<td>-32.9%</td>
</tr>
<tr>
<td>Pull</td>
<td>3</td>
<td>2.29</td>
<td>0.92</td>
<td>-1.37</td>
<td>-59.8%</td>
<td>-29.3%</td>
</tr>
<tr>
<td>Push-pull</td>
<td>1</td>
<td>7.75</td>
<td>2.36</td>
<td>-5.39</td>
<td>-69.5%</td>
<td>-39.0%</td>
</tr>
</tbody>
</table>

For the baseline data n = 8 (n = 7 for house 3) and for the intervention data n = 25.
Chapter 6

Eave screening and push-pull tactics to reduce house entry of malaria mosquitoes


Submitted for publication
Abstract

Although insecticide-treated nets and indoor residual spraying have contributed to an impressive decline in malaria over the last decade, this progress is threatened by the development of physiological and behavioural resistance of mosquitoes against the insecticides on which these interventions are based. Acknowledging the need for alternative vector-control tools in addition to insecticide-based methods, we quantified the effects of eave screening in combination with a push-pull system based on the simultaneous use of a repellent and attractant-baited traps. Two field experiments in western Kenya showed that eave screening, whether or not in combination with an attractant-baited trap, was very effective in reducing the house entry of malaria mosquitoes. The effect size was variable between different mosquito species and between the two experiments, but the reduction in house entry was always considerable (between 61% and 99%) and statistically significant. The effect of an outdoor, attractant-baited trap on house entry was not significant. However, the high number of mosquitoes trapped outdoors indicates that the attractant-baited traps could be used for removal trapping, which would enhance indoor as well as outdoor protection against mosquito bites. As eave screening was already very effective by itself, the addition of a repellent was of limited value. Nevertheless, repellents may play a role in reducing outdoor malaria transmission in the peridomestic area.
Introduction

Malaria remains one of the most deadly infectious diseases and continues to claim hundreds of thousands of lives annually, mostly of young children in sub-Saharan Africa (WHO 2014a, Murray et al. 2012). The principle prevention strategy is vector control, which largely depends on insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) (WHO 2014a). Although ITNs and IRS have contributed to an impressive decline in malaria over the last decade (Murray et al. 2012), these intradomicile measures do not target outdoor feeding mosquitoes which limits their potential to eliminate malaria completely (Killeen 2014). Moreover, the progress made is threatened by the development of physiological and behavioural resistance of mosquitoes against the insecticidal compounds used (Ranson et al. 2011, Hemingway and Ranson 2000). Recent reports confirming the rapid spread of insecticide resistance underline the need for alternative approaches in addition to insecticide-based methods (Toé et al. 2014, Mawejje et al. 2012, Kanza et al. 2012, Ochomo et al. 2012, Abilio et al. 2011, Chanda et al. 2011). In this study we aimed to quantify the effects of eave screening in combination with a push-pull system that interferes with mosquito host-seeking behaviour through the simultaneous release of attractive and repellent volatiles.

Throughout the tropics, many traditional houses are constructed with eaves, i.e. openings between the wall and the roof, which serve to increase airflow in the houses, but also form the predominant entry point for mosquitoes (Snow 1987, Lindsay and Snow 1988). Reducing mosquito house entry by screening eaves and other openings has played a well-documented role in reducing the incidence of malaria in many different countries around the globe (Lindsay et al. 2002). Numerous studies show that eave and window screens, or net ceilings, reduce mosquito house entry and in some cases anaemia (an indicator of malaria morbidity) in different African countries (Kampango et al. 2013, Kirby et al. 2009, Lindsay et al. 2003). However, house screening is difficult in many traditional houses that are commonly found in rural areas of sub-Saharan Africa. The many cracks and uneven edges hinder the complete closure of the eave, or other openings, with mesh or netting.

Push-pull is a term originally adopted in the context of agricultural pest management (Cook et al. 2007, Khan et al. 2011). A push-pull system manipulates the behaviour and/or distribution of the target species by the simultaneous use of repellent and
attractive stimuli. The design of an effective push-pull system directed at malaria vectors is a recent, and ongoing, development (Menger et al. 2014a, 2015).

Key to a functional push-pull system is the controlled, long-lasting release of attractive and repellent compounds. The invention of microencapsulation techniques allows for such a prolonged, passive release of active volatiles (Campos et al. 2013). Microcapsules can be impregnated into many different kinds of fabric to achieve a controlled release of mosquito repellent from a functional textile matrix, which could for example be used to fabricate bed nets or eave screens (Miró Specos et al. 2010, N'Guessan et al. 2008).

Menger et al. (2015) showed that a narrow strip of net fabric that was impregnated with delta-undecanolate (dUDL) and placed in the eave of traditional houses in western Kenya, reduced mosquito house entry by 50% or more. The repellent dUDL has been shown effective against anopheline and other mosquitoes in laboratory and (semi-)field settings (Menger et al. 2014a,b). Durable textiles could also be used for eave-screening, which makes it possible to integrate this repellent barrier with the physical barrier that eave screening provides. Eave screens impregnated with a long-lasting spatial repellent would not need to close off every little hole and crack in the wall as they would provide a chemical barrier as well (Menger et al. 2015). Therefore, we hypothesised that the combined application of eave-screening and push-pull might yield an intervention which is suitable for rural African areas and is superior over either approach alone.

Attractive synthetic mosquito lures, which have a similar or higher attractiveness as humans, are now available for monitoring and control of malaria vectors (Okumu et al. 2010a, Mukabana et al. 2012a, Van Loon et al. In Press). An ongoing large-scale field study explores the potential of mass-trapping malaria vectors to impede malaria transmission in an island community (Hiscox et al. 2012). By actively removing mosquitoes from the peri-domestic environment, attractant-baited traps can provide a dual protective effect: (1) a direct effect by reducing house entry of mosquitoes that would otherwise have entered (Hiscox et al. 2014, Menger et al. 2015) and (2) an indirect effect by reducing the average mosquito’s lifespan and by depleting mosquito populations through daily removal trapping (Kline 2006, Okumu et al. 2010b).

A pilot experiment in which a human-occupied house in a semi-field setup was fully
screened (eaves and other openings) and equipped with an attractant-baited trap hanging outside the house, indicated that this approach could dramatically reduce house entry of malaria vectors, enhance attractant-baited trap catches and that the effect of the full screening was superior to the passive release of a repellent from the eave without screening (data not shown).

Here, we present the results of two field experiments in which we studied the effects of eave screening using various untreated or repellent-impregnated materials, alone or in combination with an attractant-baited trap, on house entry and outdoor trap catches of malaria vectors.

The first experiment addresses the effects of traditional eave screening with wire mesh as well as the deployment of an attractant-baited trap in a malaria endemic village in western Kenya. The second experiment, in the same village, investigates the effects of repellent-impregnated versus untreated cotton net fabric, both in the absence and in the presence of outdoor, attractant-baited traps.

Material and Methods

Study site
Both experiments took place in Kigoche village, near Ahero in Nyanza province, Kenya (00°08′19″S 34°55′50″E, altitude: 1160 m) (Mukabana et al. 2012a, Menger et al. 2015). Traditional, mud-walled houses with a minimal distance of 25 m between them, were selected for the experiments. Other (unselected) houses were present around and in between.

Measuring mosquito house entry
Eight houses were selected for field experiment I and twelve houses were selected for field experiment II (see the respective sections below). Male volunteers between 18 and 28 years of age were recruited to sleep in the houses, one person per house, to attract mosquitoes. Volunteers rotated in a strict order between the houses on a nightly basis to minimize the influence of differences in individual attractiveness. Mosquito entry was measured by an unlit CDC light trap that was installed at the foot end of the bed, the top cover hanging approximately 15 cm above the mattress (Constantini et al. 1998, Lines et al. 1991). Each experimental night started at 19:30 h and finished at 6:30 h in the morning.
Species identification

Trapped mosquitoes were killed in a freezer and morphologically identified. Culicine mosquitoes were identified to genus level and anophelines were divided into An. funestus sensu lato (s.l.), An. gambiae s.l. and other Anopheles spp. Individual An. funestus s.l. and An. gambiae s.l. mosquitoes were placed into 2 ml Eppendorf tubes with silica gel and a piece of cotton wool for subsequent identification using polymerase chain reaction (PCR) (Koekemoer et al. 2002, Cohuet et al. 2003, Scott et al. 1993). The abdominal status of female mosquitoes was categorized as unfed, blood-fed or gravid.

