Odour-based strategies for surveillance and behavioural disruption of host-seeking malaria and other mosquitoes

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This research was conducted under the auspices of the C.T de Wit Graduate School for Production Ecology and Resource Conservation.

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Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof Dr M.J. Kropff,
in the presence of the
Thesis committee appointed by the Academic Board
to be defended in public
on Tuesday 20 May 2014
at 4 p.m. in the Aula.

Collins K. Mweresa
Odour-based strategies for surveillance and behavioural disruption of host-seeking malaria and other mosquitoes,
278 pages.

PhD thesis, Wageningen University, Wageningen, NL (2014)
With references, with summaries in Dutch and English

ISBN 978-90-6173-924-7

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Abstract

Malaria is one of the most important diseases that threaten the lives of a large proportion of the human population in the tropics. Currently, the application of novel technologies for malaria control is encouraged to provide reliable and robust tools for monitoring of anopheline vectors and to reduce the application of chemical insecticides. This thesis has investigated the potential of using odour-based strategies for surveillance and behavioural disruption of host-seeking malaria and other mosquitoes. All studies were done under semi-field and field conditions in western Kenya.

A study conducted in a semi-field enclosure demonstrated that nylon strips performed significantly better than low density polyethylene (LDPE) material in dispensing synthetic mosquito attractants up to 40 consecutive nights after treatment. The treated nylon strips attracted more *Anopheles gambiae sensu stricto* than LDPE sachets containing similar attractants up to one year post-treatment when re-used once a week. Additional volatile organic compounds and different bacterial populations were found on attractant-treated nylon strips after one year of intermittent exposures. Autoclaving of treated nylon strips prior to deployment did not affect their attractiveness for mosquito populations. Subsequent field results indicated that attractant-treated nylon strips and LDPE sachets deployed at weekly intervals remain attractive to indoor and outdoor host-seeking malaria vectors up to one year post-treatment. Indoor studies demonstrated that synthetic odour baits can be used to replace a human as a host stimulus in mosquito sampling devices. The interaction between visual and olfactory cues can present a more robust and reliable tool for sampling malaria and other mosquito vectors than either stimulus alone. *Anopheles arabiensis* was the most abundant member of the *An. gambiae* complex caught in Kigoche village, western Kenya. Unlike trapped *An. gambiae* s.l., a large proportion of trapped *An. funestus* had fed more on humans than bovines. One *An. funestus* tested positive for *P. falciparum* sporozoites. Addition of selected concentrations of 1-butylamine, 1-dodecanol or 2-pentadecanone to the Mbita blend + CO_2 released by fermenting sugar increased the catches of indoor-biting *An. gambiae* s.l., *An. funestus* and *Culex* mosquitoes. The majority of female *An. gambiae* s.l. caught were either unfed or gravid while *An. funestus* were predominantly unfed suggesting that the odorant cues attracted different fractions of both species. Fermentation of molasses instead of sugar provided a sustainable and additional source of CO_2 suitable for incorporation in odour baits for sampling unfed and blood-fed malaria mosquitoes. The use of locally available and regularly used textiles (i.e., cellulose + polyacrylate, cotton and polyester material) offered a similar or more efficient matrix for dispensing synthetic attractant odorants of malaria vectors compared to nylon.

In general, the high numbers of mosquitoes caught indoors and outdoors imply that deployment of odour-baited technology may provide a complementary and sustainable tool

for monitoring and possibly control of multiple malaria vectors having different abdominal conditions (i.e., unfed, blood-fed or gravid). This is likely to contribute to a reduction in mosquito bites, and transmission of malaria and other mosquito-borne diseases.

Chapter 1

General Introduction

Malaria burden

Malaria is one of the most life-threatening human diseases transmitted by infected female *Anopheles* mosquitoes. The disease is caused by five protozoan parasites namely: *Plasmodium falciparum*, *P. malariae, P. ovale, P. vivax* and *P. knowlesi* (Bigoga et al., 2007, Cox-Singh et al., 2008). Although *P. knowlesi* is a simian parasite, it was recently reported to infect humans in Malaysia and other parts of South East Asia (Cox-Singh et al., 2008). *Plasmodium falciparum* is responsible for the most severe morbidity and mortality in the world especially in sub-Saharan Africa (Kiszewski et al., 2004, Snow et al., 2005). Globally, one third of the human population lives in malaria-endemic areas where the disease remains a serious obstacle for development (Sachs and Malaney, 2002). Of the estimated 216 million episodes of malaria in 2010, approximately 81% of the cases were in the African region (WHO, 2011). Over the same year, an estimated 655,000 malaria deaths occurred globally, of which 91% were in Africa, and approximately 86% of these were children below five years of age (WHO, 2011, WHO, 2012). Thus, malaria-endemic areas are strongly correlated with a vicious cycle of disease, poverty and low rates of economic growth, but in Africa this situation is also catalysed by lack of affordable healthcare and poor infrastructure (Sachs and Malaney, 2002, Breman et al., 2004, Chuma et al., 2010).

Major malaria vectors

Human *Plasmodium* parasites are transmitted by members of the anopheline mosquito family. The mosquitoes have evolved with the *Plasmodium* parasites to establish a unique association characterized by periodic blood feeding on *Plasmodium*-susceptible hosts (Cator et al., 2012). The life cycle of human *Plasmodium* parasites relies on female *Anopheles* mosquitoes as a definitive host and humans as an intermediate host (Beier, 1998, Bigoga et al., 2007). Unlike in temperate regions, climatic conditions in most tropical areas including sub-Saharan Africa are favourable for the development of malaria parasites and survival of efficient malaria vectors (Sachs and Malaney, 2002, Snow et al., 2005, Stresman, 2010). Of the approximately 500 species of anophelines, human malaria parasites are transmitted by more than 30 anopheline mosquito species with diverse breeding and feeding habits (Kiszewski et al., 2004, Alonso et al., 2011). In Africa, the most important vectors are *An. funestus* Giles, and two members of the *An. gambiae* Giles complex (*An. gambiae* Giles *sensu stricto* (henceforth termed as *An. gambiae*) and *An. Arabiensi*s Patton (Coetzee et al., 2000, Bigoga et al., 2007). Nonetheless, *An. melas* Theobald*, An. merus* Donitz*, An. bwambae* White*, An. nili* Theobald*, An. moucheti* Evans, and *An. pharoensis* Theobald are regarded as secondary local vectors (Githeko et al., 1996, Onyabe and Conn, 2001, Dia et al., 2003, Bigoga et al., 2007). Recently, *An. coustani* Laveran and a novel *Anopheles* mosquito were reported as potential malaria vectors in East Africa (Geissbühler et al., 2009, Stevenson et al., 2012).

Whereas *An. gambiae* is predominantly anthropophilic, endophilic, endophagic and widely distributed in wet, humid and high altitude areas, *An. arabiensis* is an opportunistic and exophilic feeder that thrives in drier low altitude areas, though both species are sympatric in much of tropical Africa (Koenraadt et al., 2004, Imbahale et al., 2012). *Anopheles funestus* is a largely anthropophilic and endophagic vector that plays a more significant role during dry seasons and in areas with irrigated rice farming (Githeko et al., 1996, Takken and Knols, 1999). The resting and feeding behaviour of *An. gambiae* and *An. funestus* enhances a strong mosquito-host interaction. This makes them to search for blood in areas of high human density thereby favouring malaria transmission. A combination of such characteristics with a relatively high survival rate and innate susceptibility to *Plasmodium* infection presents both mosquitoes as the most important vectors of malaria in Africa (Charlwood et al., 1997, Takken and Knols, 1999).

There is a significant intraspecific variation in behavioural responses of *An. arabiensis* and *An. gambiae* to different host species, resting or feeding behaviours and *Plasmodium* infectivity (Bøgh et al., 2001, Killeen et al., 2001, Takken and Verhulst, 2013). These variations are attributed to genetic polymorphism, host availability and climatic factors (Snow, 1987, Touré et al., 1998, Lefèvre et al., 2009, Riehle et al., 2011). Recently, a previously unknown subgroup of exophilic *An. gambiae* that is highly susceptible to *P. falciparum* infection and sympatric with the known endophilic *An. gambiae* in the arid savanna zone of West Africa was identified in Burkina Faso (Riehle et al., 2011). This discovery confirms that reliance on indoor catches of mosquitoes, particularly *An. gambiae*, for epidemiological studies is an unbiased method as *An. gambiae* is thought to be ónaturally endophilicô. However, outdoor resting and outdoor biting behaviours have been reported and associated with the current increase of outdoor transmission of malaria especially in areas where insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) are intensively used (Bayoh et al., 2010, Russell et al., 2011, Govella and Ferguson, 2012). Because collection methods for exophilic mosquitoes are much less efficient than for indoor resting mosquitoes, more reliable tools for sampling both indoor and outdoor resting or biting malaria vectors in various abdominal conditions (i.e. unfed, blood-fed and gravid) should be sought (Mboera, 2005, Mahande et al., 2007, Bentley et al., 2009).

Malaria transmission

Gametocytes and sporozoites are the transmissible stages of *Plasmodium* spp. of malaria between mosquitoes and humans (Beier, 1998). During periodic blood feeding, susceptible malaria vectors may ingest gametocytes from infected human hosts or inject sporozoites if infected (Figure 1) (Su et al., 2007).

Upon ingestion of an infected blood meal, gametocytes undergo a series of developmental

stages to form sporozoites, which render host-seeking malaria vectors infectious to humans during subsequent blood meals (White, 1974, Beier, 1998). In humans, sporozoites develop sequentially in the liver and blood to form gametocytes. Evidence shows that human hosts infected with the gametocyte stage of *Plasmodium* parasites are more attractive to *An. gambiae* than those with asexual stages (Lacroix et al., 2005, Mukabana et al., 2007). Similarly, *An. gambiae* infected with *Plasmodium* sporozoites probe and blood-feed more frequently than when they are infected with oocytes or not infected at all (Koella et al., 2002, Cator et al., 2012). This implies that any mechanism that reduces contact between mosquitoes and humans may contribute substantially to disruption of sporozoite and gametocyte transmission thereby reducing malaria risk.

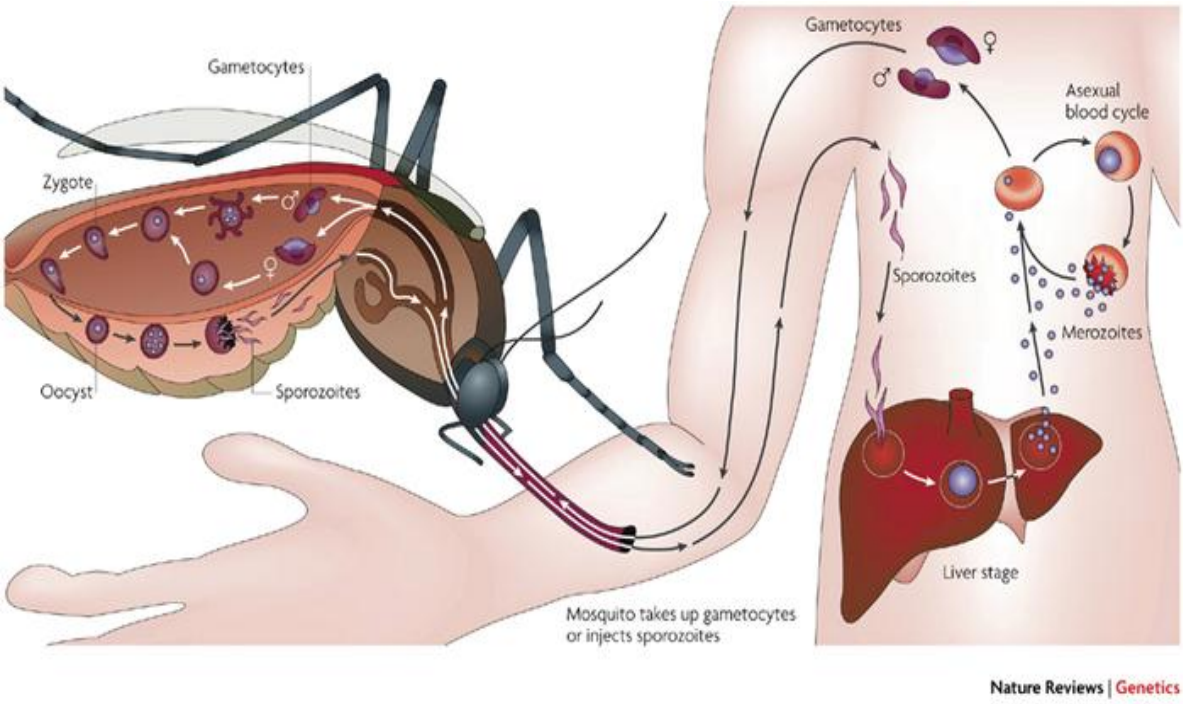


Figure 1: Life cycle of *Plasmodium falciparum* showing stages of development within a mosquito and a human (Su et al., 2007).

Host-seeking behaviour of malaria vectors

Host-seeking mosquitoes rely on interactions of olfactory, visual and physical stimuli to locate potential blood meal sources through a complex sequence of responses summarized as long-range, middle-range, and short-range (Gibson and Torr, 1999, Day, 2005). Of the stimuli, host-derived olfactory cues are the most important (Takken, 1991); however, physical cues like moisture and temperature seem to enhance responses of *Anopheles* mosquitoes to host odours (Takken and Knols, 1999, Olanga et al., 2010). Further interaction of body temperature, relative humidity and wind affects detection and location of host odour cues by creation of convection currents and structure of the odour plume to which mosquitoes respond

(Cardé and Gibson, 2010). On the other hand, the use of vision by diurnal and nocturnal species is dependent on spectral sensitivity, the blue-green range being preferred by diurnal *Aedes* and *Culex* spp. (Day, 2005, Bentley et al., 2009). It has been suggested that during flight, visual cues aid mosquitoes to orient upwind even under low-light conditions (Snow, 1987, Gibson and Torr, 1999). It is on this basis that different wavelengths of light are used as bait for monitoring haematophagous insects (Joshi et al., 1975, Mboera, 2005, Bentley et al., 2009).

Generally, long- and medium-range orientation is mediated by host-specific olfactory cues (Snow, 1987, Takken, 1991). At long range, mosquitoes receive and evaluate olfactory cues contained in volatile organic compounds (VOCs) released from the skin and breath of potential hosts (Takken, 1991, Day, 2005). Field studies have demonstrated that a human-derived odour plume influences long-range attraction of *An. gambiae* s.l., *An. funestus,* and *Culex quinquefasciatus* Say (Knols et al., 1995). Of the human-derived odorants, carbon dioxide (CO_2) activates mosquitoes to fly upwind (i.e. odour-mediated anemotaxis) and guides them towards the odour source (Gillies, 1980, Snow, 1987). It is also evident that CO_2 is a more effective kairomone for haematophagous dipterans when augmented with natural or synthetic odour cues of human or vertebrate origin, but this has a dose-dependent relationship as it varies among species (Costantini et al., 1996, Spitzen et al., 2008, Smallegange et al., 2010). During orientation, the concentration of olfactory cues diminishes at the edge of the plume and increases toward the centre and host (Cardé and Willis, 2008, Cardé and Gibson, 2010).

As mosquitoes get into closer proximity with the host, vision, heat, relative humidity, host movements, and skin odorants including those produced by skin microbial activity aid them to identify suitable hosts prior to probing and blood feeding (Takken and Knols, 1999, Day, 2005, Verhulst et al., 2011a). However, there is no consensus on precise distance ranges within which these cues influence mosquito behaviour, hence the need for further studies. Although host location and selection may depend on relative abundance and accessibility of individual hosts, selection of biting sites by members of the *An. gambiae* complex is guided by convection currents and mediated by host odours including CO_2 (Dekker et al., 1998, Dekker et al., 2002, Mukabana et al., 2004). Recent studies have demonstrated that close-range orientation behaviour of *An. gambiae* is primarily driven by host-related odour cues while relative humidity and temperature play a minor role (Olanga et al., 2010, Spitzen et al., 2013). As a result, a high degree of host preference among malaria vectors is primarily mediated by host odour profiles (Takken and Verhulst, 2013). Indeed, mosquito vectors that express strong and inherent host-selection behaviour are more important in disease transmission compared to opportunistic feeders (Day, 2005, Takken and Verhulst, 2013).

Host-seeking decision

Host-seeking decisions made by female mosquitoes depend on nutritional status and physiological stage (Scott and Takken, 2012, Takken and Verhulst, 2013). Mosquito age, body size, mating status, nutritional status, infection status and gonotrophic stage have been reported to affect temporal sensitivity of the olfactory system through expression of species-specific odour receptors (Takken and Knols, 1999, Zwiebel and Takken, 2004, Qiu and van Loon, 2010). For example, soon after emergence from the pupa, adult mosquitoes respond strongly to semiochemical cues released by nectar and mates within 24 - 48 h post-emergence (Foster and Takken, 2004). Female mosquitoes then engage in host-seeking behaviour and take a blood meal upon reaching a certain age, after which they do not respond to host-derived cues for the next 48 to 72 h until eggs have matured thereby inducing oviposition behaviour (Qiu and van Loon, 2010). However, variable results from previously related studies have been reviewed (Scott and Takken, 2012). In mosquitoes, odour receptors associated with semiochemical stimuli for host-seeking, mating, sugar feeding and oviposition are located on the antennae, maxillary palps and proboscis (Zwiebel and Takken, 2004, Qiu and van Loon, 2010). Male mosquitoes do not feed on blood and it is assumed that their odour receptors are used for detection and perception of sugar-feeding and mate-related cues, as very little is known about the extent to which they detect and use odours.