Statistical analysis

Both field experiments commenced with a baseline experiment before the interventions were installed (see below). Data from the baseline experiment allowed us to correct for initial differences in the mosquito entry rate between houses by using a difference-in-differences method (Baker 2000, Menger et al. 2015). The difference in mosquito house entry between the baseline and the intervention phase was determined for each house by calculating the percentage difference in catch size of each mosquito species compared to the baseline value: Difference (%) = (Baseline – Intervention) / Baseline * 100. Further analyses were performed using IBM SPSS Statistics 22. For field experiment I, the difference in mosquito house entry of all three intervention treatments was compared with the difference observed in the control houses during the time when the intervention was applied in other houses. Mann-Whitney U (MWU) tests corrected for multiple comparisons with the Benjamini-Hochberg procedure (false discovery rate = 0.05) were used to test for statistical significance of these differences. For field experiment II, all treatments (including the control treatment) were compared to each other using Scheffé’s post-hoc tests. This analysis was performed separately for when outdoor, attractant-baited traps were absent and for when they were present. For each separate treatment, house entry reduction without outdoor traps and with outdoor traps was compared using a MWU test. Finally, outdoor trap catches in both experiments were also compared between houses where eave screening was absent and houses where it was present, using MWU tests corrected for multiple comparisons using the Benjamini-Hochberg procedure.

Ethics statement

These experiments were part of a study that was approved by the ethical review
committee of the Kenya Medical Research Institute (KEMRI/RES/7/3/1). House owners and volunteers were informed about the purpose and procedures of the experiments and consented by signing, after having read and understood, the consent form approved by the ethics committee of KEMRI. During the study there was daily communication with the volunteers, who were screened for malaria weekly and had continuous access to artemisinin combination therapy (ACT) in case of uncomplicated malaria infection.

Field experiment I

Experimental design
The baseline experiment took place during eight nights in all eight houses (i.e. one full rotation of the eight human volunteers), while no eave screening or traps were present. The four interventions that were tested during the intervention phase were (I) the control treatment, i.e. no eave screening or attractant-baited trap, (II) eave-screening with wire mesh only, (III) an attractant-baited trap only and (IV) eave screening with wire mesh and an attractant-baited trap combined. Each intervention was randomly assigned to two houses (Supplementary Table 1). Interventions were not rotated among houses in order to allow eave screens to be installed for the full duration of the study. In all treatments the volunteers slept underneath untreated bed nets.

The whole experiment took place over a period of 33 consecutive nights in May and June 2014; eight nights for the baseline phase, one to install the eave screens and 24 nights during the intervention phase (three complete rotations of the eight human volunteers). Indoor CDC traps and outdoor Suna traps (see below) were taken to the field lab following each experimental night, after which mosquitoes were frozen and identified as described above.

Materials
In order to screen the eaves, wire mesh was cut into strips of 50 cm width, sufficiently wide to cover eaves with a width ranging from ca. 15 to 30 cm. Wire mesh was applied from the outside of the houses. It was first fixed to the lower part of the eave, using staples or nails, and then stretched upwards to the corrugated iron sheet roof and clamped around the wooden beams supporting the roof (Supplementary Figure 1). Gaps between the wooden beams and the wire mesh were filled with cotton wool.
However, due to the corrugated structure of the roof, it was not possible to close the eaves completely. To help stretch the wire mesh and hold it flush against the roof, wooden sticks were placed into the eave at regular intervals.

The attractant-baited trap chosen for this experiment was the Suna trap (Biogents AG, Regensburg, Germany), a novel type of counter-flow trap that was recently developed as a tool for mosquito monitoring and control (Hiscox et al. 2014). It was baited with an attractive five-compound odour blend released from nylon strips (Menger et al. 2014b, Van Loon et al. In Press), augmented with CO$_2$ produced by the fermentation of molasses (Mweresa et al. 2014a). Fresh odour baits were provided at the start of the experiment and left in place throughout the entire trial as previous studies have shown that the strips remain attractive for up to 52 nights after impregnation (Mukabana et al. 2012b, Mweresa et al. 2015). CO$_2$ was provided daily, at the start of each experimental night. The Suna trap was hung outside the house, next to the door, suspended from the overhanging roof with a nylon line, with the air inlet positioned at 30 cm above ground level (Hiscox et al. 2014).

**Field experiment II**

*Experimental design*

In the second field experiment, cotton net fabric was used for eave screening instead of wire mesh. The treatments tested were: (I) control, i.e. no eave-screening (II) eave-screening with untreated net fabric, (III) eave-screening with net fabric impregnated with microencapsulated dUDL (see below) and (IV) eave screening with net fabric that was treated each day with a spray-on para-menthane-3,8-diol (PMD) based repellent (see below). During the intervention phase, attractant + CO$_2$ baited traps were placed outdoors at every house including the control houses, every second night (see below).

Twelve houses were included in this experiment, seven of which had also been used in field experiment I, plus five other houses (Supplementary Table 2). The baseline experiment took place during twelve consecutive nights in all houses to allow one full rotation of all human volunteers, while none of the intervention measures were applied. Based on the mean CDC trap catch during the baseline phase, houses were classified as high, medium or low mosquito-entry houses, with four houses in each group. Within each group, one house was randomly assigned to one of the treatments (thus resulting in three houses per treatment).
The experiment consisted of 12 nights of trapping to collect baseline data, followed by 24 nights of trapping during which the interventions/treatments were installed, with attractant-baited traps being deployed every other night. Nightly indoor CDC trap catches were used as a proxy for mosquito house entry. Outdoor trap catches were counted in the morning each time after the traps had been operated overnight.

Materials
The fabric that was used for each treatment was a 100% cotton net fabric that was specially designed for this purpose (Leno structure, 65 g/m², provided by Utexbel, Ronse, Belgium). It was fixed using a staple gun, and rather than applying it to cover only the eave it was stapled to the wooden beam that forms the top of the wall and then stretched outwards and fixed on the outermost beam which supports the roof (Supplementary Figure 2). This method had the advantage that we had two solid beams to stretch the fabric between, which allowed us to work fast and efficiently and to close off the eave effectively without working around the radial beams and spaces created by the corrugation of the roof.

Microcapsules containing dUDL were produced and applied as described earlier (Menger et al. 2015). The microcapsules consisted of 31% wt. dUDL and were applied on the substrate by padding, obtaining a wet pickup of 60%. The resulting fabric contained 3 g of dry microcapsules per m².

For the PMD treatment, a commercially available repellent that contained 192 g/l Citriodiol™ (approximately 64% PMD) was sprayed on the fabric inside the eave, right before the start of each experimental night, applying 0.14 g (1 puff) per running meter.

Mosquito Magnet X (MM-X) traps (American Biophysics, North Kingstown, USA) (Njiru et al. 2006, Qiu et al. 2007) were used as outdoor traps, as these were found to better preserve the trapped mosquitoes than Suna traps. They were set up identically to the Suna traps used in the previous experiment, with the exception that the air outlet was positioned at 15 cm above ground level (Jawara et al. 2009).
Results

Experiment I

A total of 7,305 mosquitoes were trapped using CDC light traps inside the houses over the entire experiment (96% female and 4% male). Anophelines made up 62% (4,496) of the total catch and the remaining 38% (2,809) were culicines. Among anophelines 95% was *An. funestus* s.l., 5% *An. gambiae* s.l. and <0.1% other anophelines. The culicine population comprised of 99.6% *Culex* spp. and 0.4% *Mansonina* spp.

In the outdoor Suna traps, a total of 5,180 mosquitoes were caught (97% female and 3% male). Of these, 39% (1,999) were anophelines and 61% (3,181) were culicines. Among anophelines 87% was *An. funestus* s.l., 4% *An. gambiae* s.l. and 9% other anophelines (including *An. coustani*, *An. ziemanni* and other, unidentified species). The culicines comprised of 76% *Culex* spp. and 24% *Mansonina* spp.

A sub-sample of 152 *An. funestus* s.l. females collected from inside and outside houses was analysed for sub-species composition by PCR. Of the 142 samples which were successfully amplified, all were *An. funestus s.s.* Out of 158 *An. gambiae* s.l. females that were analysed with PCR, 156 were successfully amplified and all were *An. arabiensis*. Further results of intervention effects are reported for *An. funestus*, *An. arabiensis* and *Culex* spp only. For these three groups, the abdominal status and sex of the trap catches is presented in Table 1.

Mean indoor CDC trap catches for each house can be found in Supplementary Table 1. Figure 1 shows the differences in house entry between the control and each of the treatments for *An. funestus*, *An. arabiensis* and *Culex* mosquitoes. All reported values are percentage differences compared to the baseline mean. House entry of *An. funestus* in the control houses was 4% higher during the intervention period than during baseline. When Suna traps were used, mean house entry was reduced by 18%. Eave screening alone reduced house entry by 92% and the combination of a Suna trap + eave screening resulted in a mean house entry reduction of 90%. The reductions in house entry in houses that were screened, whether or not a Suna trap was present, were significantly greater than the difference observed in the control treatment (MWU tests, p < 0.001).
For An. arabiensis, indoor trap catches during the baseline phase were low (a mean of 2.1 mosquitoes per house per night), and the calculation of percentage differences in house entry led to more extreme values and greater variation in estimated means than for the other species. In the control houses, mosquito entry was 114% higher during the intervention period compared with the baseline. With a Suna trap in place, house entry was 13% lower than during baseline. Eave screening, either in combination with a Suna trap or alone, reduced house entry by 99%. The effect of both eave screening treatments was statistically significant (MWU tests, p < 0.001).

Table 1. Abdominal status and sex of indoor CDC and outdoor Suna trap catches for An. funestus, An. arabiensis and Culex spp. during experiment I.

<table>
<thead>
<tr>
<th>Species</th>
<th>Female</th>
<th>Male (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfed (%)</td>
<td>Bloodfed (%)</td>
<td>Gravid (%)</td>
</tr>
<tr>
<td>Indoor CDC trap catches</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>An. funestus</td>
<td>4037 (94.5)</td>
<td>38 (0.9)</td>
<td>42 (1.0)</td>
</tr>
<tr>
<td>An. arabiensis</td>
<td>192 (87.7)</td>
<td>10 (4.6)</td>
<td>7 (3.2)</td>
</tr>
<tr>
<td>Culex spp.</td>
<td>2592 (92.7)</td>
<td>20 (0.7)</td>
<td>23 (0.8)</td>
</tr>
<tr>
<td>Outdoor Suna trap catches</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>An. funestus</td>
<td>1641 (94.7)</td>
<td>9 (0.5)</td>
<td>6 (0.3)</td>
</tr>
<tr>
<td>An. arabiensis</td>
<td>82 (95.3)</td>
<td>3 (3.5)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Culex spp.</td>
<td>2312 (95.8)</td>
<td>6 (0.2)</td>
<td>11 (0.5)</td>
</tr>
</tbody>
</table>

For indoor CDC trap catches, numbers are the total of 32 trapping nights in eight houses, during the baseline and the intervention phase. For outdoor Suna trap catches, numbers are the total of 24 trapping nights for four houses during the intervention phase.

For Culex spp., house entry during the intervention phase was 31% lower in the control houses, compared with the baseline mean. In houses with a Suna trap placed outside, there was a decrease in house entry of 44%. Eave screening alone reduced house entry by 92%, and for the combination of a Suna trap + eave screening a reduction of 87% was measured. The reductions by the two treatments that include eave screening were significantly greater than the decrease observed in the control treatment during the same time period (MWU tests, p < 0.001).
Figure 1. Differences in house entry during the intervention phase compared with baseline, experiment I. Bars show the mean ± SEM, n = 48 trap nights for all groups. In houses that received a treatment that included eave screening, there was a significant decrease in mosquito entry compared to the control houses, * = p < 0.001 and ns = not significant (MWU tests corrected for multiple comparisons with the Benjamini-Hochberg procedure with a false discovery rate of 0.05).
Suna trap catch sizes were compared between the treatment with a Suna trap only versus a Suna trap + eave screening (Table 2). For *An. funestus*, Suna trap catches were 42% higher when the trap was deployed in addition to eave screening, compared to when the trap was installed alone (MWU, p = 0.038). For *An. arabiensis*, however, Suna trap catches were 36% lower at houses where the eaves were screened (MWU, p = 0.040), although mean trap catches were only 1.1 and 0.7 mosquitoes per house per night respectively. Suna trap catches of *Culex* spp. were 30% lower when eaves were screened, but this difference was not significant (MWU, p = 0.418).

**Table 2. Mean outdoor mosquito catch per trap per night ± SEM, with Suna trap only and Suna trap + eave screening during experiment I.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Suna only</th>
<th>Suna + eave screening</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. funestus</em></td>
<td>14.9 ± 1.5</td>
<td>21.2 ± 2.1*</td>
</tr>
<tr>
<td><em>An. arabiensis</em></td>
<td>1.1 ± 0.2</td>
<td>0.7 ± 0.2*</td>
</tr>
<tr>
<td><em>Culex</em> spp.</td>
<td>29.5 ± 4.2</td>
<td>20.8 ± 2.3</td>
</tr>
</tbody>
</table>

Values are based on 24 trapping nights with two houses per treatment (n = 48). Asterisks indicate a significant difference at α = 0.05 between treatments (MWU tests).

**Experiment II**

During the second field experiment, a total of 4,137 mosquitoes were trapped inside the houses (96% female and 4% male). Of these, 79% (3,266) were anophelines and the remaining 21% (871) were culicines. Among anophelines 75% was *An. funestus* s.l. and 25% *An. gambiae* s.l. The culicine population comprised of 97% *Culex* spp. and 3% *Mansonina* spp.

In the outdoor MM-X traps, a total of 7,471 mosquitoes were caught (88% female and 12% male). Of these, 35% (2,620) were anophelines and 65% (4,851) were culicines. The anophelines comprised of 38% *An. funestus* s.l., 48% *An. gambiae* s.l. and 13% other anophelines (including *An. coustani, An. ziemanni* and other, unidentified spp.). Among culicines 58% was *Culex* spp. and 42% *Mansonina* spp.

A sub-sample of 48 *An. funestus* s.l. individuals were analysed with PCR for sub-
species determination. All were *An. funestus* s.s. Also 48 *An. gambiae* s.l. individuals were analysed, and all 45 of those that were successfully amplified were *An. arabiensis*. Further results are reported for *An. funestus*, *An. arabiensis* and *Culex* spp. For these three groups, the abdominal status and sex of the trap catches is given in Table 3.

**Table 3. Abdominal status and sex of trap catches for *An. funestus*, *An. arabiensis* and *Culex* spp. during experiment II.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Unfed (%)</th>
<th>Female</th>
<th>Male (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. funestus</em></td>
<td>2311 (94.8)</td>
<td>48 (2.0)</td>
<td>15 (0.6)</td>
<td>63 (2.6)</td>
</tr>
<tr>
<td><em>An. arabiensis</em></td>
<td>732 (88.3)</td>
<td>29 (3.5)</td>
<td>18 (2.2)</td>
<td>50 (6.0)</td>
</tr>
<tr>
<td><em>Culex</em> spp.</td>
<td>744 (88.4)</td>
<td>40 (4.8)</td>
<td>2 (0.2)</td>
<td>56 (6.7)</td>
</tr>
</tbody>
</table>

For indoor CDC trap catches, numbers are the total of 36 trapping nights in twelve houses, during the baseline and the intervention phase. For outdoor MM-X trap catches, numbers are the total of twelve trapping nights for twelve houses during the intervention phase.

The mean indoor CDC trap catches per house during the baseline and the intervention phase are reported in Supplementary Table 2. Figure 2 shows the differences in house entry between the intervention phase and the baseline for all treatments (including the control treatment). House entry of *An. funestus* in the control houses was 11% lower during the intervention phase than during the baseline phase. Eave screening with cotton net fabric reduced house entry of *An. funestus* by 61%. Eave screening with fabric that was impregnated with microencapsulated dUDL reduced house entry by 63%. Eave screening with fabric that was sprayed with PMD before each experimental night reduced house entry by 81%. All eave screening treatments significantly reduced *An. funestus* house entry (Scheffé’s post-hoc test, Figure 2).
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Figure 2. Differences in house entry during the intervention phase compared to baseline, experiment II. Bars show the mean ± SEM, n = 36 trap nights for all groups. When houses received eave screening there was a significant reduction in the house entry of *An. funestus* and *An. arabiensis* compared to unscreened control houses; reductions in the house entry of *Culex* spp. were not significant (Scheffé’s post-hoc test, lowercase letters, bars not sharing the same letter are significantly different at α = 0.05). When an outdoor MM-X trap was present, the combination with eave screening was always more effective than the outdoor trap alone, although this effect was only significant when eave screens were treated with PMD. (Scheffé’s post-hoc test, uppercase letters, bars not sharing the same letter are significantly different at α = 0.05). For all treatments (including the control), the degree of house entry reduction was not significantly affected by the presence or absence of an MM-X trap (MWU tests, p > 0.05 for all comparisons).
With an MM-X trap in place, house entry of *An. funestus* in the unscreened control houses decreased by 22% compared to the baseline mean. An MM-X trap in combination with eave screening with cotton net fabric reduced *An. funestus* entry by 62%. The combination with fabric that was impregnated with microencapsulated dUDL reduced house entry by 35%. An MM-X trap in combination with PMD-treated fabric reduced house entry by 80%. When comparing all treatments that included an outdoor MM-X trap, only the combination with fabric that was sprayed with PMD resulted in a significantly greater house entry reduction compared to unscreened houses with an MM-X trap. However, there was no significant difference with the effects of the other eave screening treatments (Scheffé’s post-hoc test, Figure 2). The effect of adding an MM-X trap to any of the eave screening treatments or the control was not significant (MWU tests, p > 0.05 for all comparisons, not shown).