Differential attractiveness

The degree of attractiveness of human subjects to mosquitoes varies considerably, indicating that some individuals are at a greater risk of acquiring mosquito-borne pathogens because they are fed on more frequently than others (Lindsay et al., 1993, Dekker et al., 2002, Logan, 2008). These variations are mediated by signals attributed to differences in body odour profiles to which the olfactory system of mosquitoes responds (Takken and Knols, 1999, Zwiebel and Takken, 2004). These odorant signals are contained in human breath, sweat and other skin emanations (Takken and Knols, 1999, Verhulst et al., 2010). This subject has been reviewed (Takken, 1991, Kelly, 2001, Logan and Birkett, 2007) and evaluated in various studies (Lindsay et al., 1993, Lacroix et al., 2005, Logan et al., 2008). Evidence shows that differential attractiveness of humans to female *Anopheles* mosquitoes is influenced by both intrinsic and extrinsic factors (Qiu and van Loon, 2010, Takken and Verhulst, 2013). Such factors include host age, gender, size, surface area and weight, diet, alcohol consumption, pregnancy status, *Plasmodium* parasite infection status, ABO blood group type, activity and health status. Currently, it is not clear whether odour profiles associated with ónattractiveô individuals have evolved from selective pressures against potential disease vectors or whether they are by-products of underlying metabolic processes. Nonetheless, unattractive odour profiles protect humans against infective and nuisance mosquito bites, and may contribute to higher fitness (Snow et al., 2005). Occurrence of large proportions of unattractive hosts within

a population exerts intense selection pressure on mosquitoes to feed preferentially on the most accessible and least defensive host (Kelly, 2001, Smith et al., 2006, Logan, 2008). Perhaps, this partly accounts for natural and modified host-seeking behaviours observed among malaria vectors, thereby influencing sources and quality of blood meals required for their Darwinian fitness (Takken and Verhulst, 2013).

Although compounds contained in body odour that determine differential behavioural responses of mosquitoes have not been fully identified and quantified, inclusion of larger sample sizes may enhance the accuracy of such studies (Logan, 2008). This is important as VOCs associated with health disorders may be used to develop novel diagnostic techniques. Studies have revealed that synergy between carbon dioxide and body odorants such as lactic acid, ammonia and carboxylic acids may elicit different behavioural responses in mosquitoes (Logan and Birkett, 2007, Smallegange et al., 2009, Smallegange and Takken, 2010). Further exploitation of differential attractiveness could reveal novel attractants or repellents for sampling and control of mosquito vectors. For example, individual volunteers ónattractiveô to *Aedes aegypti* L. and *An. gambiae* mosquitoes release higher quantities of three aldehydes (octanal, nonanal and decanal) and two ketones (geranylacetone and 6-methyl-5-hepten-2-one), than those who are óattractiveô (Logan et al., 2008). Whereas 6-methyl-5-hepten-2-one is thought to be associated with cattle stressed by fly attacks, its application on healthy cows as a slow-release formulation reduces fly loads (Birkett et al., 2004). This may provide a new class of repellents against human-biting mosquitoes. Aldehydes like octanal and nonanal are attractive to *Cx. quinquefasciatus*, but they reduce upwind flight and landing of *Ae. aegypti*. Currently, many kairomones involved in host-seeking behaviour of *Ae. aegypti*, *Cx. quinquefasciatus* and *An. gambiae* have been identified (reviewed by Smallegange and Takken, 2010). However, the majority of the compounds or blends do not exceed the attractiveness of natural human odour except for the Ifakara blend 1 (Okumu et al., 2010) and the Mbita blend (Mukabana et al., 2012). The Ifakara blend 1 consists of a mixture of ammonia, L-lactic acid, carboxylic acids and carbon dioxide while the Mbita blend comprises ammonia, L-lactic acid, tetradecanoic acid, 3-methyl-1-butanol and carbon dioxide.

Potential of using host odour cues to monitor and disrupt malaria transmission

Since the discovery of cues involved in insect vector-host interactions, odour-baited trapping systems augmented with visual and/or physical cues have been designed for surveillance and control of vector-borne diseases (Torr, 1994, Kline, 2007, Logan and Birkett, 2007) (see also Chapter 2). The control of tsetse flies by integration of odour-baited traps and visual targets in Africa remains the most successful example of applying host-seeking cues to monitor and control haematophagous disease vectors (Vale and Torr, 2004). Currently, studies associated with olfactory and host-seeking behaviour of malaria vectors are largely laboratory-based (Smallegange and Takken, 2010). However, such studies offer opportunities for exploring the

effectiveness of putative attractants and repellents under natural conditions (Mukabana et al., 2010). For example, field studies have demonstrated that behavioural responses of *An. gambiae* s.l. and *Mansonia* spp. to a blend of CO_2, lactic acid and ammonia are enhanced by addition of tetradecanoic acid, and this combination was more attractive compared to a light trap or a more complex synthetic blend (Smallegange et al., 2005, Jawara et al., 2011). Whereas human landing catches attracted more outdoor biting *An. arabiensis* than cattle, the presence of cattle outside a house occupied by humans caused 50% reduction in house entry by the same type of malaria vector (Tirados et al., 2011). In western Kenya, odour baits constituted from synthetic compounds associated with microbial activity on human skin were attractive to outdoor biting malaria and other mosquito vectors (Verhulst et al., 2011b). The potential of a standard blend of CO_2, ammonia, lactic acid and tetradecanoic acid to attract indoor-biting African malaria vectors was significantly increased by addition of 3-methyl-1-butanol (Mukabana et al., 2012), and this new bait was more attractive compared to a synthetic mosquito odour blend of 10 compounds augmented with CO_2 (Smallegange et al., 2009, Okumu et al., 2010). Moreover, the new bait also attracted as many *An. gambiae* s.l. as were attracted to human volunteers and significantly more *An. funestus* responded to the new bait than to humans.

Although behavioural responses of female mosquitoes to semiochemicals are physiological-state-dependent, human landing catches and light traps are predominantly attractive to unfed mosquitoes (Joshi et al., 1975, Costantini et al., 1998). This unveils an opportunity to search for novel potent odour baits for blood-fed mosquitoes as they may provide a more reliable tool for estimation of malaria transmission that is currently achieved by reliance on resting collections (Mboera, 2005, Mahande et al., 2007, Bentley et al., 2009). Kairomones released from cow urine, which were shown to protect cows against potential bites of tsetse flies and tabanids (Mihok and Mulye, 2010), also affect mosquitoes. Whereas VOCs derived from ageing cow urine were highly attractive to sub-gravid and gravid *An. arabiensis* (Mahande et al., 2010), interestingly these volatiles also served as oviposition site-preference cues for anopheline and culicine mosquitoes (Kweka et al., 2011). Because semiochemicals mediate host-seeking and oviposition behaviours, the search for novel attractants and formulations of such compounds is likely to provide an alternative solution for monitoring and intervention of malaria vectors in different phases of their reproductive cycle and thus, can provide an alternative to traditional mosquito collecting tools (Service, 1993).

Problem definition and justification

In the past decade, increased funding for malaria control has contributed to a reduction of malaria morbidity and mortality by using ITNs, IRS and effective anti-malarial therapies (WHO, 2012). The three strategies were estimated to reduce global incidence of malaria by 17% since 2000, and malaria-specific mortality rates by 26% (WHO, 2011). Whereas this is a

major achievement, the reductions are lower than the internationally agreed targets of 50% by 2010. This implies that malaria remains a huge burden in many developing countries especially in sub-Saharan Africa (Sachs and Malaney, 2002, Greenwood, 2009). The burden is largely attributed to potential threats from continued emergence and spread of parasite resistance to antimalarial medicines and mosquito resistance to insecticides (WHO, 2012). Furthermore, *Plasmodium* parasites that cause human malaria are transmitted by many anopheline species with diverse breeding, resting, feeding habits and vectorial capacities coupled with lack of standardised reliable tools for monitoring vectors in different abdominal conditions (Alonso et al., 2011). The reported changes from indoor to outdoor biting behaviours and outdoor malaria transmission result in different disease patterns in various epidemiological settings (Bayoh et al., 2010, Russell et al., 2011, Govella and Ferguson, 2012). These challenges suggest that a single strategy cannot be effective in all malaria-prone regions. As a consequence, strategies used for malaria disruption and surveillance should also target indoor and outdoor biting malaria vectors in various abdominal conditions (Alonso et al., 2011). Augmenting current vector management strategies with semiochemical-based technologies can contribute to achieving these goals (Zwiebel and Takken, 2004).

The potential of applying semiochemicals as a novel tool for monitoring and control of population densities of insect pests and vectors has been investigated and deployed in many systems with promising results, especially for insect pests of crops and for tsetse flies (Vale and Torr, 2004, Cook et al., 2007, Kline, 2007). In terms of action, selected semiochemicals affect insect behaviour and may result in attraction or repellence. Currently, perceived and modelled heterogeneities in malaria risk are associated with polymorphic human odour profiles (De Jong and Knols, 1995, Dekker et al., 2002, Smith et al., 2006, Verhulst et al., 2011) and genetic variability of host-seeking mosquitoes. Such studies provide a sound rationale for the development and deployment of effective odour-baited trapping systems for mosquito surveillance and control (Mukabana et al., 2010, Smallegange and Takken, 2010). This technology was successfully demonstrated to cause a reduction in mosquito populations in the U.S.A. (Kline and Lemire, 1998, Kline, 2007). Application of the technology increases the possibility of targeting specific species, reduces the use of broad-spectrum insecticides that lead to insecticide resistance and environmental damage (Fradin, 1998). However, deployment of odour-baited trapping systems in malaria-endemic areas is challenged by lack of a synthetic lure to malaria vectors that is more potent than natural human odour. This limitation can be overcome by searching for more effective and sustainable slow-release systems for odorants, potent synthetic mosquito attractant formulations or improvement of existing ones for collection of multiple malaria vectors (Smallegange et al., 2005), efficient trapping devices that rely on cheap or renewable sources of energy (Kline, 2006), alternative sources and more effective methods of delivering carbon dioxide and other volatile kairomones (Mukabana et al., 2010, Smallegange and Takken, 2010).

Research objectives

The principle goal of this PhD study is to address the practical applications of semiochemicals for the manipulation of haematophagous insect vectors, in particular African malaria mosquitoes, to evaluate residual activity of attractant dispensing systems and explore alternative possibilities for enhancing the effectiveness of odour-baited trapping systems for surveillance and disruption of malaria transmission in western Kenya.

Specific objectives

The objectives of experiments conducted during this study were to:

1. Evaluate low density polyethylene and nylon as matrix for dispensing synthetic mosquito attractants.
2. Investigate long-lasting activity of odour-dispensing nylon strips on attraction of mosquitoes and the potential role of microbes established on nylon strips.
3. Investigate long-lasting attraction of synthetic odour baits placed indoors and outdoors to wild malaria vectors in western Kenya.
4. Evaluate odour and light as stimuli for sampling malaria vectors in a rice agro-ecosystem of western Kenya.
5. Investigate if the attractiveness of blends of synthetic odours to African malaria vectors can be further enhanced with additional candidate odourant cues.
6. Investigate whether carbon dioxide and other volatile organic compounds produced by fermentation of molasses are attractive to African malaria vectors.
7. Evaluate alternatives to nylon as substrates for dispensing synthetic mosquito attractants.

Outline of the thesis

Chapter 1: Presents an introduction to the thesis, with a focus on malaria transmission and possibility of deploying host odour cues to monitor and disrupt parasite transmission. This chapter defines the research problem, gives justification of the research, and states the objectives.

Chapter 2: Provides a review of current knowledge on the potential of using semiochemicals to disrupt the behaviour of haematophagous insect vectors of medical and veterinary importance under field conditions with emphasis on mosquitoes. Published literature on identification of semiochemicals and how haematophagous insects detect and distinguish different odorant cues, release systems, approaches for deployment and placement of semiochemical traps and targets is reviewed.

Chapter 3: Effective application of synthetic odour baits is limited by lack of robust odour-dispensing substrates that perform more reliably under field conditions. The suitability of low

density polyethylene (LDPE) and nylon strips for dispensing synthetic attractants of host-seeking *An. gambiae* mosquitoes was evaluated in a semi-field facility measuring 11 m × 7 m × 2.8 m, with the roof apex standing 3.4 m high. The residual activity of attractant-treated nylon strips and LDPE sachets containing synthetic odour baits on mosquito catches was evaluated.

Chapter 4: The need for robustness underpins the deployment of odour-baited technologies for routine sampling, surveillance and control of malaria vectors. This may be achieved by selection and utilization of suitable slow-release systems that allow for a sustained release of attractants over a long period of time. The long-lasting attraction of host-seeking *An. gambiae* to odour-treated nylon strips deployed at weekly intervals for 52 nights after treatment was investigated in a semi-field setting. The treated nylon strips already used to attract host-seeking *An. gambiae* for 52 nights post-treatment were analysed for the presence of volatile organic compounds (VOCs) and microbes. Subsequently, behavioural responses of host-seeking *An. gambiae* to volatiles produced by microbes isolated from treated nylon were evaluated. Based on previous findings, the role of microbes on attraction of malaria mosquitoes to treated nylon was also validated under semi-field and field conditions.

Chapter 5: Building on the findings reported in Chapter 4, this longitudinal study investigated whether the sustainability, robustness and cost-effectiveness of deploying odour-baited tools for sampling outdoor and indoor biting *An. gambiae* s.l. and *An. funestus* in a rice agro-ecosystem over a similar period of time could be enhanced by slow release of individual synthetic attractants from nylon strips.

Chapter 6: Although systems used for sampling of adult Afrotropical malaria vectors are mainly baited with light, odour or both cues, their reliability and effectiveness are prone to variation; hence there is a need for a more standardised bait. The experiments presented in this chapter evaluated the potential of a synthetic odour blend, light and humans as stimuli for sampling indoor biting malaria vectors in a rice agro-ecosystem in western Kenya. Effect of stimlus on the numbers of male and female *An. gambiae* s.l. and *An. funestus* mosquitoes in different abdominal conditions was investigated. Blood meal sources as well as status of *Plasmodium falciparum* sporozoite rate in *An. gambiae* s.l. and *An. funestus* were investigated.

Chapter 7: One major challenge for the deployment of odour-baited trap technologies against host-seeking malaria vectors is to find a synthetic odour bait that exceeds the attractiveness of human subjects. The capacity of the Mbita blend (ammonia + L-lactic acid + tetradecanoic acid + 3-methyl-1-butanol + carbon dioxide) to attract host-seeking African malaria mosquitoes by addition of selected dilutions of butyl-2-methylbutanoate, 2-pentadecanone, 1-dodecanol or 1-butylamine was tested under semi-field and field conditions. The effect of these supplementary compounds on the attractiveness of the Mbita blend to indoor biting female mosquitoes, and *An. gambiae* s.l. and *An. funestus* in different abdominal conditions was evaluated.

Chapter 8: This chapter presents experiments addressing the possibility of replacing refined

cane sugar with locally available molasses as a substrate for producing CO_2 and VOCs used to lure malaria vectors into traps under semi-field and field conditions. The release rates of CO_2 produced by fermentation of different ratios of molasses and dry instant yeast, their effects on behavioural responses of *An. gambiae* and interaction with the Mbita blend on mosquito attraction was compared to CO_2 obtained from fermentation of refined cane sugar. The attractiveness of the Mbita blend augmented with CO_2 derived from refined cane sugar or molasses to outdoor biting mosquitoes, mainly *An. gambiae* s.l. and *An. funestus*, was tested. The effect of the synthetic odour blends on the response of mosquitoes in various physiological condition was studied by examination of the mosquitoesô abdominal status.

Chapter 9: The potential of locally available and regularly used absorbent materials to serve as release surfaces for synthetic attractants to malaria vectors was compared to nylon under semi-natural and natural conditions. The numbers of *An. gambiae,* outdoor biting mosquitoes, and *An. gambiae* s.l. and *An. funestus* in different abdominal conditions that responded to attractant-impregnated polyester, cotton, and cellulose + polyacrylate embedded within the sanitary pad material were compared to the number responding to attractant-impregnated nylon containing the same attractants.

Chapter 10: The results of this PhD study are discussed and summarised in relation to the specific objectives outlined above. Future directions in research about the potential of enhancing various components of odour-baited technology for sampling, surveillance and intervention of malaria vectors are highlighted.

Chapter 2

Potential application of semiochemicals for monitoring and manipulation of haematophagous insects: a review

To be submitted in a slightly modified form as: Mweresa, C.K., van Loon, J.J.A., Mukabana, W.R., Dicke, M. and Takken, W. Potential application of semiochemicals for monitoring and manipulation of haematophagous insects: a review

Abstract

Semiochemicals have been deployed successfully for monitoring and control of population densities of insect pests and vectors, notably in agriculture. Semiochemical cues influence behaviour of target insects through perception by the olfactory and/or gustatory system and subsequent processing in the central nervous system. This review provides an insight in the progress reached in recent decades on validation and application of semiochemicals as an alternative tool for surveillance and control of haematophagous insects with emphasis on mosquitoes. The review includes classification of semiochemicals, the insect olfactory system, semiochemical dispersal, slow-release systems, deployment and placement of traps and targets baited with semiochemicals, monitoring and control of haematophagous insects of medical and veterinary importance. The potential future use of semiochemicals for the control of haematophagous insects is discussed.

1.0. Introduction

Insects that bite and suck blood from humans and livestock are the cause of great distress and huge economic losses due to blood loss and the transmission of pathogens (Zaim and Guillet, 2002, Townson et al., 2005, Logan and Birkett, 2007). Traditionally, control and management of adult haematophagous insects and associated diseases have largely been achieved by use of broad-spectrum insecticides and drug therapy. Reliance on both strategies has in the recent decades been threatened by increasing damage to the environment, including effects on non-target organisms, and by insecticide and drug resistance (Gubler, 1988, Zaim and Guillet, 2002, Ranson et al., 2011); hence, there is a need for alternative and complementary technologies for surveillance and control. As a result, a wide range of biological strategies including semiochemical-based technologies are being explored. Semiochemicals have been successfully exploited for control of tsetse flies and crop pests (Cardé and Minks, 1995, Foster and Harris, 1997, Cook et al., 2007), and they are currently considered for monitoring and control of mosquitoes and other haematophagous insects (Logan and Birkett, 2007, Pickett et al., 2010).

Semiochemicals modulate insect physiology and/or behaviour through the olfactory system thereby evoking a stimulatory, inhibitory or deterrent effect on a specific behaviour (Dicke and Sabelis, 1988, Foster and Harris, 1997, Cook et al., 2007). Semiochemicals form an integral part of insect communication; these compounds can be specific and selective in action, and have not been associated with resistance or toxicity to non-target organisms and the environment (Cilek et al., 2003, Weinzierl et al., 2005, Kline, 2007). It is for these reasons that semiochemicals may be applied for disruption of contact between insect vectors and their mates, blood hosts, plant sugar sources, oviposition sites and other resources (Dicke and Sabelis, 1988, Takken and Knols, 1999, Zwiebel and Takken, 2004). The definition of semiochemicals has been expanded by Dicke and Sabelis (1988) to include toxins, nutrients and information conveying chemicals referred to as infochemicals. Thus, the response elicited can be beneficial or detrimental to the interacting organisms based on cost-benefit analysis (Table 1).

The history of tsetse control in certain parts of Africa remains the most outstanding example of successful exploitation of semiochemicals for the monitoring and control of haematophagous insects (Vale and Torr, 2004, Logan and Birkett, 2007, Hassanali et al., 2008). Since then, numerous studies aimed at deploying the same technology to monitor and control vectors of medical and veterinary importance have been conducted (Kline, 2006, Kline, 2007). Many putative behaviourally active compounds with potential for manipulation of selected target species have been identified. However, most studies were limited to laboratory and semi-field investigations (reviewed in Takken and Knols, 2010). The aim of

this review is to provide an insight into what is currently known about the potential of volatile semiochemicals for the disruption of behaviour of haematophagous insects under field conditions with emphasis on mosquitoes, thereby reducing the transmission of deadly diseases.

Table 1: Common semiochemical terminologies according to Dicke and Sabelis (1988)

Terminology	Definition
Haematophagous insects	Insects that suck vertebrate blood and this action may transmit diseases or cause distress/ harm
Infochemical	A chemical information emitted by a living organism to mediate an interaction between two individuals by evoking a physiological and/or behavioural response that benefit the emitter, recipient or both.
Pheromone	An infochemical that mediates an interaction between organisms of the same species.
Allelochemical	An infochemical that mediates an interaction between two individuals of different species.
Kairomone	An allelochemical that evokes a behavioural or physiological response that benefits the receiver but not the emitter.
Allomone	An allelochemical that evokes a behavioural or physiological response that benefits the emitter but not the recipient.
Synomone	An allelochemical that evokes a behavioural or physiological response that benefits both emitter and receiver. Herbivore-infested plants may produce volatiles that attract natural enemies of the herbivore in what has been termed as ña cry for helpò (Dudareva et al., 2006, Dicke, 2009)
Apneumones	Chemical volatiles emitted by non-living materials to evoke physiologically and/or behaviourally adaptive responses in receiving organisms

2.0. Isolation, identification and optimization of semiochemicals

Active semiochemical components are extracted, identified and evaluated through elaborate chemical, electrophysiological and behavioural analyses (Masson and Mustaparta, 1990, Logan et al., 2010). The components are trapped from air or extracted from plant, insect or vertebrate sources by solvent washing, solid-phase micro extraction (SPME) or vacuum distillation (McLeod et al., 2005, Logan et al., 2010). Volatile components of interest are identified and quantified by high-resolution gas chromatography (GC) and mass spectrometry (MS). Electrophysiologically active components derived from natural extracts are detected by electro-antennography and single sensillum recordings coupled with or without GC-MS (Qiu et al., 2004, McLeod et al., 2005, Qiu and van Loon, 2010). In this way volatiles that stimulate olfactory receptors on the antennae and/or maxillary palpi can be identified.

Electrophysiologically active compounds subsequently need to be subjected to behavioural

experiments in conjunction with their authentic analogues to ascertain whether they elicit responses in intact insects (Smallegange and Takken, 2010). Behavioural experiments are conducted in an olfactometer, wind tunnel and, large indoor cage setups prior to studies under semi-field and field conditions. Behavioural bioassays provide feed-back on the effect of concentrations of the infochemicals (Smallegange et al., 2005, Smallegange and Takken, 2010). Subsequently, field studies are essential for evaluating the efficiency of candidate cues, delivery systems and trap designs before they are deployed for monitoring and control of insect pests or vectors (Athanassioua et al., 2004, Kovanci et al., 2006, Kline, 2007, Mukabana et al., 2010).

2.1. Detection and perception of semiochemicals by the olfactory system

Remarkable progress has been made over the past decade in unravelling mechanisms of insect olfaction. Indeed, insects have evolved a highly sensitive and specific system of odour discrimination to deal with a widespread occurrence of olfactory stimuli encountered in nature (Carey and Carlson, 2011). They detect and select mates, oviposition sites and nutritional sources, in spite of being exposed to complex odour profiles from other environmental sources (Hansson, 2002, Zwiebel and Takken, 2004). The olfactory system of adult insects contains thousands of olfactory receptor neurons (ORNs) situated in the lumen of olfactory sensilla and bathed in sensillum liquid. In mosquitoes, ORNs are mainly located in the sensilla of the antennae, maxillary palpi and proboscis (Hansson, 2002, Kay and Stopfer, 2006, Benton, 2009). The walls of sensilla contain numerous pores through which olfactory cues diffuse into the lumen.

During perception of semiochemicals, volatile chemical stimuli enter through pores in the sensillum wall into the lymph and selectively bind to odour or pheromone binding proteins (OBPs or PBPs). Such proteins transport and deliver stimuli to the olfactory receptor proteins located in the dendrite membrane of ORNs and trigger an action potential when a minimum flux of semiochemical molecules is exceeded (Hansson, 1995, Hansson and Stensmyr, 2011).The action potential is transmitted along the axon of the olfactory receptor neuron to the glomerulus. Processing of incoming information occurs in the antennal lobe and is transmitted to higher brain centres for integration with other sensory inputs, thereby evoking appropriate physiological and behavioural response in the insect (Kay and Stopfer, 2006, Benton, 2009).

2.2. Discrimination of odour stimuli

The olfactory sensitivity and responses in insects are enhanced by structural, functional and location differences of OBPs and PBPs in the antennae and palpi for selective detection and transport of odour stimuli (Hansson, 1995, Wojtasek et al., 1998, Carey and Carlson, 2011).

Further variations in behavioural responses to odorants is associated with polymorphisms in OBP genes and odour receptors expressed by the ORN (Carey and Carlson, 2011). Discrimination of odour stimuli is also based on the size/volatility of the compound, position of double bonds, types and positions of functional groups on the compound (Masson and Mustaparta, 1990, Galizia and Rössler, 2010). The composition, concentration, and ratio of components in synthetic and natural odour blends play a vital role during selective detection and behavioural responses of insects to olfactory stimuli. In addition, speed of air flow contributes to temporal and spatial characteristics thereby resulting in different responses (Hansson, 1995). For example, an intermittent flow of odour(s) initiates and sustains flight or walking upwind towards the source in many insects like moths, tsetse flies and mosquitoes (Murlis et al., 1992, Kuenen and Cardé, 1994).

2.3. Odour dispersion and insect navigation in odour plumes

Insects locate resources by detection and use of wind direction to navigate along wind-borne odour plumes towards the source through odour-conditioned anemotaxis. Dispersion of odour in wind is dominated by turbulent diffusion that stretches and stirs odour filaments as they are released from the source, thereby creating gaps of clean air within the plume as it expands and moves downwind (Murlis et al. 1992). A detailed description of how odour is distributed within the plume, strategies for finding an odour plume, mechanisms of detecting direction of wind flow as a directional guide to the odour source, and the kinds of orientation manoeuvres used by insects in navigation has recently been reviewed by Cardé and Gibson (2010). Such knowledge enhances our understanding of how haematophagous insects navigate towards semiochemical sources and the distance over which odour-baited tools may be attractive to target insect species under natural conditions.

2.4. Methods of dispensing semiochemicals

In order to manipulate the behaviour of haematophagous insects, semiochemicals should be released in a form that mimics the natural situation. This is achieved by selection of devices that allow (a) sustained release of optimal concentrations of biologically active volatile compounds for desired responses and duration of time, (b) control over various release parameters, (c) residual effect of odorants over a given period of time, (d) protection of active ingredients from degradation by UV light and oxygen, (e) convenience in terms of cost effectiveness and minimal environmental damage for practical application, and (f) optimal effectiveness (Foster and Harris, 1997, Torr et al., 1997). Some of the most commonly used slow-release dispensers for disrupting behaviour of insect pests and vectors include low density polyethylene sachets, glass or polyethylene vials, rubber septa, cotton wicks, and polyamide nylon (Okumu et al., 2010b, Heuskin et al., 2011). Although the amount of semiochemicals released in the atmosphere is dependent on the dispensing matrices, their

release rates are influenced by environmental parameters like air temperature, wind speed, relative humidity and physical properties of the compound (s) itself (Torr et al., 1997, Shem et al., 2009, Heuskin et al., 2011).

2.5. Approaches for application of semiochemical-baited traps and targets

Semiochemicals can be applied to control insect pests and vectors through four basic methods depending on the goal and scope of activity (Kline, 2007, Heuskin et al., 2011). The methods include (a) mass trapping where large numbers of individuals from target populations are lured by use of visual, auditory, and olfactory cues into traps. This method is viewed as an alternative to area-wide application of insecticides and is more effective if population densities are low or if initiated at the start of the season. However, it is expensive in terms of number of traps required and labour for deployment, (b) mating disruption which involves pheromonal interference with male behaviour such that females remain virgins (Cardé and Minks, 1995). The success of this approach depends on population density, degree of isolation of the target area, mating behaviour, pheromone release rate and pheromone concentrations to which males respond, (c) lure and kill is the utilization of attractants to bring large numbers of target insects into contact with toxic, sterilizing, or pathogenic agents impregnated on a resting surface or in a trap. This serves as an important method of reducing the impact of insecticides on non-target organisms (Day and Sjogren, 1994). Both mass trapping and lure and kill approaches have been reported for screwworm flies, *Cochliomyia hominivorax* Coquerel and *C. macellaria* Fabricius, tsetse flies, and on a limited spatial and temporal scale for mosquitoes (Foster and Harris, 1997, Vale and Torr 2004 , Kline, 2007), and (d) integration of repellents and attractants in a push-pull strategy for directing movement and management of haematophagous dipterans (Cook et al., 2007, Hassanali et al., 2008). In this case, attractant cues may include aggregation pheromones, sex pheromones, host (plant or animal) volatiles, oviposition stimulants, while deterrents or repellents may comprise of anti-aggregation and alarm pheromones, non-host volatiles, or repellents (Foster and Harris, 1997).

2.6. Placement of semiochemical-baited traps and targets

Strategic placement of traps and targets for monitoring of pest insect populations and protection of a potential host or resource is important in decision making as it enhances efficiency and cost-effectiveness (Day and Sjogren, 1994, Sumaye et al., 2012). This can be achieved by (a) formation of a perimeter barrier around an area of protection with a highly aggregated population surrounded by potential habitats of haematophagous insects (Kline and Lemire, 1998), (b) deployment of individual traps and targets within a protection area that has few but identifiable hot spots of vectors, and (c) intercepting vectors during dispersal from breeding sites or resting sites surrounded by potential hosts.

3.0. Monitoring and control of haematophagous insects

Haematophagous insects are attracted to sources of CO_2, moisture, heat and odours emanating from hosts including physical, visual and chemical cues encountered in their environments (Gibson and Torr, 1999). Vertebrate-derived kairomones are contained in breath, urine, faeces, skin and volatiles produced from microbial activity on the skin (Braks et al., 1999, Gibson and Torr, 1999, Verhulst et al., 2011). This section highlights the potential of applying semiochemical-based technology to monitor and disrupt the transmission of vector-borne diseases and distress associated with adult tsetse flies, horse flies, stable flies, myiasis causing flies, triatomine bugs, biting midges, sand flies, and mosquitoes (Gibson and Torr, 1999, Townson et al., 2005, Logan and Birkett, 2007, Pickett et al., 2010). A large number of trapping tools used for collection of insect pests and vectors have been reported by Qiu et al. (2007a).

3.1. Tsetse flies (Diptera: Glossinidae)

Tsetse flies transmit *Trypanosoma* parasites responsible for Human African Trypanosomiasis (HAT, also known as sleeping sickness) and African Animal Trypanosomiasis (AAT) termed nagana in sub-Saharan Africa (Rayaisse et al., 2010). The control of tsetse flies is the most successful example of mass-trapping, and lure and kill approaches against haematophagous insects (Vale and Torr, 2004). The achievements were largely attributed to long-term multidisciplinary research and sustained support from private and government sectors (Kline, 2007). Unlike other haematophagous insects, the control of tsetse flies was relatively amenable as they have a naturally low reproductive rate because single females produce less than 10 offspring (Vale and Torr, 2004). As a result, fewer individuals needed to be eliminated to achieve a measurable effect, though still trap efficacy needed to be improved.

During intervention, a combination of attractant-baited traps, insecticide-impregnated blue and black cotton targets, and the sterile insect technique (SIT) were used (Vale, 1993). Blue colour increased attractiveness, black was favourable for landing whilst smaller targets were preferred for concentration of flies at one point for removal (Gibson and Torr, 1999). Synthetic attractants applied included CO_2, acetone, butanone, 1-octen-3-ol, 4-methyl phenol and 3-*n*-propyl phenol and repellents such as pentanoic acid and 2-methoxy phenol (Vale and Torr, 2004). Whereas targets baited with a blend of acetone and 1-octen-3-ol augmented with CO_2 are presently used to monitor and control *Glossina morsitans* species Westwood, a standard blend for the attraction of *G. pallidipes* Austen, comprises acetone, 1-octen-3-ol, 4-methyl phenol and 3-*n*-propylphenol (Torr and Solano, 2010). Nonetheless, highly potent novel odour baits for the riverine *Glossina* species are required to reduce transmission of HAT (Pickett et al., 2010). Consequently, field studies have demonstrated that attractants derived from monitor lizard, *Varanus niloticus*, are potentially important for

designing odour-baited traps for *G. fuscipes fuscipes* Newstead known to transmit HAT (Omolo et al., 2009). In West Africa a blend of POCA (P = 3-*n*-propylphenol; O = 1-octen-3-ol; C = 4-methylphenol; A = acetone) alone or synthetic cattle odour (acetone, 1-octen-3-ol, 4 -methylphenol and 3-*n*-propylphenol with CO_2) was more attractive for *G. tachinoides* Westwood than natural cattle odour (Rayaisse et al., 2010). Similarly, POCA-containing traps and targets were significantly and consistently attractive to *G. p. gambiensis* Vanderplank while equivalent host doses of CO_2 sustained high catches of *G. p. palpalis* Robineau-Desvoidy. However, only 50% of the three *Glossina* spp. attracted to the vicinity of the traps were caught implying that better trap designs incorporated with visual and novel short-range cues are required (Rayaisse et al., 2010).

Repellents including the 4-methyl-substituted analogue (4-methylguaiacol) of guaiacol (2-methoxyphenol), a mild repellent constituent of bovine odours provides an additional protection and a promising tool in the arsenal of techniques required to control trypanosomiasis in cattle. The application of 4-methylguaiacol to approximately 75% of cattle herds protected entire herds and substantially reduced feeding responses of tsetse flies, especially *G. pallidipes* (Saini and Hassanali, 2007). In order to improve the efficacy of repellents, preliminary push-pull studies have shown that better protection of cattle against tsetse flies could be achieved by deploying attractant-baited traps adjacent to cows sprayed with synthetic repellents (Hassanali et al., 2008). Treatment of cattle with insecticides against tsetse flies is considered to be the most cost-effective technique, but this can be compromised by patchy distribution of livestock and potential aggravation of tick resistance to acaricides (Vale and Torr, 2004). Such effects can be overcome by deployment of odour-baited traps and insecticide-treated targets (Torr and Vale, 2011).

3.2. Horse flies (Diptera: Tabanidae)

Tabanids are a serious nuisance, they irritate and cause blood loss in livestock and are largely incriminated for mechanical transmission of Loasis in humans, and animal diseases such as surra, anaplasmosis, equine infectious anaemia and besnoitiosis (Baldacchino et al., 2012). Both tabanids and muscids alight on the legs and can also be sampled by use of sticky traps or electrocuting grids (Mohamed-Ahmed and Mihok, 2009). Like tsetse flies, tabanids are mainly diurnal, rely on visual stimuli for final orientation in host location and on olfaction for long and short-range orientation coupled with black surfaces for landing (Gibson and Torr, 1999, Krandļmar et al., 2006). Apart from ketones including acetone, CO_2, aged cow urine, 1-octen-3-ol and phenols (3- and 4-methylphenol) are prime attractants of tabanids, however, variation occurs among species (Mihok et al., 2007, Mihok and Mulye, 2010). As a kairomone, 1-octen-3-ol is a suitable cue for tabanids that feed preferentially on mammals such as *Chrysops relictus* Meigen but also for *Aedes* mosquitoes (Nilssen, 1998). Although CO_2 or 1-octen-3-ol supplemented with CO_2 are considered effective lures for tabanids,

mosquitoes and the biting midge *Culicoides furens* (Poey), the attractiveness is greatly reduced by addition of butanone (Kline et al., 1990).

Additional findings from Croatia indicate that volatiles released from aged urine of cows, horses, sheep and pigs, 4-methylphenol, and a mixture of 1-octen-3-ol, acetone and ammonia were effective attractants for different species of tabanids (Krandļmar et al., 2006, Krļmar, 2007). In south-eastern France, Nzi traps baited with aged cow, horse or sheep urine were predominantly attractive to *Tabanus bromius* Linnaeus and *Atylotus quadrifarius* Loew. Whereas aged horse and sheep urine were relatively more attractive to *A. quadrifarius*, aged cow urine was equally attractive to both species of tabanids (Baldacchino et al., 2012). However, in Croatia, there was no difference between the number of *Haematopota pluvialis* Linnaeus attracted to aged cow and horse urine suggesting a strong effect of species sensitivity (Krandļmar et al., 2006). In addition, synergy between ammonia and phenols commonly found in aged urine has also been demonstrated for the attraction of *Hybomitra* species (Mihok and Lange, 2012). By contrast, ageing raw urine seems to be a good bait for sub-gravid and gravid *An. arabiensis* (Mahande et al., 2010) and also produces oviposition cues for other *Anopheles* spp. and culicine mosquitoes (Kweka et al., 2011).