House entry of *An. arabiensis* was 11% lower in the control houses during the intervention phase. Eave screening led to a reduction of 72%. Eave screening with dUDL reduced house entry by 83%. Eave screening with PMD-treated fabric reduced house entry by 89%. All eave screening treatments significantly reduced house entry of *An. arabiensis* (Scheffé’s post-hoc test, Figure 2).

With an MM-X trap in place house entry of *An. arabiensis* into unscreened control houses decreased by 30%. An MM-X trap in combination with eave screening led to a house entry reduction of 65%. The combination of an MM-X trap with dUDL-impregnated fabric reduced house entry by 55%. With PMD-treated fabric and an MM-X trap, the reduction was 80%. Only the combination with PMD- treated fabric reduced house entry of *An. arabiensis* significantly when compared to the control houses with an outdoor MM-X trap. However, the difference with the effects of the other eave screening treatments was not significant (Scheffé’s post-hoc test, Figure 2). There was no significant effect of the presence or absence of an MM-X trap on house entry reduction for any of the treatments or the control (MWU tests, p > 0.05 for all comparisons, not shown).

House entry of *Culex* spp. was 36% lower in the control houses during the intervention phase compared to the baseline mean. With eave screening, house entry of *Culex* spp. was 54% lower. When dUDL-impregnated fabric was used, the reduction was 35%. Eave screening with PMD-treated fabric reduced house entry by 84%. None of these reductions was significant compared to the difference observed in the
control houses, notwithstanding the relatively large effect size of the treatment with PMD treated fabric (Scheffé’s post-hoc test, Figure 2).

In houses with an MM-X trap placed outside, the decrease in house entry of *Culex* spp. was 43%. For houses that received both eave screening and an MM-X trap, a reduction of 74% was observed. When dUDL-impregnated fabric was used in combination with an MM-X trap, the reduction was 59%. An MM-X trap in combination with PMD-treated fabric reduced house entry by 83%. The combination of an MM-X trap and fabric that was sprayed with PMD reduced house entry significantly more than the MM-X trap used alone at unscreened houses. However, there was no significant difference with the effects of the other eave screening treatments (Scheffé’s post-hoc test, Figure 2). For all treatments, the degree of house entry reduction with or without an MM-X trap was similar (MWU tests, p > 0.05 for all comparisons, not shown).

MM-X trap catches were compared for the four treatments that included the placement of an MM-X trap. For all species MM-X trap catches were higher when the treatment included eave screening compared to when the trap was used at unscreened control houses (see Table 4 for statistical significance). The increase in mosquito catches ranged from 36% up to 110%.

**Table 4. Mean outdoor mosquito catch per trap per night ± SEM in MM-X traps placed outdoors next to control houses and next to houses with various types of eave screening during experiment II.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Control</th>
<th>Eave screening</th>
<th>Eave screening dUDL</th>
<th>Eave screening PMD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. funestus</em></td>
<td>5.0 ± 0.7</td>
<td>8.1 ± 1.5</td>
<td>8.2 ± 1.2</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td><em>An. arabiensis</em></td>
<td>5.9 ± 0.9</td>
<td>8.2 ± 1.1</td>
<td>12.4 ± 1.9*</td>
<td>8.6 ± 1.2</td>
</tr>
<tr>
<td><em>Culex</em> spp.</td>
<td>14.5 ± 3.7</td>
<td>20.9 ± 3.3*</td>
<td>22.9 ± 3.2*</td>
<td>20.0 ± 3.4*</td>
</tr>
</tbody>
</table>

Values are based on twelve trapping nights, with three replicates per treatment per night (n = 36). Asterisks indicate a significant difference compared to the control group (MWU tests, after Benjamini-Hochberg procedure with a false discovery rate of 0.05).
Discussion

Both of the field experiments showed that eave screening, whether or not it was used in combination with an attractant-baited trap, was very effective in reducing the house entry of malaria mosquitoes. The effect size was variable between the different mosquito species and between the two experiments, but the effect was always considerable (between 61% and 99%) and statistically significant.

For *Culex* spp. house entry was reduced to a similar degree whenever eave screens were installed, although the reductions measured in the second field experiment were substantially smaller than in the first and not statistically significant. Partly, this may be explained by *Culex* mosquitoes being less affected by eave screening than *Anopheles* mosquitoes, as observed by Njie et al. (2009). However, the limited effect size and lack of statistical significance in the second experiment can also be explained by a much lower overall house entry of *Culex* spp. during the intervention phase compared to baseline. Most likely these observations were caused by natural population fluctuations occurring during periods with heavy showers, as the second field experiment took place during the start of the short rainy season in 2014. *Culex* spp. are known to be very sensitive to rains, in terms of population dynamics as well as larval survival rates (Day and Curtis 1994, Koenraadt and Harrington 2008). Although *Culex* spp. are not vectors of malaria, they are able to transmit lymphatic filariasis and they are nuisance biters, which makes their control important both from a medical viewpoint and for the social acceptability of the intervention (WHO 2014b).

Other studies that looked into the use of physical barriers to reduce mosquito house entry also report consistent though variable reductions. Lindsay et al. (2003) found that installing ceilings made of different materials reduced mosquito house entry by 59% to 80%. Kirby et al. (2009) reported that full house screening or the installation of screened ceilings reduced house entry of *An. gambiae* s.l. by around 50% and, moreover, was associated with significantly reduced anaemia in children. Several later studies confirmed that screening the house entry points of mosquitoes can significantly reduce the entry of malaria vectors and other mosquitoes, although the effect size differs per species and according to the method of screening used (Ogoma et al. 2010, Kamphango et al. 2013).

The method of screening that was used in field experiment I (wire mesh, see material
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and methods section for details on the application technique) yielded house entry reductions between 87% and 99%, whereas the method that was used in field experiment II (cotton net fabric, see material and methods), resulted in reductions of between 61% and 74%. In part, this difference may be explained by the application of cotton wool to gaps between the house’s structure and the wire mesh in field experiment I. However, it was also observed that the net fabric would tear at the places where it was fixed, if it was not applied as a double layer. Besides, it would lose its elasticity after a number of days, which resulted in the formation of narrow openings at the top of the wall and at the wooden beams.

The performance of the net fabric could probably be improved by using doubled up fabric as a standard and by stapling it to the wood at shorter intervals. However, when only eave screening, without the application of repellents is the aim, then wire mesh would probably be the superior material, based on its durability and robustness. A similar conclusion was drawn by Kirby et al. (2010), who recommended future research on house screening to focus on materials with a high robustness.

When the net fabric eave screen was impregnated with microencapsulated dUDL, no significant changes in mosquito house entry were observed. Menger et al. (2015) observed that the application of a narrow strip of dUDL-impregnated fabric reduced house entry of anopheline mosquitoes by around 50%. The reason that dUDL did not improve the effect of eave screening may be that eave screening itself was already so effective that most of the mosquitoes trapped inside screened houses did not enter via the eave or surrounding gaps and cracks, but through other openings which were outside the spatial range of dUDL (e.g. through the door if it was left open during the early evening or not properly closed during night-time and through gaps between the sheets of corrugated iron that made up the roofs of the selected houses). Assuming this was the case, we can deduct that although dUDL impregnated fabric has a spatial effect as shown in Menger et al. (2015), this repellent effect is not large enough to completely prevent house entry when the fabric is only applied inside the eave. Based on the width of the eaves (approximately 30 cm) and the size of the houses, we can then roughly estimate the spatial effect of the dUDL fabric to be between 20 and 100 cm; a more precise experiment would be needed to confirm this estimation.

Spraying the cotton eave screen with the repellent PMD was associated with a greater house entry reduction of all analysed mosquito species. Although this effect was
consistent, it was too small to be significant, probably partly because there was little room for improvement as the untreated cotton was already a quite effective mechanical barrier by itself. As PMD was sprayed on to the fabric at the onset of each experimental night, the concentration of volatile PMD in the surrounding air must have been relatively high during the first hours of the night. Indeed, volunteers reported that the smell of PMD was clearly present around the house after application, which was not the case for dUDL.

The addition of an outdoor, attractant-baited trap to unscreened control houses, was associated with reductions in house entry for all mosquito species in both field experiments (not significant). When added to houses with the various types of eave screening, the effect of an outdoor trap on mosquito house entry was variable. In none of the cases, the effect of the outdoor trap was statistically significant. Previous studies reported effects of outdoor, attractant-baited traps on house entry of mosquitoes ranging from absent (Jawara et al. 2009) to reductions of around 40% (Menger et al. 2015). In several semi-field studies, reductions of between 33% and 82% were observed, although this may partly be explained by the limited number of mosquitoes released in such setups (Hiscox et al. 2014, Menger et al. 2014a). The main aim of installing attractant-baited traps, however, would be to deplete mosquito populations through daily removal trapping (Day and Sjogren 1994, Kline 2006, Okumu et al. 2010b). In both field experiments, outdoor traps caught considerable numbers of malaria vectors and other mosquitoes. In field experiment II, MM-X traps hung outside houses to which a type of eave screening had been applied, trapped consistently more mosquitoes of all species than traps outside unscreened houses. The results of field experiment I were more variable, however. When taken together, higher outdoor trap catches were associated with eave screening in ten out of twelve cases, with increases up to 110%. This is noteworthy, because in studies in which only a repellent barrier was used to reduce mosquito house entry, no increases in outdoor trap catches were observed (Menger et al. 2014a, 2015).