Nzi traps baited with 1-octen-3-ol, phenols, and acetone collected very high numbers of tsetse flies, tabanids, and stable flies during field studies conducted in both North America and Africa (Mihok et al., 2007). Such trapping systems could actually suppress larger numbers of biting flies that land on traps if a lure and kill or lure and contaminate strategy was adopted (Cook et al., 2007, Pickett et al., 2010). Since most traps designed for female tabanids incorporate both host-related odours and visual cues, males are rarely caught. Male tabanids, though, can be sampled by the use of sticky traps baited with ethane-ethiol or swormlure-4 for collection of calliphorid flies (Hall et al., 1998).

3.3. Stable flies and Horn flies (Diptera: Muscidae)

The stable flies of the genus *Stomoxy*s (L.) and several species of nuisance, biting and disease -transmitting flies that settle or feed on grazing livestock contribute to increased disease incidence, reproductive failure, reduced meat and milk yields, thus translating into significant economic losses (Birkett et al., 2004). Like tsetse flies, tabanids and biting midges, *Stomoxys calcitrans* Linnaeus are also attracted to CO_2, acetone, 1-octen-3-ol, phenols and black targets (Logan and Birkett, 2007a). Intercontinental studies by Mihok et al. (2007) demonstrated a significant increase in the numbers of tabanids and *S. calcitrans* attracted to 1-octen-3-ol alone in both Canada and Ethiopia. In Ethiopia, more *G. pallidipes* responded to acetone, urine or 1-octen-3-ol, or any combination of these, but increased catches of the tsetse fly *G. morsitans submorsitans* were observed for individual baits. Whereas CO_2 or a combination of acetone, cattle urine and octenol attracted more *G. pallidipes, G. longipennis* Corti*, S. niger*

Macquart, *niger* and *S. niger bilineatus* Macquart, in Kenya, variations occurred among species.

Besides use of attractants, Birkett et al. (2004) demonstrated that application of a slow-release formulation of 6-methyl-5-hepten-2-one (MHO) on heifers results in a significant reduction in attraction of various fly species viz. *Musca autumnalis* De Geer, *Haematobia irritans* Linnaeus, *Hydrotaea irritans* Fallen, *S. calcitrans* and *Wohlfahrtia magnifica* (Schiner) (Diptera: Sarcophagidae). These findings suggest that host-derived repellents can be applied singly or in a push-pull system to protect livestock from biting and nuisance effects. Subsequent results from Chile have also shown that different concentrations of host-derived compounds, 2-decanone and 2-undecanone elicit significant attraction or repellent responses of obligate blood-feeding pests of cattle, *H. irritans* (Oyarzún et al., 2009). What these results imply is that cows and other vertebrates produce volatiles that mask them against biting and nuisance flies. The concentration-dependent effect of 2-decanone and 2-undecanone is a clear indication that they could be optimized for a push-pull system (Cook et al., 2007). Additional studies have also shown that a 50% dilution of farm-grade blackstrap molasses in deionized water, or its hexane-extract may offer a cost-effective attractant for the control and monitoring of house fly populations (Quinn et al., 2007).

3.4. Screwworm flies and blowflies (Diptera: Calliphoridae)

The screwworm flies, *C. hominivorax* in tropical America, and *C. macellaria* in North Africa lay eggs in wounds of livestock thereby causing myiasis. Besides the sterile insect technique, the use of carrion, and raw meat, especially a combination of liver and sodium sulphide as bait for monitoring and control of screwworm flies has been reported (Foster and Harris, 1997). Attractants have also been identified from rotting meat and used to formulate a potent lure called swormlure-4. Use of electric nets demonstrated that a large visual target was not necessary for the precise location of a swormlure source by *C. hominivorax;* instead, blue-black targets or traps were important for landing responses (Torr and Hall, 1992). A combination of swormlure derivatives, dried blood, insecticide and a feeding stimulant (sugar) were applied in the form of pellets to control or eradicate screwworms and this was coupled with the sterile male technique (Foster and Harris, 1997). Nevertheless, the effect of this approach is restricted by resistance and environmental threat imposed by insecticide usage. Consequently, sticky traps baited with ethane-ethiol or swormlure-4 have also shown a higher potential of trapping calliphorid flies (Hall et al., 1998). Currently, a new fish-baited trap has shown a high potential for sampling the African latrine fly, *Chrysomya putoria* Wiedemann, known for transmission of pathogens associated with enteric infections in humans (Lindsay et al., 2012). Furthermore, synthetic dimethyl trisulphide-baited flight traps provided an effective monitoring and control tool for the myiasis-causing larvae of sheep blow fly, *Lucilia sericata* Meigen *and L. cuprina* Wiedemann. The advantage of such traps is that they also

attract other calliphorid and muscid flies associated with nuisance, myiasis, and mechanical transmission of pathogens (Nilssen et al., 1996).

3.5. Triatomine bugs (Heteroptera: Reduviidae)

Triatomine bugs are considered in this review because they transmit *Trypanosoma cruzi* parasites responsible for Chagas disease (Vitta et al., 2007). The bugs are highly diverse in terms of species and habitats, therefore, multiple and specific tools are required to disrupt transmission, and supplement residual insecticide spraying and wall improvement of rural houses. Triatomines respond to volatiles present in their faeces, physical cues including dark colours of their resting sites, heat, water vapour and host-associated chemical cues (Vitta et al., 2007, Lazzari and Lorenzo, 2009).

According to Lazzari and Lorenzo (2009), aggregation signals present in faecal volatiles from triatomine bugs seem to be the most promising source of odour baits. This is because: (i) faecal samples can be obtained easily from rearing or resting facilities, (ii) chemical composition of faecal pheromones can be unravelled using available modern technology and subsequently provide synthetic analogues of the same; (iii) the effective period can last up to two weeks, (iv) the actual mechanism of communication by use of pheromones found in the faeces of triatomine bugs is already known, (v) faecal pheromones are used in the context of finding shelters, and (vi) can be applied to attract and assemble triatomines in any fashion (Vitta et al., 2007). Apparently, the chemical composition of aggregation cue is common to several species of triatomines, and similar synthetic blends have demonstrated a potential for luring triatomines into artificial shelters. However, attempts to use CO_2 produced by baker's yeast as a lure was met with limited effects as this is a short-lived source and it does not incorporate the heat component of the host (Pires et al., 2000). Although light traps are used for sampling, surveillance and control may be enhanced by developing a tool that incorporates dark surfaces similar to their resting sites, host odour including their faeces and light, but this should be standardised to cater for species diversity. To date, live hosts as bait have been used in a simple device known as a ñNoireau's trapõõ for capturing sylvatic triatomines in natural ecotopes, but it would be convenient if synthetic alternative baits were available as a replacement (Lazzari and Lorenzo, 2009). Currently, the prospects of deploying sticky traps baited with different concentrations of hexanal, benzaldehyde, octanal and nonanal for collection of high numbers of *Triatoma infestans* Klug, than *T. sordida* Stal has been reported (Rojas de Arias et al., 2012).

3.6. Biting midges (Diptera: Ceratopogonidae)

Biting midges, *Culicoides* spp., are mainly known for transmission of two viral pathogens of non-human animals, African horse sickness virus and bluetongue virus in ruminants, as well

as because of nuisance bites to humans and animals (Logan and Birkett, 2007). Repellents are widely applied against biting midges whilst various models of suction light traps including UV light traps are primarily used for monitoring (Logan et al., 2010). In recent years, the use of kairomones associated with humans and livestock such as CO_2, 1-octen-3-ol, and acetone from breath, and a mixture of body odours has been suggested as a potential tool for sampling *Culicoides* spp. (Gibson and Torr, 1999a, Logan et al., 2010). A study conducted in Florida, USA, showed that attractants tested for mosquitoes lured *Culicoides* and tabanids into traps as well (Kline et al., 1990). 1-octen-3-ol was the most attractive to *C. furens,* followed by phenol compared to L-lactic acid or CO_2 alone. Two blends of 1-octen-3-ol in conjunction with phenol, and 1-octen-3-ol supplemented with CO_2 increased trap catches of *C. furens* by 100-fold compared to CO_2 alone (Kline et al., 1990). This indicates that synergy between CO_2 and 1-octen-3-ol enhances trap collections and this combination of olfactory cues may serve as a suitable bait for *C. furens* (Gillies, 1980, Kline et al., 1990). Similarly, more biting midges were collected in MM-pro or MM-X traps baited with heat, 1-octen-3-ol, and CO_2, than in CDC light traps (Qiu et al., 2007b, Logan et al., 2010). Whereas CO_2 was an effective attractant for coastal female *C. furens, C. hollensis* Melander and Brues, and *C.melleus* Coquillett at optimal concentrations, 1-octen-3-ol alone or in combination with CO_2 was more attractive to *C. furens* but repellent to the other two species (Kline et al., 1994).

Removal trapping of biting midges was also demonstrated by a reduction in the populations of *C. furens* in two delimited areas along the Atlantic coast in South Florida and on an island in the Bahamas. This was achieved by the use of insecticide-treated fabric targets containing CO_2 and 1-octen-3-ol (Day et al., 2001). Traps baited with CO_2 and a 4:1:8 mixture of 1-octen -3-ol, 3-*n*-propylphenol, and 4-methylphenol, respectively, collected many *C. mississippiensis* Hoffman, *C. barbosai* Wirth & Blanton,, *C. melleus*, *C. furens*, and *C hollensis* of which *C. mississippiensis* was the predominant species (Cilek et al., 2003). In both studies by Day et al. (2001) and Cilek et al. (2003), attractant-baited trapping systems demonstrated a threshold of consistent reduction. In Scotland, the biting midge, *C. impunctatus* Goetghebuer is a generalist feeder of large mammals including cattle, water buffalo, sheep, red deer and pony (Mands et al., 2004). This species is highly attracted to CO_2, 1-octen-3-ol and acetone but an effective large scale control method has not been reported (Logan and Birkett, 2007). Traps baited with 1-octen-3-ol and CO_2 generated from propane fuel were deployed for localized removal of biting midges, demonstrating that *C. vexans* Staeger, *C. delta* Edwards, *C. pulicaris* Linnaeus, *C. lupicaris* Campbell & Pelham-Clinton, *C. albicans* Winnertz, and *C. impunctatus* were the dominant species (Mands et al., 2004). Optimization of acetone and CO_2 concentrations may provide highly potent lures for *Culicoides* spp. It is likely that development of novel commercial repellents (such as 6-methyl-5-hepten-2-one and geranylacetone) identified from odours of unattractive volunteers may offer an alternative protection against bites of *C. impunctatus* (Logan et al., 2009) and other haematophagous insects such as mosquitoes. Currently, the MM-Liberty trap and lures containing either 1-

octen-3-ol, L-actic acid or L-lactic acid augmented with ammonium carbonate, or a combination of L-lactic acid, ammonia and propionic acid are commercially available for surveillance and control of *Culicoides* spp. (Logan et al., 2010).

3.7. Sand flies (Diptera: Psychodidae)

Host-seeking behaviour of female sand fly vectors of leishmaniasis in humans is primarily influenced by host cues (Gibson and Torr, 1999). Male *Lutzomyia longipalpis* Lutz & Neiva do not feed on blood, but they produce sex pheromones which may be used to enhance trap collections of virgin females (Bray and Hamilton, 2007). Odours from Syrian hamster, *Mesocricetus auratus* are highly attractive to virgin female *L. longipalpis,* but this response is greatly reduced by addition of hexane and enhanced by sex pheromone emitted from males (Bray and Hamilton, 2007).

Traps baited with pheromones emitted by male *L. longipalpis* pheromones are more effective when placed adjacent to host animals or synthetic odours. Like other blood-feeding dipterans, phlebotomine sand flies display variable dose-response effects to CO_2, ammonia, lactic acid, 1 -octen-3-ol, and hexanoic acid (Gibson and Torr, 1999, Logan and Birkett, 2007). As a universal attractant, CO_2 released from dry ice acts synergistically with light traps for enhanced collections of phlebotomine sand flies (Andrade et al., 2008). Therefore, animal baits used to attract sand flies may be replaced by synthetic odorants. Moreover, attraction of *L. intermedia* Lutz & Neiva to light-baited traps is enhanced by addition of 1-octen-3-ol whereas *L. longipalpis* is highly responsive to light in the presence of a synthetic blend of L-lactic acid, hexanoic acid and ammonia (Andrade et al., 2008). However, female *L. intermedia* were not affected by the release of individual components of the same blend and no synergistic effect occurred between 1-octen-3-ol and the blend, hence the need for further studies. Some biting flies, including mosquitoes, blackflies and *Culicoides* midges, are believed to produce a pheromone while blood feeding. As observed in other dipterans, sand flies respond with high levels of species-specificity to cues associated with sugar sources, implying that control could be achieved by inducing toxin secretion in nectar of highly-preferred plants (Schlein and Jacobson, 2008). Extracts of rabbit food and the oviposition pheromone of *L. longipalpis* increase collections of gravid females and number of eggs laid in oviposition traps (Dougherty et al., 1993). Similarly, *Similium damnosum* Theobald (Diptera: Simulidae) oviposit communally in response to an aggregation pheromone produced by their recently laid eggs (McCall, 1995). This creates a good opportunity for interference because aggregation pheromones concentrate vectors within a limited space and increase the efficacy of using larvicides, insecticides or entomopathogenic fungi as a strategy of integrated vector management.

3.8. Mosquitoes (Diptera: Culicidae)

Mosquitoes transmit more vector-borne diseases than any other group of haematophagous insects. Mosquito-borne diseases such as dengue haemorrhagic fever, encephalitis, West Nile virus (WNV), malaria and filariasis remain a major source of illness and death world-wide, particularly in tropical and sub-tropical climates (Goddard, 2008).

3.8.1. Manipulation of mosquitoes by use of cues associated with sugar feeding

Plant-derived sugars contribute to mosquito energy requirements for survival, flight, maintenance of nutritional reserves and reproduction. Sugar feeding is critical for male mosquitoes of all species and occurs frequently, while females sugar on feed just after emergence and then intermittently after a first blood meal (Foster and Takken, 2004). While semiochemicals from vertebrates have been tested extensively and exploited as attractants in traps, little is known about the composition of volatiles released from host plants attractive to mosquitoes (Foster, 2008). The limitation of deploying host-derived attractants as trap lures for surveillance and control is that they lack the capacity to attract mosquitoes of both sexes, females of varying physiological states, ages, feeding and resting behaviours (Foster and Takken, 2004, Foster, 2008). However, in spite of potential challenges of competing volatiles, narrow plant-host specificity, and a weaker behavioural response to phytochemical cues, plant -based attractants may provide more appealing lures for mosquitoes.

In view of the underlying needs, many field studies have shown that densities of adult mosquitoes (Müller and Schlein, 2008) and sand flies (Müller and Schlein, 2011) can be reduced remarkably by the use of attractive toxic sugar baits (ATSB). The baits contain natural plant-derived kairomones such as natural sugary juices combined with an oral insecticide or a component like boric acid to kill mosquitoes upon ingestion. Indeed, a single application of toxic sugar bait by plant-spraying methods can substantially decrease the population densities and longevity of malaria vectors in the *An. gambiae* complex (Müller et al., 2010a). Operationally, ATSB methods for malaria vector control appear highly effective in arid environments regardless of competitive, highly attractive natural sugar sources in their outdoor-environments (Beier et al., 2012). A mixture of sugar baits and boric acid can also be applied to attract and kill, respectively thereby reducing population densities of *Aedes albopictus* Skuse (Naranjo et al., 2013). This mosquito species is known for nuisance bites, and transmission of arboviruses including dengue virus and WNV. In a field trial conducted in Florida, boric-acid containing sugar baits reduced adult *Ae. albopictus* populations up to day 21 post-treatment while causing a significant reduction in oviposition at day 7 and 14 post-application (Naranjo et al., 2013). Similarly, populations of *Cx. pipiens* Linneaus and other mosquito species were reduced dramatically after vegetation surrounding larval habitats were sprayed with a fruit-based sugar bait containing an insecticide (Schlein and Müller, 2008,

Müller et al., 2010b). In mosquitoes, odour is important for long-range attraction while visual cues play a role at shorter range (Gibson and Torr, 1999). Thus, blossoms of *Tamarix jordanis* baited on a CDC trap without light were highly effective in attraction of *Cx. pipiens*, and populations were reduced significantly by insecticide-treated blossoms (Schlein and Müller, 2008).

There is a high likelihood of using ATSB strategies to provide a new and robust tool for the control of mosquito-borne infections because they are highly effective, technologically simple, inexpensive, and environmentally relatively safer than insecticides. Effectiveness of this method may be improved by (a) optimizing the performance of ATSB plant-spraying and bait stations, by determining required coverage of plant spraying and density of bait stations, (b) equipping bait stations with covers and re-applications during the rainy seasons to compensate for the ATSB washed off by heavy rains, and (c) ensuring that ATSB use confers minimal risks to humans and non-target organisms such as pollinators, beneficial predators, and hence the need to spray on non-target plants.

As the usefulness of human kairomone blends has already been demonstrated for host-seeking mosquitoes (Okumu et al., 2010a, Mukabana et al., 2012), volatile compounds used to modulate sugar-feeding behaviour of mosquitoes remain largely unknown and should be identified. Laboratory-based evidence has shown that synthetic floral blends could potentially be used in trapping devices to sample adult mosquito populations (Nyasembe et al., 2012). As part of the recent progress, a three-component blend consisting of benzaldehyde, phenylacetaldehyde, and (*E*)-2-nonenal was shown to attract both male and female *Cx. pipiens* during a behavioural bioassay (Otienoburu et al., 2012). However, it remains to be seen whether different mosquito species rely on similar plant-based chemical cues. Future research should seek additional attractants, determine optimal blends, release rates, odour-dispensing systems and trap designs for maximal trap collections under different field conditions.

3.8.2. Manipulation of mosquitoes by use of vertebrate host-derived cues

Since the realization that host-seeking behaviour of mosquitoes is influenced by vertebrate host odours, studies aimed at discovering potent synthetic odour baits for surveillance and control have been conducted (Eiras et al., 2010, Mukabana et al., 2010, Smallegange and Takken, 2010). Carbon dioxide acts as a universal attractant for mosquitoes, and has been shown to act as a synergist with other host-derived kairomones (Gillies, 1980, Constantini et al., 1998). Thus, CO_2 is added to vertebrate-derived odour baits to increase trap catches (Logan and Birkett, 2007). Apart from the need for effective attractants, availability of effective mosquito trap designs is also critical as it has been demonstrated to influence mosquito catches (Murlis et al., 1992, Dekker et al., 2001, Gibson and Torr, 1999). The

recently-developed Biogents (BG) sentinel trap baited with the BG-lure (hexanoic acid, L-lactic acid and ammonia) and CO_2 (Eiras et al., 2010) is equally efficacious as the Mosquito Magnet Liberty (MML) trap and human landing catch for the collection of *Aedes* and *Culex* species (Krockel et al., 2006b, Qiu et al., 2007b, Graig et al., 2006, Eiras et al., 2010). Although such traps have not been specifically designed for anopheline mosquitoes, it has been shown that the MM-X trap (an odour-baited counterflow trap) is an adequate trap for catching several anopheline species (Qiu et al., 2007b, Jawara et al., 2009).

Mosquitoes are quite prolific, vary in terms of feeding behaviour, travel great distances and are highly diversified and widely distributed (Kline et al., 1990, Kline, 2006, Torr and Solano, 2010). Thus, different kairomone combinations, slow release systems, and trap designs have been tested for enhanced attraction and trap collections of different adult mosquito populations (Table 2). To date, the most practical application of odour-baited traps and insecticide-treated targets for mass trapping and management of mosquito populations was successfully demonstrated on Key Island, FL, USA, where populations of *Ochlerotatus taeniorhynchus* Wiedemann as the most abundant species were reduced (Kline and Lemire, 1998, Kline, 2007).

Recently, synthetic odour blends for the attraction of malaria vectors were developed (Chapter 1, Okumu et al., 2010, Mukabana et al., 2012) and shown to be more or as attractive as human odorants. The availability of such attractive baits suggests that these can be employed for the purpose of mass trapping and/or population reduction. Recently, an area-wide study was initiated using odour-baited traps for the elimination of malaria from Rusinga Island in Kenya, to demonstrate the feasibility of this technology for malaria vectors (Hiscox et al., 2012).

3.8.3. Manipulation of mosquitoes by use of oviposition cues

The decision to select a site for oviposition is influenced by various semiochemicals including pheromones, as well as contact stimulants and deterrents from decomposing organic materials found in larval habitats (Eiras et al., 2010, Seenivasagan and Vijayaraghavan, 2010) (Table 3). The association between microbial breakdown of organic matter found in breeding sites and mediation of oviposition responses of *Aedes* and *Culex* mosquito populations has been demonstrated (Trexler et al. 2003, Ponnusamy et al. 2008). For example, carboxylic acids and methyl esters associated with bacterial breakdown of organic materials have been identified as oviposition cues (Ponnusamy et al., 2008). Similarly, oviposition responses are elicited in gravid mosquitoes exposed to pheromones associated with eggs, water in which conspecific larvae were reared or water from natural mosquito production sites (Allan and Kline, 1995).

Organic infusions derived from fermented plant materials and animal waste products are frequently used to increase the attraction of gravid mosquitoes and collections of laid eggs (Mboera et al., 2000b, Ritchie et al., 2004, Graig et al., 2006, Qiu et al., 2007a). Currently, volatile compounds (3-methylindole, 4-methylphenol, 4-ethylphenol, indole, and phenol) isolated from hay and Bermuda grass infusion (Millar et al., 1992) are known to elicit oviposition in *Culex* mosquitoes (Allan and Kline, 1995). Thus, the oviposition kairomone, (5*R*,6*S*)-6-acetoxy-5-hexadecanolide, and synthetic oviposition attractant compounds; indole, 3-methylindole (skatole) and 4-methylindole, have been evaluated for monitoring and control of *Culex* and *Aedes* mosquitoes (Beehler et al., 1994, Mboera et al., 2000b, Trexler et al., 2003). However, application of pheromones for vector control is limited by sub-optimal activity of site-derived cues, and by financial and technical challenges associated with identification of new potent cues. Possibly, traps complemented with site-derived and local oviposition site cues, specific biolarvicides, the insect growth regulator pyriproxyfen, a juvenile hormone analogue, or larva-specific pathogens are likely to provide a more effective and sustainable tool than oviposition kairomones alone (Otieno et al., 1988, Fillinger et al., 2003, Logan and Birkett, 2007, Pickett et al., 2010, Seenivasagan and Vijayaraghavan, 2010).

Various designs of MosquiTRAP and attractants have been optimized to provide a supplementary tool for surveillance of yellow fever as well as dengue fever, chikungunya fever, filariasis, encephalitis, and West Nile encephalomyelitis (Fávaro et al., 2006, Burkett-Cadena and Mullen, 2007, Eiras et al., 2010). Although many studies have been reported for *Culex* and *Aedes* mosquitoes, appropriate investigations are required for anopheline mosquitoes as they may lead to the discovery of suitable synthetic attractants for sampling and control of gravid and infected malaria vectors.

4.0. Prospects and Future Challenges

The application of semiochemicals is a promising method for surveillance and intervention of haematophagous insects and could be utilised equally or more effectively for population management in areas where insect densities are relatively low, build up gradually or for species that are confined to defined sites. Unlike insecticides, this novel method targets selected species, relies on cues that are naturally exploited by haematophagous insects and is harmless to the environment. As a kairomone, CO_2 enhances trap catches of all host-seeking disease vectors (Mboera and Takken, 1998). The responses of tsetse flies, biting midges, tabanids and stomoxinae are basically influenced by 1-octen-3-ol, CO_2 and host urine. Volatile compounds from host urine seem to affect oviposition site preference and trap catches of gravid malaria vectors as well. Synergy between ammonia and L-lactic acid enhances the behavioural response of host-seeking mosquitoes, a blend of L-lactic acid,

ammonia, and hexanoic acid is a potent lure for *Ae. aegypti* (Krockel et al., 2006) whereas a tripartite synergism among L-lactic acid, ammonia and tetradecanoic acid was demonstrated for *An. gambiae* (Smallegange et al., 2005). The identification of host-derived repellents, such as 6-methyl-5-hepten-2-one and geranylacetone from human beings (Logan et al., 2008) may provide new repellents that are highly active.

Based on the tsetse fly control programs in Africa and the mass trapping of mosquitoes conducted in the USA, it is evident that odour-baited technologies alone are not sufficiently effective to reduce target populations to acceptable levels. Instead, robust control can be achieved through lure and kill, push-pull strategies, use of biological larvicides, long-lasting repellents that are harmless to the environment and development of effective drugs against parasitic diseases. Preliminary multidisciplinary studies are important for successful deployment of this technology for different life stages of various species. In terms of control, initial area-wide deployment and sustainability will require a high financial input and attractants or attractant blends that are exceedingly more potent than natural hosts or sources. Moreover, repellents with a spatial and long-lasting activity against target insects are currently non-existent. Consequently, there is need for novel potent attractants and repellent combinations, formulations and effective odour-dispensing systems that allow sustained release of optimal doses over a desired operational period coupled with appropriate trap designs and a cheap source of CO_2 (Herbache, 1992, Cook et al., 2007, Kline, 2006).

The majority of haematophagous insects are sampled with light traps, but their effectiveness and selectivity can be improved by incorporating long- and short-range odour cues with appropriate trap designs. Depending on species, effectiveness of odour-baited devices is also enhanced by use of visual and physical cues especially for day biting vectors (Gibson and Torr, 1999, Kline, 2007, Rayaisse et al., 2010). Whereas electrically-powered trapping devices have demonstrated high efficiency for monitoring and control of mosquitoes, their reliance on electricity makes them less convenient in rural areas (Eiras et al., 2010, Qiu et al., 2007b), hence the need for alternative sources of energy and development of attractive non-mechanical traps. As a result, the feasibility of using solar-powered odour-baited traps for mass trapping of mosquitoes has been implemented for the elimination of malaria from Rusinga Island in western Kenya (Hiscox et al., 2012).

A push-pull strategy has been reported for the control of animal trypanosomiasis (Hassanali et al., 2008), but this may not be realised in the short run for other haematophagous insects of medical importance. Effective diversion of vectors to more attractive animals or utilization of readily available plant-derived repellents has been proposed. In Africa and Asia, keeping of livestock close to human dwellings is associated with increased malaria risk, however, it is also considered an alternative way of diverting opportunistic malaria mosquitoes from humans (Tirados et al., 2011). Thus, this aspect of animal husbandry and malaria risk should

Table 2: Effect of host-seeking cues on responses of mosquitoes under field conditions

Cue	Objective (country of study)	Results	Reference
CO_2	-Comparison of CO_2-baited traps for monitoring outdoor biting mosquitoes (Tanzania)	CFG and ENT performed better than CDC light trap. *Anopheles gambiae* Giles *sensu stricto* was the most abundant species in Njagi while *Cx. quinquefasciatus* was dominant in Muheza.	(Mboera et al., 2000a)
	Evaluate efficiency of BG-sentinel, CDC backpack aspirator and CO_2-baited EVS trap for collection of *Ae. aegypti* (Australia)	BG-sentinel was the most suitable trap for collection of *Ae. aegypti.* CDC back pack aspirator collected most blood-fed *Ae. aegypti*	(Graig et al., 2006)
CO_2 and nonanal	Compare responses of *Culex* mosquitoes to a human- and bird-derived attractants (USA)	Attraction of *Culex* mosquitoes was enhanced by synergy between nonanal and CO_2	(Syed and Leal, 2009)
CO_2 and 1-octen-3-ol	Compare host-seeking behaviour of mosquitoes to baited traps in a malarious area of the Republic of Korea	Abundance and diversity of caught mosquitoes was dependent on trap and bait type Mosquito magnet and CFG traps captured highest mosquito numbers. 1-octen-3-ol-baited traps were less attractive to *Cx. pipiens* than those without.	(Burkett et al., 2001)
	Evaluate attractiveness of CO_2 and 1-octen-3-ol baited trap to mosquitoes (Australia)	More *Oc. Vigilax* Skuse, *M. uniformis* Theobald and *Coquillettidia xanthogaster* Edwards were attracted to 1 -octen-3-ol + CO_2 Carbon dioxide was highly attractive to *Anopheles* mosquitoes	(Miller et al., 2005)

Table 2: continued

Cue	Objective (country of study)	Results	Reference
CO_2, 1-octen-3-ol, light	Evaluate responses of *Aedes* and *Culex* mosquitoes to 1-octen-3-ol or light combined with CO_2 (Australia)	Attraction of *Ae. vigilax* to CO_2 was enhanced by addition of 1-octen-3-ol but not light. Attraction of *Cx. annulirostris* Skuse and *Cx. sitiens* Wiedemann was affected by addition of light or 1-octen-3-ol to CO_2 Lower responses of *Cx. sitiens* to 1-octen-3-ol than to CO_2 alone.	(Vanessen et al., 1994)
CO_2, 1-octen-3-ol, vapour and heat	Evaluate mosquito control on Atsena Otie islands by mass trapping (USA)	MM-Pro caught more *Oc. taeniorhynchus* mosquitoes than mosquito magnets trap. Drastic reduction in mosquito populations and biting pressure	(Kline, 2006b)
CO_2, water vapour and heat	Evaluate the efficiency of mosquito traps in Gainesville, FL.USA	CFG captured more *An. crucians* Wiedemann while MM-Pro trap caught more *Culex* mosquitoes	(Mboera et al., 1997)
	Evaluate the efficacy of commercially available mosquito traps to capture vectors of West Nile Virus (WNV) (USA)	MM-Pro, MM-X, and CDC caught more mosquitoes than MM Liberty in both 30-min. and overnight trapping trials. All traps caught *Cx. salinarius* Coquillett, *Cx. erraticus* Dyar and Knab, and *An. crucians.*	(Campbell, 2003)
CO_2 , butanone, honey, 1-octen-3-ol, L-lactic acid and mixed phenols	Evaluate attractiveness of candidate compounds to mosquito populations (USA)	Carbon dioxide increased attractiveness of all compounds Addition of L-lactic acid and/or octenol to CO_2 increased catches of *Ae. taeniorhynchus*, *An. atropos* Dyar & Knab, and *An. crucians*, *Cx. nigripalpus* Theobald. Combinations of 1-octen-3-ol + phenol and 1-octen-3-ol + 200 ml/min CO_2 was the most attractive to *Cx. furens*	(Kline et al., 1990)

A blend of CO_2, L-lactic acid, ammonia, candidate carboxylic compounds	Determine responses of wild malaria vectors to synthetic odour baits (The Gambia)	Responses of *An. gambiae* s.l. and *Mansonia* spp. to a blend of CO_2, L-lactic acid and ammonia was enhanced by addition of tetradecanoic acid and were higher compared to light or a more complex synthetic blend	(Jawara et al., 2011)
CO_2 + a synthetic blend of 10 compounds derived from skin bacteria	Evaluate efficacy of odour baits derived from human skin microbiota to attract malaria and other mosquito populations (Kenya)	Odour baits increased mosquito catches but had no significant effect trap collections of *An. gambiae* s.l.	(Verhulst et al., 2011)
CO_2, worn nylon socks, a synthetic blend of ammonia and carboxylic acids	Evaluate suitability of nylon strips to dispense synthetic mosquito attractants for sampling *An. gambiae* (Tanzania)	Treated nylon strips attracted more mosquitoes than open glass vials or LDPE sachets.	(Okumu et al., 2010b)
Hexane-extracted from *Apium graveolens* and DEET	Compare mosquito responses to repellent properties of hexane-extract versus DEET (Thailand)	Hexane-extracted from *A. graveolens* was strongly repellent and protective against *Aedes.* spp. *Anopheles* spp*, Culex* spp. and *Mansonia uniformis.*	(Tuetun et al., 2005)
Synthetic attractant - AtrAedesTM Ecovec Ltd	Evaluate efficiency of MosquiTrap model 3, and BG-Sentinel trap for sampling host seeking *Ae. aegypti* in Brazil	Both MosquiTrap and BG-sentinel traps are effective sampling tools for host seeking *Ae. aegypti*	(Eiras et al., 2010)
Human odour, industrial CO_2 and yeast-produced CO_2	Evaluate efficacy of CO_2 produced by yeast-fermented sugar to attract malaria vectors	- Similar responses of *An. arabiensis* to traps baited with CO_2 produced by yeast-fermented refined sugar and industrial CO_2	(Smallegange et al., 2010)

Table 2: continued

Cue	Objective (country of study)	Results	Reference
Human odour, CO_2	Evaluate efficiency of CO_2 baited trapping devices versus humans to collect malaria vector *An. aquasalis* Curry in Suriname	Majority of *An. aquasalis* were collected by human landing catch (HLC) compared to CO_2	(Hiwat et al., 2011)
Human, CO_2, worn socks	To optimize odour-baited trap methods for collection of mosquitoes during the malaria season in The Gambia	Catches of *An. gambiae* s.l. and other mosquito species were dependent on CO_2 and not presence of a person in the hut Trap catches not dependent on number of traps around a hut. Entry of mosquitoes into huts was not affected by number of outdoor traps	(Jawara et al., 2009)
Human, lactic acid, ammonia, and caproic acid,	Evaluate monitoring tools for adult *Ae. aegypti* (Brazil)	HLC was the most effective followed by BG-sentinel trap for collection of *Ae. aegypti*	(Krockel et al., 2006b)
Human, CO_2 , ammonia, L-carboxylic acids,	To test the efficacy of a synthetic mosquito lure against human (Tanzania)	Synthetic blend attracted 3 to 5 times more mosquitoes than humans when placed in different experimental huts (10 - 100 m apart) Synthetic blend was equally or less attractive than humans when compared side by side within same huts.	(Okumu et al., 2010a)
Human and synthetic odour	Compare attractiveness of MM-X traps baited with human or synthetic odour to mosquitoes in The Gambia	MM-X traps collected more Mansonia than Anopheles and Culex mosquito populations CDC traps caught more *Mansonia* than, *An. gambiae* s.l. *An. ziemanni* Grünberg, and *Culex* spp. Synthetic blends were more attractive than human odours	Qiu et al., 2007a)

Human, ox odour, blend of acetone, 1-octen-3-ol, 4-methylphenol and 3-n-propylphenol	Compare orientation responses of *An. arabiensis* Patton and *An. quadriannulatus* Theobald to odour baited traps and targets (Tanzania)	Trap entry responses of *An. arabiensis* was stronger with human odour than with ox odour or a mixture of cattle and human odours	(Torr et al., 2008)
Humans, synthetic odour blends augmented with CO_2	Evaluate the attractiveness of selected synthetic blends and human hosts to host-seeking mosquitoes (Kenya).,	Addition of 3-methyl-1-butanol to the standard blend of ammonia, lactic acid and tetradecanoic acid augmented with CO_2 enhanced attraction of female *An. gambiae, An. gambiae* s.l., *An. funestus* Giles and other mosquito species of public health importance. Enhanced attractiveness of the standard blend to malaria vectors was greater than the Ifakara blend 1. The enhanced standard blend caught equal numbers of female *An. gambiae* s.l. as humans and the blend was more attractive to *An. funestus* than humans.	Mukabana et al., 2012
Human	Evaluate efficiency of Mbita trap to sample anopheline vectors (Madagascar)	Mbita trap collected fewer mosquitoes and was unreliable compared to HLC.	(Laganier et al., 2003)
	Evaluate efficiency of Mbita trap for sampling anopheline vectors (Kenya)	Mbita trap was less sensitive than either HLC or CDC light trap.	(Mathenge et al., 2005)
	Compare HLC and odour-baited entry traps for sampling malaria vectors (Senegal)	HLC was the most suitable for collection of anophelines indoors. Both methods were effective for *An. gambiae s.s., An. arabiensis* and *An. funestus.* Age of mosquitoes and infectivity rates were not influenced by the method of sampling.	(Dia et al., 2005)
	Evaluate new tent traps for sampling exophagic and endophagic *An. gambiae* s.l. (Tanzania)	Both Ifakara B and HLC were suitable for monitoring of endophagic and exophagic Afrotropical malaria vectors.	(Govella et al., 2009)