Compared to CDC trap catches inside the houses, both types of outdoor traps caught relatively more *Culex* spp. and less *An. funestus*, while catches of *An. arabiensis* were relatively similar both indoors and outdoors. In experiment I, the percentage of blood-fed and gravid mosquitoes trapped outdoors was lower than indoors for all species. In experiment II, the same was true for gravid mosquitoes of all species and blood-fed *An. funestus*, while relatively more *An. gambiae* and *Culex* spp. were trapped in
outdoor, compared with indoor traps. The species composition of indoor trap catches in field experiments I and II also differed. While the fraction of An. funestus remained constant, the proportion of An. arabiensis was much higher in field experiment II compared with experiment I (20% instead of 3% respectively), while the proportion of Culex mosquitoes in experiment II was lower (20% instead of 38%). An explanation may be found in the availability of more temporal breeding sites during experiment II, which took place during the short rainy season, as these are easily colonized by members of the An. gambiae complex (Fillinger et al. 2004).

This study shows that, in order to reduce house entry of malaria and other mosquitoes, eave screening was already very effective by itself and the addition of a repellent (dUDL or PMD) was of limited value. As eave-screening does not kill mosquitoes, it would be advisable to combine it with an attractant-baited trap for population reduction. Eave screening also increased outdoor trap catches, an effect which has not been observed for repellent barriers. Population reduction would increase indoor as well as outdoor protection, but to achieve this effect the degree of trap coverage would probably have to be high, depending on mosquito abundance and the attractiveness of the trap. A currently ongoing study should produce valuable insights in the feasibility and efficacy of the deployment of attractant-baited traps as a tool to reduce malaria transmission (Hiscox et al. 2012).

The combination of eave screening and attractant-baited traps would be complementary to insecticide-based vector control tools such as ITNs and IRS. Using this combination, the efficacy of the trap will be enhanced by the presence of the eave screen. With robust eave screens and traps that can operate independently for prolonged periods of time, the system would be user-friendly and practical for real-world implementation. For the eave screening part, this would mean that long-lasting materials should be used, such as wire mesh. As for the traps, long-lasting formulations of blends with a high attractiveness already exist (Mukabana et al. 2012b, Mweresa et al. 2015). Remaining issues are cost versus effectiveness and the continuous supply of CO₂ and electricity on a large scale, although recent advances in the use and storage of solar energy may resolve the latter in the near future (Hiscox et al. 2012).

The possible benefit of impregnating the eave screening material with a repellent would be small and probably not weigh up to the extra costs. However, this does not
imply that repellents may not play a role in the control of malaria mosquitoes. On the contrary, repellents may still play a key role in reducing outdoor transmission in the peridomestic area. A concern regarding the use of topical repellents is the diversion of mosquitoes from repellent users to unprotected individuals (Maia et al. 2013). However, in an environment in which many traps are deployed, mosquitoes may be diverted to the traps instead. When, in addition, houses are screened, rendering the occupants inaccessible to endophagic mosquitoes, this effect would presumably be enhanced.

Both PMD and dUDL remain interesting candidates for future usage in this context. PMD is derived from the essential oil of *Corymbia citriodora* and is marketed as a natural alternative to DEET. PMD-based repellents have been shown to have an efficacy similar to that of DEET against mosquitoes of several genera, including vectors of human disease (Carroll & Loye, 2006 and references therein). dUDL was first identified in a structure-activity study on olfactory receptor proteins of *An. gambiae s.s.* (Pask et al. 2013) and was later shown to be a good repellent against anopheline and other mosquitoes in laboratory and (semi-)field settings (Menger et al. 2014a,b). It is a natural product that is also found in food sources and has an odour which is generally described as pleasant (Lin and Wilkens 1970, Mahajan et al. 2004). Microencapsulated dUDL or other repellents may be used to impregnate garments or could be added to soaps and shampoos. Repellents with a spatial effect may also provide a degree of protection to outdoor spaces such as cooking areas which are otherwise hard to protect.

Especially in areas where insecticide resistance is widespread, the introduction of eave screening, alone or in combination with attractant-baited traps, could provide an important addition to currently used vector control methods. When a significant proportion of malaria transmission occurs outdoors, the addition of topical and/or spatial repellents may contribute to enhance protection against mosquito biting. The efficacy of such integrated approaches to reduce malaria transmission should be determined in long-term field experiments, preferably in different ecosystems.
Chapter 6

Acknowledgements

The authors wish to thank the volunteers and house owners of Kigoche village who made this study possible. The study was funded by the COmON Foundation, The Netherlands.

References


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Chapter 6


Njie M, Dilger E, Lindsay SW, Kirby MJ (2009) Importance of eaves to house entry by anopheline, but not culicine, mosquitoes. *Journal of Medical Entomology* **46**: 505-510


Supplementary Table 1. Mean indoor CDC trap catches per house during baseline and intervention phase, experiment I.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49.4 ± 11.4</td>
<td>1.9 ± 0.8</td>
<td>27.5 ± 4.6</td>
<td>Suna trap + eave screening</td>
<td>1.4 ± 0.3</td>
<td>0.0 ± 0.0</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>16.5 ± 3.1</td>
<td>0.3 ± 0.2</td>
<td>19.0 ± 2.3</td>
<td>Control</td>
<td>19.8 ± 2.3</td>
<td>1.0 ± 0.3</td>
<td>12.2 ± 1.7</td>
</tr>
<tr>
<td>3</td>
<td>44.3 ± 8.7</td>
<td>2.1 ± 0.7</td>
<td>29.4 ± 13.2</td>
<td>Eave screening</td>
<td>3.4 ± 1.1</td>
<td>0.04 ± 0.04</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>45.6 ± 8.1</td>
<td>0.4 ± 0.3</td>
<td>15.5 ± 3.1</td>
<td>Suna trap</td>
<td>40.9 ± 5.2</td>
<td>0.6 ± 0.2</td>
<td>9.8 ± 1.8</td>
</tr>
<tr>
<td>5</td>
<td>48.4 ± 9.7</td>
<td>4.1 ± 0.9</td>
<td>44.5 ± 8.8</td>
<td>Eave screening</td>
<td>3.8 ± 0.8</td>
<td>0.0</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>6</td>
<td>12.9 ± 4.5</td>
<td>3.8 ± 1.8</td>
<td>15.1 ± 3.5</td>
<td>Suna trap + eave screening</td>
<td>9.5 ± 3.1</td>
<td>0.7 ± 0.2</td>
<td>7.2 ± 1.8</td>
</tr>
<tr>
<td>7</td>
<td>24.1 ± 5.2</td>
<td>2.9 ± 1.0</td>
<td>17.3 ± 4.6</td>
<td>Suna trap + eave screening</td>
<td>4.3 ± 1.2</td>
<td>0.1 ± 0.1</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>8</td>
<td>12.0 ± 3.4</td>
<td>1.4 ± 0.5</td>
<td>9.5 ± 2.4</td>
<td>Control</td>
<td>10.6 ± 1.6</td>
<td>1.1 ± 0.3</td>
<td>7.0 ± 1.3</td>
</tr>
</tbody>
</table>

Values indicate mean ± SEM, based on n = 8 trapping nights for the baseline phase and n = 24 trapping nights for the intervention phase.
Supplementary Table 2 (continues on the next page). Mean indoor CDC trap catches per house\(^1\) during baseline and intervention phase, experiment II.