Table 2: continued

Cue	Objective (country of study)	Results	Reference
Human and cow	Investigate preferential attraction of mosquitoes to calf and human odour (Burkina Faso).	Human odour was preferred by *An. gambiae* s.l *and An. pharoensis* Theobald - *Culex antennatus* Becker*, An. rufipes* Gough*, Cx. nebulosus* Theobald, preferred calf odour *Anopheles funestus, M. africana* Theobald*, Ae. dalzieli* Theobald *and Ae. hirsutus* Theobald preferred human	(Costantini et al., 1998b).
	Test zoophily of *An. arabiensis* and *An. gambiae s.s* in Madagascar.	*An. arabiensis* and *An.gambiae s.s.* were predominantly zoophilic unlike on African continent *Anopheles funestus* was mainly anthropophillic	(Duchemin et al., 2001)
	Assess responses of female *An. arabiensis* to a human and cattle-baited trap in Ethiopia	No differences in attraction and *Plasmodium* infection of *An. arabiensis* lured by human and cattle-baited trap Vectorial capacity of *An. arabiensis* not affected by insecticide-treated cattle	(Tirados et al., 2006)
	- Assess mosquito sampling techniques in rice irrigation schemes of lower Moshi, Tanzania	Odour-baited entry traps and pit shelters collected more mosquitoes Our-baited entry trap and cow attracted more culicine and *An. arabiensis*	Kweka and Mahanda, 2009
	Evaluate protective efficacy of keeping cattle around human dwellings from malaria vectored by *An. arabiensis* (Ethiopia)	Human landing catches attracted more outdoor biting *An. arabiensis* than cattle. Presence of cattle outside a house occupied by human reduced house entry of *An. arabiensis* by a half	(Tirados et al., 2011)
Human and calf odour, CO_2	Investigate the response of host-seeking mosquitoes to odour-baited trap (Burkina Faso)	*Anopheles gambiae* s.l, *An. funestus* and *Mansonia* spp were the most abundant species. Diversity of caught mosquitoes was dependent on odour type.	(Costantini et al., 1993)

Human and light	Compare methods used to sample malaria vectors in Tanzania.	CDC light trap placed next to a bed net and the Mbita trap occupied by human bait were more attractive to malaria vectors than HLC.	(Okumu et al., 2008)
Human, cattle, sheep, goat and pig	Evaluate responses of Anopheline mosquitoes to different odour sources (Tanzania)	Cattle odour was the most attractive to *An. arabiensis* compared to human, sheep, goat or pig.	(Mahande et al., 2007)
Cows urine	Optimize odour-baited resting boxes for sampling malaria vector, *An. ararabiensis* (Tanzania)	Resting box baited with cattle urine odour was efficient for collection of female *An. arabiensis* and *Cx. quinquefasciatus.* CDC light traps more efficient for species density and diversity. Baited resting boxes sampled more blood-fed and gravid mosquitoes	(Kweka et al., 2010)
Odours from fresh and decaying cattle urine	Evaluate efficacy of resting boxes baited with ageing raw cattle urine to sample *Anopheles arabiensis* (Tanzania)	More *An. arabiensis* were attracted to raw than 3- or 7-day old cattle urine Most catches were endophilic In general, urine volatiles were highly attractive to semi-gravid and gravid compared to unfed and blood-fed *An. arabiensis*	(Mahande et al., 2010)

Table 3: Effect of oviposition cues on responses of gravid mosquitoes under field conditions

Oviposition cue	Objective (country)	Result	Reference
Nonanoic acid (C9) and octanoic acid (C10).	Evaluate repellency of gravid mosquitoes to ponds treated with nonanoic acid and octanoic acid (USA)	Nonanoic acid was a better repellent to gravid *Ae. aegypti, Cx. quinquefasciatus*, and *Cx. tarsalis* Coquillett than octanoic acid.	(Schultz et al., 1982)
Synthetic oviposition pheromone, 6-acetoxy-5-hexadecanolide in the active (-) -(5R,6S)-isomer	Evaluate oviposition responses of gravid *Cx. quinquefasciatus* to a synthetic oviposition attractant pheromone and insect growth regulator, pyriproxyfen (Kenya)	Gravid *Cx. quinquefasciatus* was highly attracted to the pheromone Pheromone activity persisted for four days of post-application Insect growth regulator, killed all larvae and pupae but had no effect on oviposition responses.	(Otieno et al., 1988)
Synthetic oviposition pheromone (*5R,6S*)-6-acetoxy-5-hexadecanolide) and grass infusions	Assess responses of gravid *Cx. quinquefasciatus* to traps baited with a synthetic oviposition pheromone versus grass infusions (Tanzania)	Gravid *Cx. quinquefasciatus* were highly attracted to outdoor CFG traps baited with either grass infusion or pheromone Pheromone + grass infusion was more attractive compared to either grass infusion or pheromone alone	(Mboera et al., 2000b)
Synthetic oviposition pheromone erythro-6-acetoxy-5-hexadecanolide, egg rafts, grass infusion and *Bacillus sphaericus*	Evaluate attractiveness of ovitrap (BR-OVT) based on physical and chemical stimuli for gravid *Cx. quinquefasciatus* (Brazil)	Choice of oviposition site was not affected by combination of *B. sphaericus* and grass infusion. BR-OVT was more effective for indoor egg collections.	(Barbosa et al., 2007)
6-acetoxy-5-hexadecanolide derived from *Kochia scoparia* plant, synthetic oviposition Pheromone,3-methylindole (skatole)	Compare responses of *Cx. quinquefasciatus* to gravid traps baited with plant-derived oviposition pheromone and a synthetic pheromone (UK)	Both pheromones were equally attractive. Synergy was observed.	(Olagbemiro et al., 2004)

3-methylindole, Dimethyl disulphide, indole, 4-methylphenol, trimethylamine, and untreated water	Compare attractiveness of *Ae. albopictus* to ovitraps baited with synthetic oviposition attractants in controlled release packets (USA)	No significant differences in attraction of *Ae. albopictus* to either attractants or untreated water	(Trexler et al., 2003)
Synthetic attractant (AtrAedesTM Ecovec), derived from *Panicum maximum* grass infusions	Optimize indoor location for both MosquiTRAPs and sticky ovitraps for attraction and collection of female *Ae. aegypti* females (Brazil)	Outdoor MosquiTRAPs caught more females than indoor traps. Number of females captured was not influenced by indoor or outdoor locations	(Fávaro et al., 2006)
Volatile compounds produced by crude substrates			
Aged tap water	Optimize the use of ovitrap for *Ae. aegypti* in Cairns, Queensland, (Australia)	Four traps per house were optimal for mosquito catches Sticky ovitraps deployed at ground level captured more female *Ae. aegypti* than at 1.75 m high Traps on leeward side of the houses were more effective during a dry than wet season.	(Graig et al., 2006)
Cow grass infusion (*Panicum maximum*).	Evaluate potential of sticky MosquiTRAPÊ for detection of *Ae. aegypti* during dry season in Belo Horizonte (Brazil).	MosquiTRAPÊ caught more mosquitoes and was more sensitive to *Ae. aegypti* mosquitoes than larval survey.	(Gama et al., 2007)
Grass infusions of *Panicum maximum*	Evaluate ovitraps baited with grass infusions as oviposition attractants for *Ae. aegypti* (Brazil).	Ovitraps baited with infusions of *P. maximum* collected more eggs than water. Anaerobic fermentation of grass infusions attracted more *Ae. aegyypti* than aerobic fermentation	(Santôana et al., 2006)

Table 3: Continued

Oviposition cue	Objective (country)	Result	Reference
Nonanal, trimethylamine (TMA), and 3-methylindole	Evaluate attractants for monitoring gravid populations of *Cx. quinquefasciatus* in human dwellings (Brazil)	A combination of TMA and nonanal was equally attractive as the currently used infusion-based lure.	(Leal et al., 2008)
3-methylindole and untreated pond water	Test oviposition responses of gravid *Culex* mosquitoes to 3-methylindole (USA)	*Culex quinquefasciatus, Cx. tarsalis*, and *Cx. stigmatosoma* Dyar and thriambus Dyar preferred ponds with 3-methylindole to those without	(Beehler et al., 1994)
3-methylindole, 4-methylphenol, indole, and phenol, larval rearing water, larval field water, hay infusion compounds,	Compare oviposition responses of gravid *Ae. aegypti* and *Ae. albopictus* to natural organic infusions of hay and oviposition water (USA)	3-methylindole was more attractive than hay infusion compounds. *Aedes aegypti* responded strongly to larval water than 3-methylindole. *Aedes.albopictus* were more responsive to oviposition cues than *Ae. aegypti*	(Allan and Kline, 1995)
CO_2, grass infusion	Evaluate sampling techniques for surveillance of *Cx. quinquefasciatus* and other mosquitoes (Kenya)	*Culex. quinquefasciatus* and *Cx. annulioris* Theobald were highly attracted to CO_2-baited CDC light traps *Anopheles arabiensis* and *An. funestus* were not affected by technique used. More *Cx. quinquefasciatus* responded to grass infusion than to eggs CO_2-baited and CDC light trap without odour caught more unfed than gravid mosquitoes	(Muturi et al., 2007)

Oak leaf infusion	Test attractiveness of gravid *Ae. albopictus* and *Ae. triseriatus* to oviposition attractants from oak leaf infusions (USA).	*Aedes albopictus* oviposited more eggs in ovitraps with infusion *Aedes triseriatus* preferred both water and 80% infusion	(Trexler et al., 1998)
Hay and grass infusions from different species	Compare the attractiveness of selected gravid-trap infusions to ovipositing female mosquitoes (USA)	*Culex restuans*Theobald and *Cx. quinquefasciatus* were highly attracted to hay than grass infusions *Culex quinquefasciatus* and *Cx. restuans* were more selective than *Ae. albopictus*.	(Burkett-Cadena and Mullen, 2007)
Infusions of alfalfa hay, alfalfa pellets, Bermuda hay, oak leaves, and typha leaves, cow manure infusion, well water, diluted dairy effluents, larval water.	Evaluate oviposition preferences of female *Culex* mosquitoes to oviposition substrates and organic infusions (USA)	Oviposition preferences of gravid females were not affected by infusion type. Dilutions of dairy effluent were the most attractive, while cow manure infusion were the least attractive to *Culex* mosquitoes	(Allan et al., 2005)
Grass, manure, hay, and rabbit infusions	Compare oviposition preferences of *Cx. restuans* and *Cx. pipiens* for selected infusions in oviposition traps and gravid traps (USA)	Hay and grass infusions were highly preferred by *Cx. pipiens*. Hay infusion was attractive to both *Cx. pipiens* and *Cx. restuans*	(Jackson et al., 2005)
Larvae of *Anopheles gambiae s. s.*	To evaluate oviposition reponses of *An. gambiae s,s.* to conspecific larval density	Oviposition stimulated by low larval density Oviposition inhibited by high larval density	(Sumba et al., 2008)

Table 3: Continued

Oviposition cue	Objective (country)	Result	Reference
Oak leaves, pine straw, red (dyed) hardwood mulch, and composted manure infusions	Compare attractiveness of gravid traps baited with infusions made from commercial garden products to ovipositing female mosquitoes (USA)	Most *Ae. albopictus*, *Cx. nigripalpus* Theobald, and *Cx. restuans* preferred light traps to gravid traps. Gravid traps attracted more *Cx. quinquefasciatus*	(Burkett-Cadena and Mullen, 2008)
Aqueous infusion of a fungus found on wood (*Polyporus* spp.)	Evaluate oviposition responses of mosquito vectors to indoors and outdoors infusion derived from a wood inhabiting fungus (India)	Both indoor and outdoor locations were highly attractive to gravid *Ae. aegypti.* Treated pots were highly attractive to gravid *An. subpictus* Few eggs of *Cx. quinquefasciatus* were laid in fungal infusion treated pots	(Sivagnaname et al., 2001)
Raw and decomposing cow urine	Investigate the effect of cattle urine on oviposition responses of *Anopheles* and Culicine mosquitoes	Cow urine was more attractive to *An. gambiae* s.l. in the first four days, but attraction of *Cx. quinquefasciatus* increased from day 4-30 during dry season	(Kweka et al., 2011)

be investigated further. It has been reported that production of repellents in cattle is genetically determined and could provide an opportunity for the selection of unattractive individuals without compromising existing advantageous traits (Logan and Birkett, 2007).

Odour-baited stations have been reported for the control and monitoring of screwworm flies, tsetse flies and malaria vectors but this is highly dependent on spatial attributes of the host and insect vectors. Unlike for *Culex* and *Aedes* mosquitoes, field application of oviposition cues is highly limited for anopheline mosquitoes, thus the need for appropriate studies. In nature, stimulants and deterrents from decomposing organic materials found in potential breeding habitats are key to mediation of oviposition decision in mosquitoes. This is why volatile compounds produced by crude substrates are frequently used in studies associated with oviposition. However, further research is important in selecting an optimum infusion concentration at which optimal attraction to the oviposition cue(s) can be attained. Future studies should also identify the responsible bacteria and volatile compounds from organic infusions that attract mosquitoes to allow mass-production for surveillance and intervention (Lindh et al., 2008).

Similarly, very little has been reported about the exploitation of cues used during mating and sugar feeding. Whereas host-seeking kairomones are highly attractive to unfed female mosquitoes, plant-derived attractants may serve as lures for surveillance and intervention of both sexes, females of varying physiological states, ages, feeding and resting behaviours of mosquito populations. Many field studies have shown that adult mosquito densities (Müller and Schlein, 2008) including sand flies (Müller and Schlein, 2011) can be reduced significantly by use of ATSB. However, a majority of volatile compounds used to modulate sugar-feeding behaviour of mosquitoes remain largely unknown, and hence the need for additional investigations. Although the semiochemical-based technology is largely in a preliminary stage, with further improvement, effective tools may be developed to reduce over -reliance on insecticides and the risks imposed by haematophagous insects on man and his resources.

Conclusion

The examples discussed in this review demonstrate that semiochemicals can be exploited as a complementary or alternative tool for the monitoring and control of haematophagous insects, in particular disease vectors and nuisance species. To-date the behavioural response to semiochemicals of only few species of this important group of insects has been studied in detail, and future investigations are required to make this technology available wherever these insects cause a health risk. This requires a multidisciplinary study approach. Such studies should also focus on the efficacy of selected trapping systems and how semiochemicals can

be applied to manipulate sugar-feeding, mating, host-seeking, and oviposition behaviour of target populations. By so doing, odour-baited devices may be employed more intensively to mimic the natural situation and improve trapping probability of haematophagous insects thereby reducing transmission and the burden imposed on societies by vector-borne diseases of medical and veterinary importance.

Acknowledgements

This research was funded by a grant from the Foundation for the National Institutes of Health (FNIH) through the Grand Challenges in Global Health initiative (GCGH #121) and a sandwich Ph.D. scholarship of Wageningen University and Research Centre, The Netherlands (CKM). CKM was hosted by ICIPEï TRO campus as a Dissertation Research Internship Program (DRIP) scholar.

Chapter 3

Evaluation of low density polyethylene and nylon for dispensing synthetic mosquito attractants

Published as: Mukabana, R. W., Mweresa, C.K., Omusula, P., Otieno, B., Orindi, B.O., Smallegange, R.C., van Loon, J.J.A. and Takken, W. 2012. Evaluation of low density polyethylene and nylon for dispensing synthetic mosquito attractants. Parasites & Vectors 5: 202

Abstract

Synthetic odour baits present an unexploited potential for sampling, surveillance and control of malaria and other mosquito vectors. However, application of such baits is impeded by the unavailability of robust odour-dispensing devices that perform reliably under field conditions. In the present study the suitability of low density polyethylene (LDPE) and nylon strips for dispensing synthetic attractants of host-seeking *Anopheles gambiae* mosquitoes was evaluated. Baseline experiments assessed the numbers of *An. gambiae* mosquitoes caught in response to low density polyethylene (LDPE) sachets filled with attractants, attractant-treated nylon strips, control LDPE sachets, and control nylon strips placed in separate MM-X traps. Residual attraction of *An. gambiae* to attractant-treated nylon strips was determined subsequently. The effects of sheet thickness and surface area on numbers of mosquitoes caught in MM-X traps containing the synthetic kairomone blend dispensed from LDPE sachets and nylon strips were also evaluated. Various treatments were tested through randomized 4 × 4 Latin Square experimental designs under semi-field conditions in western Kenya. Attractant-treated nylon strips collected 5.6 times more *An. gambiae* mosquitoes than LDPE sachets filled with the same attractants. The attractant-impregnated nylon strips were consistently more attractive (76.95%; n = 9,120) than sachets containing the same attractants (18.59%; n = 2,203), control nylon strips (2.17%; n = 257) and control LDPE sachets (2.29%; n = 271) up to 40 days post-treatment ($P < 0.001$). The higher catches of mosquitoes achieved with nylon strips were unrelated to differences in surface area between nylon strips and LDPE sachets. The proportion of mosquitoes trapped when individual components of the attractant were dispensed in LDPE sachets of optimized sheet thicknesses was significantly higher than when 0.03 mm-sachets were used ($P < 0.001$). Nylon strips continuously dispense synthetic mosquito attractants several weeks post-treatment. This, added to the superior performance of nylon strips relative to LDPE material in dispensing synthetic mosquito attractants, opens up the opportunity for showcasing the effectiveness of odour-baited devices for sampling, surveillance and control of disease vectors.

Introduction

The effectiveness of odour-baited tools for sampling, surveillance and control of insect vectors is strongly influenced by the selected odour-dispensing device (Torr et al., 1997, Cork, 2004). Low density polyethylene (LDPE) materials have proved useful because odour baits are released at predictable rates and do not need to be replenished over prolonged periods of time (Torr et al., 1997, Okumu et al., 2010a). However, these attributes may not guarantee maximal mosquito trap catches without prior optimization of sheet thickness and surface area (Torr et al., 1997, Cork, 2004, Smallegange et al., 2012). Since LDPE sachets are prone to leakage, further searches for slow-release materials and techniques is warranted for the optimal release of odorants. In a previous eight-day study we reported on the efficacy of nylon fabric (90% polyamide and 10% spandex) as a tool for dispensing odours (Okumu et al., 2010a). A potent synthetic mosquito attractant namely Ifakara blend 1 (hereafter referred to as blend IB1) was used to evaluate open glass vials, LDPE and nylon as dispensing tools. Nylon strips impregnated with blend IB1 attracted 5.83 and 1.78 times more *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *An. gambiae*) mosquitoes than solutions of attractants dispensed from glass vials and LDPE sachets, respectively (Okumu et al., 2012a). However, in the case of nylon strips each chemical component of the attractant was applied at its optimal concentration whereas such optimization had not been implemented in advance for LDPE sachets.

In this study we re-evaluated the suitability of nylon versus LDPE as materials for dispensing synthetic mosquito attractants. We pursued four specific aims i.e. (i) comparison of nylon strips and LDPE sachets as materials for releasing synthetic mosquito attractants, (ii) assessment of the residual activity of attractant-baited nylon strips and LDPE sachets on host-seeking mosquitoes, (iii) determination of the effect of LDPE sheet thickness on attraction of mosquitoes to synthetic attractants, and (iv) comparison of surface area effects on attraction of mosquitoes to attractants administered through nylon strips versus LDPE sachets.

Materials and Methods

The study was carried out at the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology (*icipe*) located near Mbita Point Township in western Kenya between April 2010 and January 2011.

Mosquitoes

The Mbita strain of *An. gambiae* was used for all experiments. For maintenance of this strain, mosquito eggs were placed in plastic trays containing filtered water from Lake Victoria.

Larvae were fed on Tetramin® baby fish food three times per day. Pupae were collected daily, put in clean cups half-filled with filtered lake water and then placed in mesh-covered cages (30 × 30 × 30 cm). Emerging adult mosquitoes were fed on 6% glucose solution.

General procedures

The experiments were conducted under semi-field conditions in a screen-walled greenhouse measuring 11 m × 7 m × 2.8 m, with the roof apex standing 3.4 m high. Four treatments including two negative controls were evaluated in each experimental run. A total of 200 adult female mosquitoes aged 3-5 days old were utilized for individual bioassays conducted between 20:00 and 06:30 h. Experiments were started at 20.00 h as it was completely dark, to exclude the possibility of experimental mosquitoes released from one central point from responding to a unidirectional light source at sunset. The mosquitoes were starved for 8 h with no prior access to blood meals. Only water presented on cotton towels on top of mosquito holding cups was provided. Mosquitoes attracted to each treatment were sampled using MM-X traps (American Biophysics, North Kingstown, RI, USA). The nylon strips and LDPE sachets were suspended inside the plume tubes of separate traps where a fan blew air over them to expel the attractant plume as indicated in our previous study (Okumu et al., 2010a). Latex gloves were worn when hanging odour dispensers in the traps to avoid contamination.

Trap positions were rotated to minimise positional effects. The traps were placed 1 m away from the edges of the greenhouse (Smallegange et al., 2010, Verhulst et al., 2011a, Smallegange et al., 2012). Each trap was marked and used for one specific treatment throughout the experiments. The number of mosquitoes collected per trap was counted and used both as an estimate for the attractiveness of the baits and an indicator for the suitability of dispensing materials. Each morning the traps were cleaned using 70% methanol solution. Mosquitoes that were not trapped were recaptured from the green house using manual aspirators and killed. Temperature and relative humidity (RH) in the greenhouse were recorded using data loggers (Tinytag®). Whereas all experiments were conducted for 12 nights, responses of mosquitoes to residual release from attractant-treated nylon strips were evaluated for 40 nights and repeated three times.

Response of mosquitoes to attractant-treated nylon strips versus LDPE sachets

A 4 × 4 Latin square experimental design was conducted incorporating LDPE sachets filled with IB1, IBI-treated nylon strips, LDPE sachets filled with water (hereafter termed control LDPE sachets) and water-treated nylon strips (hereafter termed control nylon strips) as treatments. Sheet thicknesses of LDPE sachets each measuring 2.5 cm × 2.5 cm (surface area 12.5 cm^2) were optimized for individual chemical components of blend IB1 (Okumu et al., 2010b). These were 0.2 mm (distilled water, propionic, butanoic, pentanoic, and 3-

methylbutanoic acid), 0.1 mm (heptanoic and octanoic acid), 0.05 mm (lactic acid) and 0.03 mm (tetradecanoic acid and ammonia solution). Depending on treatment, LDPE sachets were filled with either 1 ml of the attractant compound or solvent. Individual nylon strips measuring 26.5 cm × 1 cm (surface area 53 cm^2) were separately soaked in 1 ml of each of the chemical constituents of blend IB1 at their optimal concentrations (Okumu et al., 2010a, Okumu et al., 2010b). The strips were air-dried at room temperature for 5 h before the start of experiments. Whereas attractant-treated nylon strips were freshly prepared each day, LDPE sachets filled with IB1 were re-used throughout the 12 days of the study and replaced upon leakage or depletion of individual components. Carbon dioxide, produced from 250 g of sucrose dissolved in 2 L of tap water containing 17.5 g of yeast (Okumu et al., 2010a, Smallegange et al., 2010, Mukabana et al., 2012) was supplied through silicon gas tubing at a flow rate of approximately 63 ml/min into traps baited with IB1-treated nylon strips or LDPE sachets filled with IB1 only and not with control nylon or LDPE sachets. Individual LDPE sachets containing chemicals were weighed before and after each experiment to determine how much of the individual components of the blend had been released. Control LDPE sachets and LDPE sachets filled with IB1 were stored in the refrigerator at 4^oC between experimental runs.

Residual activity of attractant-treated nylon strips on host-seeking mosquitoes

In our previous study we noted the potential disadvantage of nylon strips i.e. that they tend to dry up quickly so no more active ingredient may be available following long hours of trap operation (Okumu et al., 2010a). We designed experiments aimed at addressing this shortcoming. A 4 × 4 Latin square experimental design was used to evaluate residual attraction of *An. gambiae* to IB1-treated nylon strips and LDPE sachets filled with IB1. The four treatments included (i) LDPE sachets filled with IB1, (ii) IB1-treated nylon strips, (iii) control LDPE sachets and (iv) control nylon strips. The number of mosquitoes attracted to each treatment over a period of 40 nights was recorded daily and proportions trapped were calculated. The experiment was replicated three times. Analysis of data revealed no need to prepare fresh nylon strips daily. Thus, nylon strips were re-used in subsequent experiments. Whereas control LDPE sachets and IB1-filled LDPE sachets were also re-used, individual sachets were replenished upon depletion of contents. Sachets containing butanoic, pentanoic, 3-methylbutanoic, heptanoic and octanoic acid were replaced after every 10 - 14 nights.

Effect of sheet thickness of LDPE sachets containing attractants on *An. gambiae c*atches

Direct exposure of IB1-treated nylon to environmental conditions may have led to higher release rates of attractant volatiles resulting in more mosquitoes being attracted relative to LDPE sachets of optimal sheet thicknesses containing the same attractants. We hypothesized that increasing release rates for all components in the blend using IB1-filled LDPE sachets of

0.03 mm sheet thickness for all components in the blend (hereafter indicated as 0.03 mm-LDPE or 0.03 mm-sachet) could enhance numbers of mosquitoes attracted. A sheet thickness of 0.03 mm was selected, because it was the thinnest available LDPE material and had been used in our previous investigations (Okumu et al., 2010b). This hypothesis was tested by comparing *An. gambiae* mosquito capture rates with sachets of variable thickness versus 0.03 mm sachets. The sachets were weighed daily before and after each experiment to verify differences in volatile release rates. The carbon dioxide component of the blend was delivered separately through silicon tubing. A randomised 4 × 4 Latin square experimental design was adopted. The treatments included (a) LDPE sachets with optimized sheet thicknesses for all components of IB1, (b) each component of IB1 dispensed in LDPE sachets of 0.03 mm sheet thickness, (c) control LDPE sachets with optimal sheet thicknesses for all components of IB1, and (d) control LDPE sachets with 0.03 mm sheet thickness.

Response of mosquitoes to attractants applied on nylon versus 0.03 mm LDPE sachets

In addition to investigating the effect of volatile release rates on mosquito behaviour, we compared numbers of *An. gambiae* mosquitoes attracted to IB1-filled in LDPE sachets of uniform sheet thickness (0.03 mm) or applied on nylon strips. The following treatments were tested (a) IB1-treated nylon strips, (b) each component of IB1 dispensed in 0.03 mm-LDPE sachets, (c) control nylon strips and (d) control 0.03 mm-LDPE sachets. A randomised 4 × 4 Latin square experimental design was adopted. The sachets and nylon strips had surface areas of 12.5 cm^2 and 53 cm^2, respectively.

Effects of dispenser surface area on attraction of mosquitoes

As higher mosquito catches associated with IB1-treated nylon strips could not be explained by the strips being freshly treated prior to each experiment, we tested whether variations in mosquito catches were due to differences in surface area. The LDPE sachets and nylon strips used in previous experiments of this study had surface areas of 12.5 cm^2 and 53 cm^2, respectively. Thus, the strips released odorants over a larger surface area than the LDPE sachets. We designed two sets of 4 × 4 Latin square experiments to test whether the larger surface area of nylon strips was responsible for the higher mosquito catches. The four treatments included (a) IB1-treated nylon strips, (b) LDPE sachets filled with IB1, (c) control nylon strips and (d) control LDPE sachets. Enlarged LDPE sachets were similarly filled with one ml of attractant or solvent. In the first set of experiments, surface areas of control and attractant-filled LDPE sachets were enlarged (2.5 cm wide × 10.6 cm long × 2 sides of the sachet) to equal the surface area of nylon strips. In the second set of experiments, a piece of absorbent material (nylon strip) was placed inside enlarged (53 cm^2) control and attractant-filled LDPE sachets to ensure that blend IB1 was evenly spread over the entire inner surface of the sachets. Each set of experiments was replicated 12 times. All other experimental

procedures were similar to those described in previous sections.

Data analysis

The relative efficacy of each treatment was defined as a percentage of female mosquitoes caught in the traps containing either of the two release materials impregnated or filled with synthetic attractants or solvent. In order to investigate the effect of residual activity of attractant-treated materials on capture rates, we used the baseline-category logit model (Agresti, 2002). The nominal response variable was defined as the attractant type with four categories: IB1-containing LDPE sachets, IB1-treated nylon strips, control nylon strips, and control LDPE sachets with day and trap position as covariates. We estimated the odds that mosquitoes chose other attractants instead of IB1-treated nylon strips over time, while adjusting for trap position. The Mann Whitney-U test was used to estimate the effect of sheet thickness of LDPE sachets on release rates of IB1 components except carbon dioxide. To investigate the effect of surface area and sheet thickness on the release material on mosquito catches, we fitted a Poisson regression model controlling for trap position. The analyses were performed using SAS v9.2 (SAS Institute Inc.) with tests performed at 5% level.

Results

Response of mosquitoes to attractant-treated nylon strips versus LDPE sachets

The 12-day period over which experiments were conducted was characterized by a mean temperature and RH of 22.18 ± 0.1^{o}C and 86.15 ± 1.56%, respectively, within the screen-walled greenhouse. Out of 2,400 female *An. gambiae* mosquitoes released, 51.9% (n = 1,235) were caught in the four treatment traps. Of these catches, 77.7%, 18.6%, 1.8% and 1.9% were trapped by IB1-treated nylon strips, LDPE sachets filled with IB1, control nylon strips and control LDPE sachets, respectively (Figure 1). Baseline-category logit model results revealed that IB1-impregnated nylon strips attracted, on average, 5.6 times more mosquitoes than LDPE sachets filled with IB1 ($P < 0.001$). Whereas there was no significant difference in the proportion of mosquitoes attracted to control nylon strips and control LDPE sachets ($P = 0.44$), these treatments attracted significantly fewer mosquitoes than nylon and LDPE sachets containing blend IB1 ($P < 0.001$). Day effect was not significant ($P = 0.06$), and was therefore excluded from the final model. However, trap position was an important determinant of mosquito catches ($P < 0.001$). These experiments provided baseline information for subsequent investigations conducted during the study.

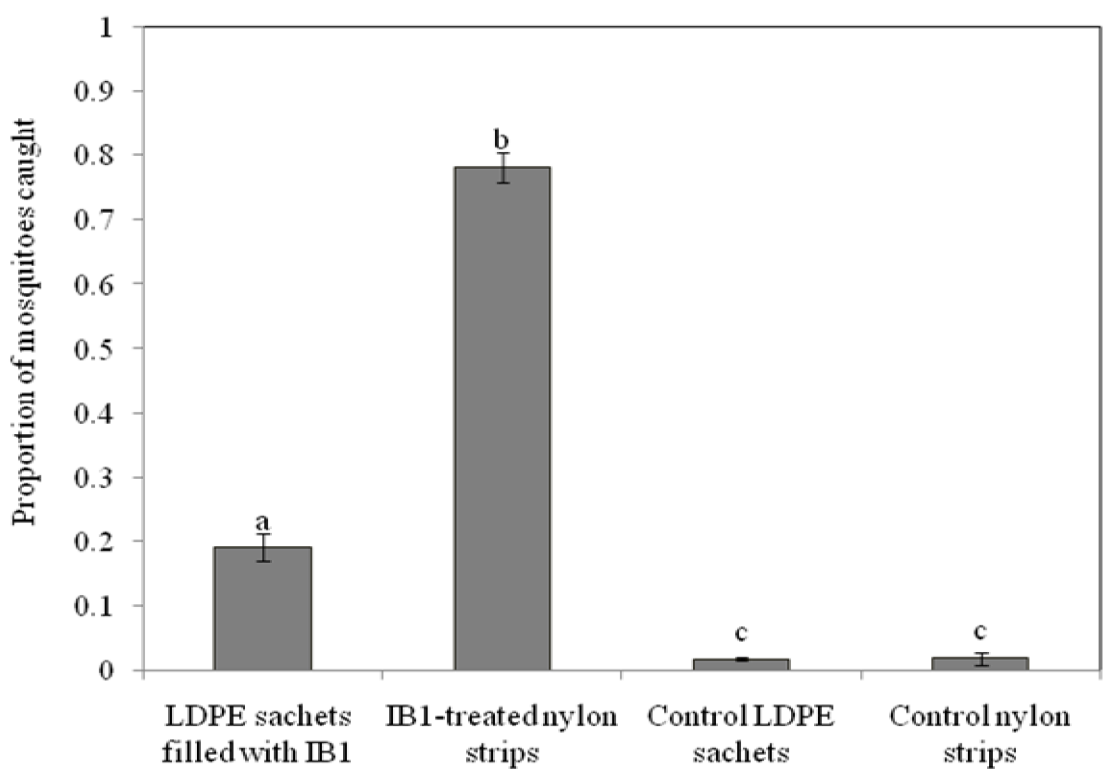


Figure 1: Proportions of mosquitoes caught in MM-X traps containing LDPE sachets filled with blend IB1, IB1 -treated nylon strips, control LDPE sachets and control nylon strips. Mean mosquito catches represented by bars with different letters are significantly different ($P < 0.05$). Error bars represent the standard error of the mean proportion of mosquito caught.

Residual activity of attractant-treated nylon strips on host-seeking mosquitoes

A total of 11,851 (49.38%) mosquitoes were attracted and collected over 120 nights (i.e. three replicates of 40 days each). The proportions of mosquitoes caught over time differed among treatments ($P < 0.001$) (Figure 2). Attractant-treated nylon strips repeatedly trapped the highest proportion of mosquitoes without re-applying the attractant blend up to 40 days post-treatment. During this period the treated nylon strips, LDPE sachets filled with IB1, control nylon strips and control LDPE sachets attracted 77.0% (n = 9,120), 18.6% (n = 2,203), 2.2% (n = 257) and 2.3% (n = 271) of the mosquitoes, respectively. There was also a significant increase over time in the proportion of mosquitoes choosing LDPE sachets filled with IB1 ($P < 0.001$), but not for control nylon strips ($P = 0.051$) and control LDPE sachets ($P = 0.07$). In contrast, the numbers of mosquitoes attracted to IB1-impregnated nylon strips decreased considerably over time ($P < 0.002$). However, they were consistently preferred to LDPE sachets filled with IB1 (Figure 2).

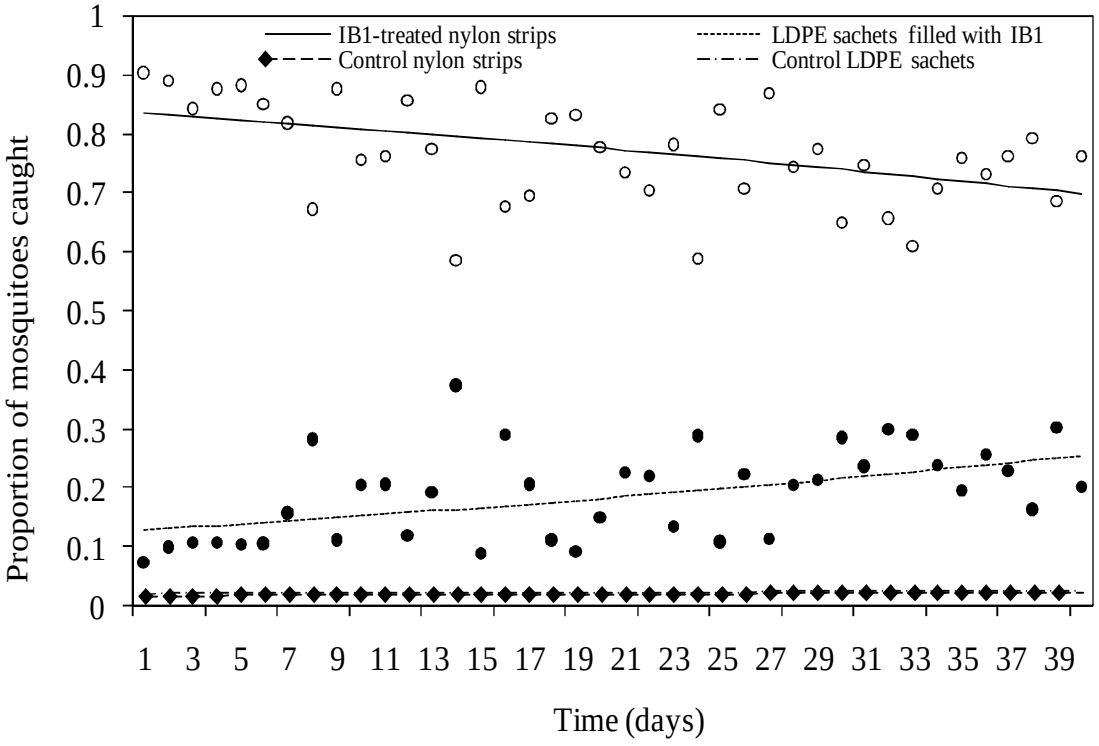


Figure 2: Proportions of mosquitoes caught in traps containing IB1-treated nylon strips (−--), LDPE sachets filled with blend IB1 (------), control nylon strips (ð Dž) and control LDPE sachets (Ž - Ž), over 40 consecutive nights post-treatment. Lines and symbols representing mosquito catches due to control nylon strips and control LDPE sachets are superimposed over each other. Open (IB1-treated nylon strips) and closed circles (LDPE sachets filled with IB1) represent observed values. Lines represent the Baseline-category logit model fit showing trends of proportions of mosquitoes attracted over time.