<table>
<thead>
<tr>
<th>House no.(^1)</th>
<th>An. funestus</th>
<th>An. arabiensis</th>
<th>Culex spp.</th>
<th>Treatment(^2)</th>
<th>An. funestus</th>
<th>An. arabiensis</th>
<th>Culex spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1)</td>
<td>23.1 ± 4.2</td>
<td>10.1 ± 2.2</td>
<td>3.3 ± 0.6</td>
<td>Eave scr PMD</td>
<td>0.2 ± 0.2</td>
<td>0.33 ± 0.14</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>2 (2)</td>
<td>5 ± 0.9</td>
<td>1.4 ± 0.5</td>
<td>5.8 ± 2.3</td>
<td>Eave scr PMD</td>
<td>2.25 ± 0.52</td>
<td>0.42 ± 0.15</td>
<td>1.8 ± 8.2</td>
</tr>
<tr>
<td>3 (3)</td>
<td>10.2 ± 2.9</td>
<td>3.3 ± 0.5</td>
<td>11.6 ± 3.8</td>
<td>Control</td>
<td>6.83 ± 1.22</td>
<td>2.08 ± 0.48</td>
<td>6.0 ± 3.0</td>
</tr>
<tr>
<td>4 (4)</td>
<td>24.8 ± 5.0</td>
<td>2.0 ± 0.5</td>
<td>6.6 ± 1.4</td>
<td>Eave scr</td>
<td>5.25 ± 1.71</td>
<td>0.25 ± 0.18</td>
<td>2.8 ± 1.7</td>
</tr>
<tr>
<td>5 (-)</td>
<td>2.3 ± 0.4</td>
<td>1.9 ± 0.5</td>
<td>1.7 ± 0.4</td>
<td>Eave scr dUDL</td>
<td>1.33 ± 0.62</td>
<td>0.58 ± 0.29</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>6 (-)</td>
<td>4.3 ± 0.6</td>
<td>2.8 ± 0.5</td>
<td>0.9 ± 0.3</td>
<td>Eave scr</td>
<td>2.25 ± 0.68</td>
<td>1.17 ± 0.39</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>7 (-)</td>
<td>4.1 ± 1.0</td>
<td>2.3 ± 0.4</td>
<td>0.8 ± 0.4</td>
<td>Control</td>
<td>3.75 ± 1.07</td>
<td>1.83 ± 0.51</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>8 (5)</td>
<td>8.1 ± 1.4</td>
<td>3.3 ± 0.9</td>
<td>2.4 ± 0.3</td>
<td>Control</td>
<td>8.83 ± 2.51</td>
<td>4.1 ± 1.2</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>9 (-)</td>
<td>4.3 ± 0.8</td>
<td>4.8 ± 0.9</td>
<td>1.4 ± 0.5</td>
<td>Eave scr</td>
<td>1.83 ± 0.66</td>
<td>1.4 ± 0.6</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>10 (-)</td>
<td>17.5 ± 5.0</td>
<td>2.0 ± 1.0</td>
<td>1.9 ± 0.7</td>
<td>Eave scr dUDL</td>
<td>5.17 ± 1.57</td>
<td>0.1 ± 0.1</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>11 (7)</td>
<td>12.2 ± 2.3</td>
<td>5.7 ± 1.7</td>
<td>2.1 ± 0.5</td>
<td>Eave scr dUDL</td>
<td>2.92 ± 0.90</td>
<td>1.0 ± 0.4</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>12 (8)</td>
<td>3.9 ± 0.6</td>
<td>2.7 ± 0.7</td>
<td>0.8 ± 0.3</td>
<td>Eave scr PMD</td>
<td>0.42 ± 0.23</td>
<td>0.0</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Values indicate mean ± SEM, based on n = 12 trapping nights for each phase: baseline phase, intervention phase without MMX and intervention phase with MMX.

\(^1\)House no. in brackets refers to the number the house had in field experiment I.

\(^2\)Eave scr: Eave screening.
Supplementary Table 2 (continuation of the previous page). Mean indoor CDC trap catches per house\(^1\) during baseline and intervention phase, experiment II.

<table>
<thead>
<tr>
<th></th>
<th>Intervention phase with MMX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>An. funestus</td>
</tr>
<tr>
<td>1.2 ± 0.5</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>2.6 ± 0.5</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>7.2 ± 1.2</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>4.5 ± 1.2</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>2.9 ± 1.1</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>2.6 ± 0.7</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>3.7 ± 1.2</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>6.0 ± 1.0</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>1.6 ± 0.7</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>5.3 ± 1.5</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>4.8 ± 1.5</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Values indicate mean ± SEM, based on n = 12 trapping nights for each phase: baseline phase, intervention phase without MMX and intervention phase with MMX.

\(^1\)House no. in brackets refers to the number the house had in field experiment I.

\(^2\)Eave scr: Eave screening.
Supplementary Figure 1. Eave screening with wire mesh, experiment I. Wire mesh was applied from the outside. It was first fixed to the lower part of the eave and then stretched upwards towards the corrugated iron sheet roof and clamped around the wooden beams supporting the roof. Gaps between the wooden beams and the wire mesh were filled with cotton wool. To help stretch the wire mesh and hold it flush against the roof, wooden sticks were placed into the eave at regular intervals.
Supplementary Figure 2. Eave screening with cotton net fabric, experiment II. The fabric was fixed using a staple gun, and rather than applying it to cover only the eave it was stapled to the wooden beam that forms the top of the wall and then stretched outwards and fixed on the outermost wooden beam which supports the roof.
Chapter 7

General Discussion

David J. Menger
With 198 million cases and around 600,000 deaths per year, the battle against malaria is long but over (WHO 2014a). Over the last decade, much progress has been made with improved diagnostics and artemisinin-based combination treatments in conjunction with insecticide-based vector control methods (White et al. 2013). However, it now becomes increasingly clear that the main tools, insecticide treated nets (ITNs) and indoor residual spraying (IRS), will not be sufficiently effective for malaria elimination in all regions and under all circumstances (malERA 2011, Killeen 2014). Some vector species feed and/or rest outdoors, which makes it hard to target them with methods that focus on the intra-domiciliary environment (Besansky et al. 2004). Other malaria vector species display opportunistic feeding behaviour, taking blood from animal hosts besides humans (Takken and Verhulst 2013). In addition, ITNs and IRS face widespread physiological resistance of mosquitoes against all main classes of insecticides, while the enormous selection pressure they cause results in changes in host-seeking behaviour of originally endophilic, nocturnal vectors as well as changes in the composition of vector populations (Van den Berg 2009, Ranson et al. 2011, Reddy et al. 2011, Russell et al. 2013, Lwetoijera et al. 2014). Therefore, additional tools are required in areas where malaria transmission is high despite high coverage with ITNs and IRS and/or vector populations have reduced susceptibility to insecticide-based methods.

In this thesis, the use of push-pull tactics to control malaria mosquitoes through behavioural disruption, and thereby reduce malaria transmission, has been investigated. The research focussed on a push-pull system that functions through the simultaneous use of repellent and attractive volatiles which interfere with the mosquito’s host-seeking behaviour, reduce human-vector contact and deplete vector populations. Such a system would be complementary to the main insecticide-based methods while addressing the challenges mentioned above. I have described how existing tools could potentially be combined in such a push-pull system, identified novel repellents in the laboratory, tested a prototype push-pull system in a semi-field setup, improved the system and evaluated its functioning in a malaria endemic field setting and compared and combined the push-pull concept with the existing practice of eave screening.

The main conclusions of this work are: (1) it is possible to reduce house entry of malaria and other mosquitoes using (spatial) repellents and/or attractant-baited traps; (2) the effect of repellents on house entry is larger and more consistent than
the effect of attractant-baited traps; (3) the main function of the attractant-baited traps is to deplete mosquito populations through removal trapping; (4) the attractive and repellent components of the push-pull system complement each other and there is no or very little interaction between them; (5) a push-pull system based on repellent and attractive volatiles can be expected to reduce malaria transmission through a strong decrease of the entomological inoculation rate; (6) eave screening is a highly efficient method to reduce house entry of malaria and other mosquitoes and increases outdoor trap catches, while there is little added value in impregnating screening material with a repellent.

Based on the experimental work described in this thesis and on the outcomes of previous research on repellents, odour-baits, trapping devices and the development of resistance, there are a number of considerations to be taken into account regarding the selection, development and use of push-pull tools against malaria vectors.

Successfully repelling a mosquito from a potential host also means depriving it of a blood meal. Therefore, high coverage with a repellent may decrease the reproductive success of affected mosquitoes and result in a selective force favouring mosquitoes which are less sensitive to the specific compound. Variation in sensitivity to DEET (N,N-diethyl-3-methylbenzamide) has been reported for *Ae. aegypti* and has a genetic basis (Stanczyk et al. 2010). Although the selection pressure exercised by a repellent would be less than that of an insecticide, which in a sufficient dose would kill the mosquito and thus exclude any further reproduction, it may prove a problem in the practical use of push-pull systems. As a precaution it would therefore be advisable to use repellent formulations containing more than one active compound if area-wide coverage is considered. However, this would only reduce the risk of selecting for insensitivity if the included compounds act on different molecular targets. For a long time the mode of action of popular mosquito repellents, especially DEET, has been a matter of debate (Kain et al. 2013, DeGennaro et al. 2013, Syed and Leal 2008). However, a recent publication by Xu et al. (2014) convincingly showed that the mode of action of not only DEET, but also of picaridin, IR 3535, and PMD (p-menthane-3,8-diol) is activation of one specific odorant receptor (labelled OR136 in the southern house mosquito, *Cx. quinquefasciatus*). As mosquitoes of different genera react similarly to these compounds (Costantini et al. 2004, Badolo et al. 2004, Carroll and Loye 2006), the underlying molecular mechanism may also be similar for these
species (Bohbot et al. 2010, Bohbot and Dickens 2012). In An. gambiae DEET is a ligand of OR40 (J.R. Carlson, unpublished results), whereas Pask et al. (2013) demonstrated that it is AgOR48 which has a high binding affinity to delta-decalactone, delta-undecalactone (dUDL) and delta-dodecalactone. This implies that the repellent dUDL, which was used in most studies described in this thesis, would be a good candidate for inclusion in a blend with DEET, PMD, or one of the other popular repellents. Structure-activity studies could determine which ORs are activated by other compounds of which the repellent action has already been demonstrated (Bohbot et al. 2014). Future studies on potential new repellents should take into account the mode of action of the candidate compounds, in order to identify those which act on other molecular targets than existing products. Molecular techniques such as automated high-throughput screening of large numbers of compounds against a single OR could help to identify specific compounds that target an OR which is different from the ones activated by already identified compounds (Tauxe et al. 2013, Bohbot et al. 2014).

Besides taking into account the mode of action of the individual compounds in a repellent blend, a favourable safety record should be one of the compound’s essential features. Even if a repellent as a component of a push-pull system would not be applied topically, low concentrations of the volatile or vaporized compound may be inhaled during extended periods. It is for this reason, as well as for concerns regarding the spread of already developed resistance, that vaporized or volatile pyrethroids or DDT (dichlorodiphenyltrichloroethane) are not recommended for inclusion in a push-pull system aimed at malaria vectors (Aneck-Hahn et al. 2007, Koureas et al. 2012).

Although I have mainly focussed on selective forces which would render repellents less useful, the usage of repellents might trigger selective forces in favour of the human host population. If humans become even less accessible, opportunistic feeders such as An. arabiensis may be diverted to other host species such as livestock (mainly cattle, sheep and goats) instead. Individuals with a genetically based preference for non-human hosts may have an advantage over more anthropophillic individuals, which would promote the spread of their genes in the population. However, the inherent host preference of an individual mosquito may be overruled by its nutritional status as eventually the primary need is to get any blood meal in order to reproduce (Takken and Verhulst 2013).
The efficacy of attractant-baited trapping devices will depend on how their attractiveness compares with the attractiveness of the mosquito’s natural hosts. As with repellents, blends of attractants, rather than single compounds should be used, to avoid the development of insensitivity. Since a trapped mosquito dies a certain death, the selection for insensitive individuals would be stronger than in the case of repellents. Host-seeking behaviour, however, has been shown to depend on synergisms between attractants, implying that host seeking would be compromised if mosquitoes could not detect certain specific compounds (Smallegange et al. 2005). Moreover, most odour blends are constituted of a selection of the same compounds as those which are produced by humans. Mosquitoes which would not be attracted to these blends, may therefore also not be attracted to humans. Several multi-compound blends, with a high attractiveness and long-lasting effects, have already been developed (Mukabana et al. 2012, Mweresa et al. 2015, Van Loon et al. In Press).

Two remaining challenges in the large-scale deployment of traps are the dependence on electricity and the need for a continuous supply of carbon dioxide (CO₂). Most trapping devices require a source of electricity which is not necessarily present in many of the rural tropical regions where malaria is most prevalent. Solar energy may solve this matter in the future (Hiscox et al., 2012). If attractant-baited traps are to be employed for mass-trapping of host-seeking mosquitoes, CO₂ will most likely be an essential constituent of the attractive blend to lure mosquitoes into the vicinity of the traps (Qiu et al. 2007, Jawara et al. 2009). As outlined in Chapter 2, most sources of CO₂ are unsuitable for practical use at a large-scale, even low-tech methods based on the fermentation of sugar or molasses by yeast have limited practicality due to their labour-intensive preparation method. Further research on possible CO₂ mimics and field testing of identified candidate compounds such as 2-butanone would therefore be extremely relevant (Turner et al. 2011).

The principles of behavioural disruption on which push-pull tactics are based make the technique potentially suitable to target a wider selection of arthropod vectors of disease than malaria mosquitoes alone. Many disease vectors are bloodsucking insects that rely on olfactory cues to find their hosts. Besides Anopheles spp. which transmit malaria and in some areas lymphatic filariasis, these include other mosquito species, especially of the genera Aedes (dengue fever, yellow fever, chikungunya, Rift Valley fever) and Culex (lymphatic filariasis, West Nile fever, Japanese encephalitis) as
well as other dipterans such as sand flies of the subfamily Phlebotominae (leishmaniasis) and tsetse flies (Glossina spp., human and animal trypanosomiasis) (WHO 2014b). Presumably it would only be a small step to translate a push-pull system directed at malaria vectors to target Aedes spp. or Culex spp. The behaviour of several of the main vector species, e.g. Ae. aegypti, Ae. albopictus, Cx. quinquefasciatus and Cx. pipiens is reasonably well studied and attractive odour blends have been developed which can be used to trap host-seeking females (Molaei et al. 2006, Mathew et al. 2013, Cilek et al. 2011). Repellents such as DEET and PMD are effective against a wide variety of insects, albeit with varying efficacy (Costantini et al. 2004, Badolo et al. 2004, Carroll and Loye 2006). Also delta-undecalactone had a similar effect on Ae. aegypti as on An. gambiae in the laboratory (Chapter 3). Indeed, several authors have addressed the possibility of using push-pull tactics against Ae. aegypti to reduce dengue transmission (Paz-Soldan et al. 2011, Tainchum et al. 2013). Nevertheless, it should be borne in mind that vector species differ greatly in their ecology and behavioural patterns, including their blood feeding habits. Behavioural characteristics such as daytime feeding (e.g. Ae. aegypti, Scott et al. 2000) or a primary dependence on avian hosts (Culex spp., Molaei et al. 2006) may compromise the efficacy of attractant-baited traps and repellents and require innovative solutions. Push-pull tactics could not only be used to protect humans, but also to protect animal species. Repellent collars to keep tsetse flies away from cattle have been field-tested with limited success (Bett et al. 2010). However, recent research has identified repellent compounds from waterbuck body odour on the basis of which a multi-compound repellent blend was composed (a key-component of which, interestingly, is a lactone: delta-octalactone) (Bett et al. 2015). In a set of in vivo experiments including a live ox this blend reduced blood feeding by more than 90%. Such progress may well lead to the development of more effective repellent devices, which could be employed besides attractant-baited traps (Vale 1993).

Although push-pull tactics are potentially applicable against a wide variety of insect vectors of disease, there is no such thing as one silver bullet when it comes to vector control. Rather, it seems likely that future vector control strategies will consist of the integration of many different approaches, of which push-pull tactics may be one. Regarding malaria control, there are several methods, besides insecticide-based tools, which have also been shown to be successful in recent and historical programmes (Takken and Knols 2009, Alonso et al. 2011). Typically, these are measures that are knowledge-intensive, requiring detailed understanding of the ecology of vector
species and strong programme management. However, once such programmes are in place, they are more sustainable than insecticide-based methods, as they are not or less compromised by the possible development of resistance or insensitivity.

Environmental management is an approach which consists of the modification or manipulation of the environment to reduce the availability of vector habitats or make them less suitable, for example by filling or draining breeding sites (Ault 1994). In the past, vector control programmes that used environmental management have been highly effective in reducing malaria-induced morbidity and mortality (Keiser et al., 2005). Larval control by treating larval habitats with larvicides can also greatly reduce transmission, especially when the number of habitats and their spread is limited (Fillinger and Lindsay 2011). Although there are chemical larvicides, some of the most successful examples that are reported use the biological agent Bacillus thuringiensis israelensis (Bti), which produces several endotoxins and is highly selective (Federici et al. 2006, Ben-Dov 2014). Another method that has proven its value in different settings and regions is the screening of mosquito entry points in houses, e.g. by screening eaves and windows or by installing a ceiling (Lindsay et al. 2002). Such house improvements are often desired by inhabitants and have in some cases been shown to reduce anaemia, an indicator of malaria morbidity (Ogoma et al. 2009, Kirby et al. 2009).

Although many control programmes understandably prioritise increasing the coverage with ITNs, it is equally important to look at times and places when or where malaria is sustained through residual transmission, if the goal of elimination is to be met. This thesis shows the potential of a push-pull system to further reduce malaria transmission through a combination of house entry reduction and mass trapping of mosquitoes. However, from the field experiment described in Chapter 6, it also became clear that the added value of a repellent (dUDL or PMD) is very limited when eaves are already screened, since screening by itself proved to be very efficient as a mechanical barrier against mosquitoes trying to enter houses. In a vector-control programme in which house improvement is a component of the strategy, the release of a (spatial) repellent around the house would therefore not be advisable. However, repellents may still play a central role in control programmes, especially when outdoor transmission is concerned. Repellents have been shown to considerably reduce man-biting rates and although their impact on malaria prevalence is uncertain (Wilson et al. 2014), this may be explained by a diversion effect, i.e. non-users
receiving more bites when mosquitoes are diverted from users (Maia et al. 2013). A high density of attractant-baited traps to which mosquitoes may be diverted instead could tackle this complication. Moreover, attractant-baited traps reduce the vector population through removal trapping, which reduces indoor as well as outdoor biting. Further population reduction measures such as environmental management and larval control would complete a robust control programme.

By augmenting ITNs and/or IRS with a selection of complementary approaches such as improving housing, the use of topical or spatial repellents and attractant-baited traps, environmental management and larval control, it will be possible to mitigate the development of resistance while targeting vectors in different life stages, uncompromised by changing behavioural patterns and the changing composition of vector populations. This requires an integrated view on vector control, knowledge on the ecology of vectors and the political will to invest in programmes that focus on long term sustainable control.

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Chapter 7

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Summary

Malaria remains one of the deadliest infectious diseases and the most important disease that is transmitted by an arthropod vector: mosquitoes of the genus *Anopheles*. The efficacy of the main vector control tools, insecticide treated bed nets (ITNs) and indoor residual spraying (IRS), is compromised by the development of physiological and behavioural resistance in the target species and by changes in the species composition of vector populations. These developments underline the need to move away from interventions based on a single active compound and the reliance on insecticides. New strategies should be designed in such a way that they are complementary to existing methods, but less prone to the development of resistance; e.g. by using blends of active compounds, biological agents or mechanical measures. In this thesis, push-pull tactics, which would be complementary to existing methods are considered as a potential vector control tool that addresses some of the challenges named above. By the simultaneous use of repellent and attractive volatiles, a push-pull system disrupts the host-seeking behaviour of malaria mosquitoes in order to reduce human-vector contact and deplete vector populations.

Chapter 2 describes how the push-pull concept, originally designed for agricultural pest control, may be translated in a system that targets *Anopheles* mosquitoes. The chapter suggests how and which existing tools, such as repellents (push) and traps baited with attractive odour blends (pull) may be combined in a tool directed at malaria vectors. It is concluded that, within a push-pull system, there would be need for a safe, effective repellent that could provide protection at a spatial scale for a prolonged period of time. Developments like microencapsulation and the impregnation of fabrics with long-lasting formulations may yield repellent-based tools that can effectively be deployed in a push-pull system. Although little theoretical work has been done on the degree of trapping that will be required for population control, it is expected that trapping female mosquitoes at the stage when they are host-seeking or gravid is the most effective approach towards population control. To effectively reduce mosquito populations, baited traps should be able to compete with the mosquito’s actual hosts in terms of attractiveness. The importance of odour blends which exhibit similar or greater attractiveness than humans is highlighted. It is expected that an increased understanding of the host-seeking behaviour of malaria mosquitoes will lead to the development of more effective trapping devices in the future.
Summary

In Chapter 3, I present a new bioassay for the accurate assessment of candidate repellent compounds, using a synthetic odour that mimics the odour blend released by human skin. Using DEET (N,N-diethyl-meta-toluamide) and PMD (p-menthane-3,8-diol) as reference compounds, nine candidate repellents were tested, of which five showed significant repellency to the malaria mosquito *Anopheles coluzzii* (formerly *An. gambiae sensu stricto* M form). These included: 2-nonanone, 6-methyl-5-hepten-2-one, linalool, delta-decalactone and delta-undecalactone. The lactones were also tested on the yellow fever mosquito *Aedes aegypti* (*Stegomyia aegypti*), against which they showed similar degrees of repellency. It is concluded that the lactones are highly promising repellents, the more so because these compounds are pleasantly smelling, natural products that are also present in human food sources.

In Chapter 4 a push-pull system is presented that operates by the simultaneous use of repellent and attractive volatile odorants. Experiments were carried out in a semi-field setup; a traditional house which was constructed inside a screenhouse. The release of different repellent compounds from the four corners of the house resulted in significant reductions of 45% to 81.5% in house entry of host-seeking malaria mosquitoes. The highest reductions in house entry (up to 95.5%) were achieved by simultaneously repelling mosquitoes from the house (push) and removing them from the experimental setup using attractant-baited traps (pull). We conclude that reductions in house entry of malaria vectors, of the magnitude that was achieved in these experiments, would likely affect malaria transmission. Recommendations are provided for the practical implementation of the system in the field. This system may also be considered in areas where most of malaria transmission occurs outdoors, where it is expected to increase the efficacy of existing methods such as ITNs and IRS that do not target host-seeking mosquitoes outside the house.

In Chapter 5, the push-pull system is modified and taken to the field, where the effect on the house entry of wild mosquitoes is determined. The combination of a trap baited with a five-compound attractant and a strip of net-fabric impregnated with micro-encapsulated repellent and placed in the eaves of houses, was tested in a malaria-endemic village in western Kenya. Using the repellent delta-undecalactone, mosquito house entry was reduced by more than 50%, while the traps caught large numbers of outdoor flying mosquitoes. By adjusting an existing mathematical model, the impact of adding the push-pull system to existing vector-control tools on malaria transmission is predicted. Assuming area-wide coverage, the addition of a push-pull system to existing prevention efforts is predicted to result in up to 20-fold reductions in the entomological inoculation rate. Reductions of such magnitude are also
predicted when mosquitoes exhibit a high resistance against insecticides. I conclude that a push-pull system based on non-insecticidal volatiles provides an important addition to existing strategies for malaria prevention.

In Chapter 6, the effects of eave screening in combination with a push-pull system based on the release of a repellent and attractant-baited traps are quantified. Two field experiments in western Kenya showed that eave screening, whether or not in combination with an attractant-baited trap, was very effective in reducing the house entry of malaria mosquitoes. The effect size was variable between the different mosquito species and between the two experiments, but the reduction in house entry was always considerable (between 61% and 99%) and statistically significant. The effect of an outdoor, attractant-baited trap on house entry was not significant. However, the large number of mosquitoes trapped outdoors indicates that the attractant-baited traps could be used for removal trapping, which would enhance indoor as well as outdoor protection against mosquito bites. As eave screening was already very effective by itself, the addition of a repellent was of limited value. Nevertheless, repellents may play a role in reducing outdoor malaria transmission in the peridomestic area.

In the final chapter, the conclusions of this thesis are summarized and a number of considerations regarding the use of push-pull techniques is addressed. Firstly, the issue of selection for insensitivity to the used compounds is discussed, as well as methods how to manage this. Furthermore, it is described how the principles of behavioural disruption on which push-pull tactics are based make the technique potentially suitable to target a wider selection of arthropod vectors of disease than malaria mosquitoes alone.

Finally, I express the expectation that future vector control strategies will consist of the integration of many different approaches, of which push-pull tactics may be one. Several other vector control tools, besides ITNs and IRS, which have also been shown to be successful in recent or historical programmes are discussed. It is concluded that by integrating different approaches, it will be possible to mitigate the development of resistance while targeting vectors in different life stages, uncompromised by changing behavioural patterns and changes in the composition of vector populations. This would require an integrated view on vector control, knowledge on the ecology of vectors and the political will to invest in programmes that focus on long term sustainable control.
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When I started as a research assistant at Entomology nearly four years ago, I did not expect that my work on malaria mosquitoes would grow into a PhD thesis. Part of the reason that it did, is that during these years numerous people contributed, in many different ways, to the realization of this effort. Perhaps even more importantly, they made it enjoyable.

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David
Curriculum Vitae

Since David Jan Menger began his journey on earth, now nearly 30 years ago, he has explored reality in a variety of ways. He first came into contact with the scientific approach as a pupil at the Bonhoeffer College in Enschede. As it was clear that his greatest interest was the living nature, he decided to focus on how our understanding thereof can help us to answer the challenges of our age. Therefore, he started to study general biology at Wageningen University in 2005. For his thesis he explored the concept of Analogue Forestry: an agroforestry-based concept that aims to functionally restore tropical forest ecosystems while providing local communities with a means of existence. On the side he took a minor in philosophy & ethics, fuelling his interest in thinking about thinking by learning about the great thinkers of the past and present. After obtaining his BSc, he continued with an MSc programme to specialise in terrestrial ecology. For his thesis he spent three months in Ethiopia to do fieldwork on the endangered tree species *Boswellia papyrifera*; the thesis formed the basis for a publication in Annals of Botany. On the side he took a minor in education that provided him with basic teaching experience in secondary schools. For his internship at Stichting ARK he worked on a nature development & ecotourism project in southeast Bulgaria. After graduating, he worked as a research associate at Wageningen University, exploring novel, ecology-based methods to control mosquitoes in order to prevent the transmission of mosquito-borne infectious diseases such as malaria. It resulted in the PhD thesis which you are now holding in your hands.
List of publications