Effect of sheet thickness of LDPE sachets containing attractants on *An. gambiae* catches

Here LDPE sachets with sheet thickness optimized (Okumu et al., 2010b) or kept uniform (0.03 mm) for each chemical constituent of the attractant were evaluated. Out of 2,400 mosquitoes released, 51.2% were trapped (Table 1). Whereas trap position was not a significant factor ($P = 0.18$), attraction of mosquitoes to different traps was influenced by LDPE sheet thickness ($P < 0.001$). Dispensing of attractant components through sachets with optimized sheet thicknesses resulted in a significant increase in mosquito catches as opposed to uniform 0.03 mm-sachets ($P < 0.001$). There was no difference in mosquito catches between both types of control LDPE sachets ($P = 0.11$). The effect of porosity due to differences in sheet thickness of LDPE sachets on release rates of various chemicals emitted from blend IB1 was also investigated. Mann Whitney-U tests indicated that sheet thickness had a significant effect on the release rates of propionic acid, pentanoic acid, heptanoic acid, distilled water and lactic acid ($P = 0.04$, 0.03, 0.02, 0.01 and 0.02, respectively). However, release rates of butanoic acid, 3-methylbutanoic acid, octanoic acid, tetradecanoic acid, and

ammonia were not dependent on sheet thickness of LDPE sachets (P = 0.72, 0.97, 0.30, 0.23, and 0.87, respectively) (Figure 3).

Table 1: Effect of polyethylene sheet thickness on attraction of *An. gambiae* to sachets filled with attractants.

Treatment	N	Proportion of mosquitoes caught	
		n	Mean ±S.E
IB1 in sachets with optimal sheet thicknesses	12	633	52.75 ± 4.5[a]
IB1 in sachets with thinner-sheets (0.03mm)	12	495	41.25 ± 5.2[b]
Control sachets with optimal sheet thicknesses	12	58	4.83 ± 0.9[c]
Control sachets with thinner-sheets (0.03mm)	12	42	3.50 ± 0.8[c]

N refers to the number of replicates and n to the total number of mosquitoes trapped. Mean (±S.E) numbers of mosquitoes trapped with different letter superscripts differ significantly at P < 0.05 (Generalized Linear Models).

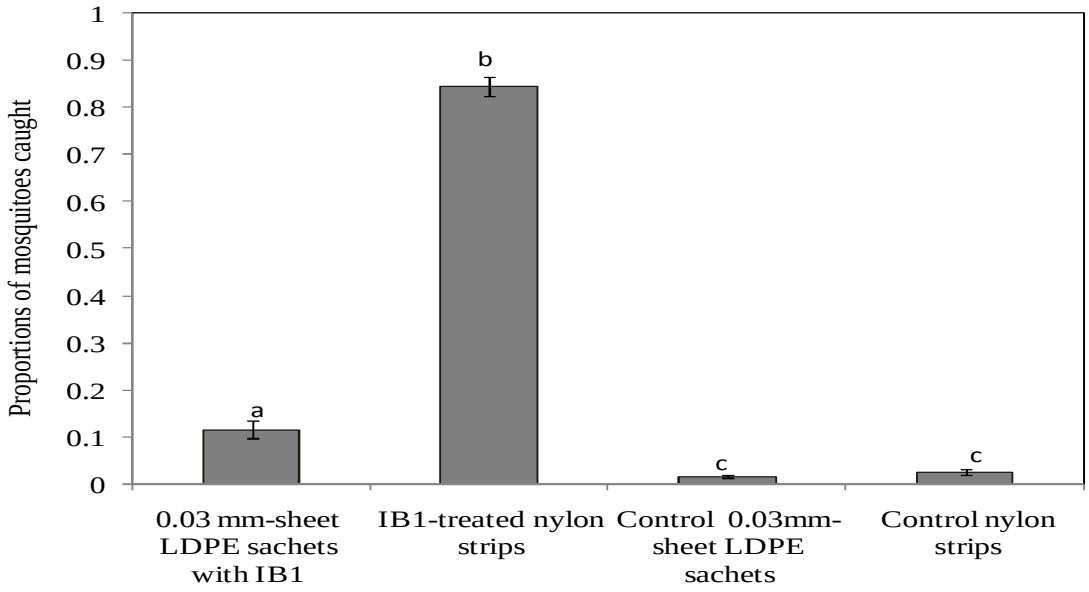


Figure 3: Proportions of mosquitoes caught by traps containing IB1-treated nylon strips, 0.03 mm-LDPE sachets filled with blend IB1, control 0.03 mm-LDPE sachets and control nylon strips. Mosquito catches represented by bars with different letters differ significantly (P < 0.05). Error bars represent the standard error of the mean proportion of mosquito caught.

Response of mosquitoes to attractant-treated nylon versus 0.03 mm LDPE sachets

Additional studies confirmed that mosquitoes preferred attractant-treated nylon strips compared to attractants contained in 0.03 mm-LDPE sachets (P < 0.001). Overall, 49.6% (n = 1191) of released mosquitoes were recaptured. Of these, 84.5%, 11.1%, 2.3%, and 1.7% were found in traps baited with attractant-treated nylon strips, LDPE sachets (0.03 mm) filled with

IB1, control nylon strips and control LDPE sachets (0.03 mm), respectively (Figure 4). The numbers of mosquitoes caught by control strips and sachets were not significantly different (P = 0.31).

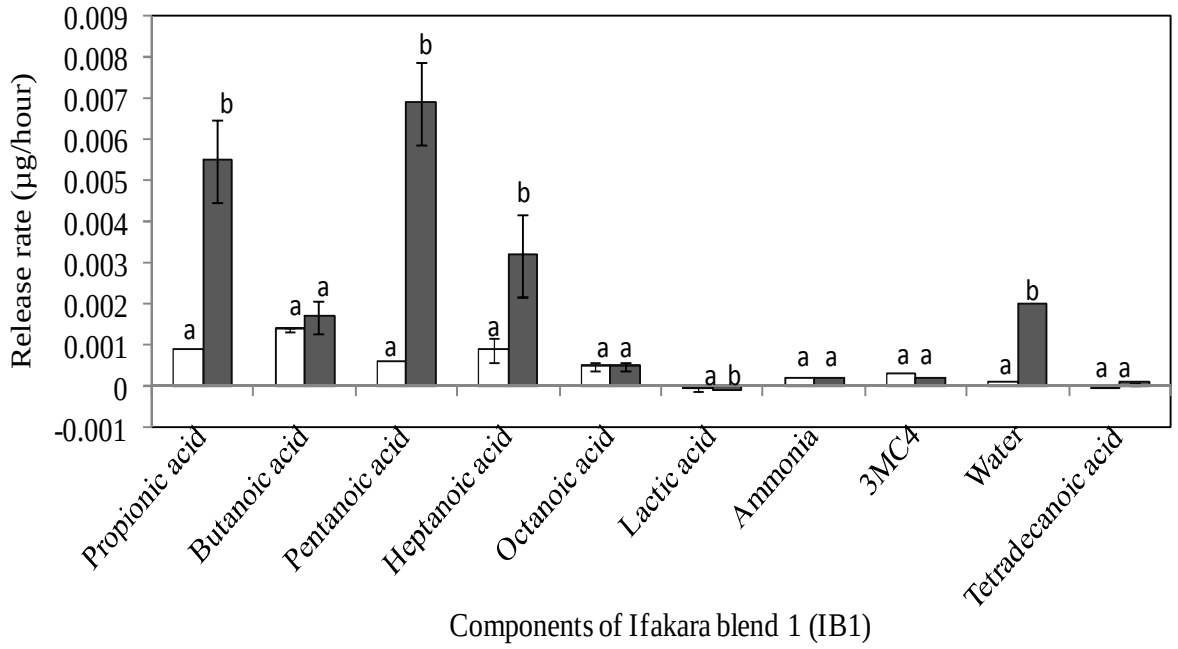


Figure 4: Effect of LDPE sheet thickness on release rates of chemical constituents contained in the mosquito attractant Ifakara blend 1 (IB1). Release rates from sachets, the sheet thickness of which had been optimised for all chemicals components of the blend (open bars) or kept uniform (0.03 mm-sheets) for all the chemical constituents (shaded bars), are shown. The optimal LDPE sheet thicknesses were 0.2 mm [distilled water (H_2O), propionic (C3), butanoic (C4), pentanoic (C5), and 3-methylbutanoic acid (3MC4)], 0.1 mm [heptanoic (C7) and octanoic acid (C8)], 0.05 mm [lactic acid (LA)] and 0.03 mm [tetradecanoic acid (C14) and ammonia solution (NH_3)]. Odour release rates represented by bars with different letters differ significantly ($P < 0.05$). Error bars represent the standard error of the mean odour release rates measured in ng/h.

Effects of dispenser surface area on attraction of mosquitoes

The LDPE sachets and nylon strips used to dispense blend IB1 in preceding experiments of this study had total surface areas of 12.5 cm^2 and 53 cm^2, respectively. Follow-up experiments were conducted in which LDPE sachets were enlarged (2.5 cm × 10.6 cm × 2) to equal the surface area of the nylon strips. Attractant-treated nylon strips caught significantly more mosquitoes than attractants contained in enlarged LDPE sachets with ($P < 0.001$) and without an inner lining of absorbent material ($P < 0.001$) (Table 2). Thus, higher attraction of mosquitoes to IB1-treated nylon strips was not neutralized by equalized surface area or uniform spread of attractants over the inner surface area of LDPE sachets. Mosquito responses to traps containing control nylon strips versus control LDPE sachets with or

without the absorbent nylon material were not different ((P = 0.17 and P = 0.56, respectively). Position had a significant effect on trap catches (P < 0 .001 in both cases).

Table 2: Behavioural responses of mosquitoes towards attractant treated polyethylene sachets lined with nylon versus nylon strips treated with a similar attractant.

Treatment	N	Proportions of mosquitoes caught			
		No absorbent in LDPE sachet		LDPE sachets with absorbent	
		n	Mean ±S.E	n	Mean ±S.E
IB1-treated nylon strips	12	968	78.20 ± 2.6[a]	1066	86.02 ± 2.7[a]
IB1-filled LDPE sachets	12	282	22.78 ± 1.4[b]	320	25.82 ± 1.5[b]
Control nylon strips	12	28	2.35 ± 0.44[c]	26	2.10 ± 0.41[c]
Control LDPE sachets	12	35	2.73 ± 0.46[c]	17	1.37 ± 0.33[c]

N refers to the number of replicates and n to the total number of mosquitoes trapped. Mean (±S.E) numbers of mosquitoes trapped in the same column with different letter superscripts differ significantly at P < 0.05 (Generalized Linear Models).

Discussion

This study demonstrates that nylon strips can act as a sustainable matrix for dispensing synthetic attractants of host-seeking *An. gambiae* mosquitoes, performing much better than low density polyethylene (LDPE) sachets. It was remarkable that attractant-treated nylon strips continued to attract mosquitoes without re-application and remained consistently more attractive than LDPE sachets filled with the same attractants over a period of 40 nights post-treatment. The higher catches of mosquitoes associated with nylon strips were apparently not due to smaller surface area, uneven spread of the attractant on inner surfaces or LDPE sheet thickness.

The baseline experiments reported herein confirm findings of our previous studies in which nylon strips were found to provide a better release matrix for dispensing synthetic attractants of host-seeking *An. gambiae* mosquitoes than did LDPE sachets or open glass vials (Okumu et al., 2010a). Nylon and LDPE differ in physico-chemical characteristics such as porosity and chemical binding affinity that may explain the observed differences in mosquito catches through their effects on the release rate of odorant volatiles (Torr et al., 1997, Dekker et al., 2002, Shem et al., 2009). Although the use of LDPE sachets allows the adjustment of attractant release rates, release rates from nylon have yet to be determined e.g. through headspace sampling at the trap outlet.

That IB1-treated nylon strips remained consistently more attractive to host-seeking *An. gambiae* mosquitoes than LDPE sachets filled with the same attractants for a period of up to 40 days post-treatment is a definitive proof of inherent residual activity. This finding corroborates that of related studies where nylon stockings impregnated with human emanations remained attractive to *An. gambiae* mosquitoes for several weeks (Qiu et al., 2004, Njiru et al., 2006, Pates et al., 2011). Blend IB1 impregnated on nylon strips may have been subject to bacterial degradation over the prolonged experimental time. This may have resulted in the release of additional components than were originally present on the nylon strips (Braks et al., 1999, Verhulst et al., 2009, Verhulst et al., 2011b). However, the present study did not investigate the presence of microbes or additional attractant compounds on aging IB1-treated nylon strips.

The current study shows that, attractant-treated nylon strips can be re-used for at least 40 consecutive days as baits for host-seeking *An. gambiae* mosquitoes, thereby reducing costs of odorants and nylon strips, time and labour used to prepare fresh baits. These attributes are consistent with those associated with the long-lasting fabric materials impregnated with mosquito repellents or insecticides (Yates et al., 2005, N'guessan et al., 2006). The availability of long-lasting mosquito-attractant fabrics is interesting as these can potentially be combined with mosquito pathogens such as entomopathogenic fungi or bacteria (Mnyone et al., 2012). Thus, a cheap and effective tool for intercepting and eliminating host-seeking mosquitoes can be exploited for vector-borne disease control. However, further testing is needed to examine the maximal duration of residual activity of the attractant-treated strips. Contrary to our expectations, LDPE sachets optimized for release rates and surface area caught fewer mosquitoes than nylon strips. The release rate of some compounds (propanoic, pentanoic, heptanoic, lactic acid and water) was significantly increased when uniformly thinner-sheeted sachets were utilized. Because sheet thickness of LDPE sachets is a determinant of volatile release rate, the composition of the volatile blend released may have changed so as to negatively affect attractiveness to *An. gambiae* mosquitoes (Torr et al., 1997, Smallegange et al., 2009). We conclude that, blend ratio and concentration affects orientation and capture rates of insect vectors with odour-baited systems (Cooperband and Cardé, 2006, Cardé and Willis, 2008).

Although LDPE sachets have been effectively used to release attractants for tsetse flies and other insect pests (Torr et al., 1997, Cork, 2004), they attracted fewer mosquitoes compared to nylon strips when both were treated or filled with the same blend of attractants. This could be explained by differences in optimized sheet thicknesses of LDPE sachets and physical and chemical characteristics of the odorants used for attraction of tsetse flies versus those used for mosquitoes (Mihok et al., 2007). Moreover, trap designs used for collection of both insect vectors were also different (Jawara et al., 2009, Okumu et al., 2010a). Dispensing of synthetic

attractant components through sachets with standardized sheet thickness and surface area have demonstrated consistent mosquito catches under laboratory and semi-field conditions (Verhulst et al., 2011b). Whereas nylon strips were associated with higher mosquito catches, we currently lack information on the release rates of the odorants dispensed. Accurate release rates have been established for odorants dispensed through LDPE sachets (Smallegange et al., 2012), and such chemical measurements should also be done for nylon, as this allows for a direct comparison of the active aerial odorant concentration that host-seeking mosquitoes encounter.

Conclusion

This study demonstrates that nylon strips present a potent and sustainable release material for dispensing synthetic mosquito attractants. Apparently, attractant-treated nylon strips can be used over prolonged time without re-applying the attractant blend. Treatment of nylon surfaces with attractants presents an opportunity for use in long-lasting odour-baited devices for sampling, surveillance and control of disease vectors.

Acknowledgements

We are grateful to Mr. David Odhiambo Alila for rearing mosquitoes used for our experiments. This work was supported by a grant from the Foundation for the National Institutes of Health (FNIH) through the Grand Challenges in Global Health initiative (GCGH #121) and a sandwich Ph.D. scholarship of Wageningen University and Research Centre, The Netherlands to MCK. MCK was hosted by *icipe*-TRO campus as a dissertation research internship scholar

Chapter 4

Long-lasting behavioural response of malaria mosquitoes to attractant-impregnated nylon: potential role of microbes

To be submitted in a slightly modified form as: Mweresa, C.K., Otieno, B., Omusula, P., Weldegergis, B.T., Verhulst, N.O., Dicke, M., van Loon, J.J.A., Takken, W. and Mukabana, W. R. Long-lasting behavioural response of malaria mosquitoes to attractant-impregnated nylon: potential role of microbes

Abstract

The deployment of odour-baited technologies for surveillance and control of malaria vectors requires sampling tools that are more robust. We evaluated the residual activity of a novel synthetic attractant blend to host-seeking *Anopheles gambiae* Giles *sensu stricto* at weekly intervals for one year. The attractant blend was dispensed from either nylon strips or low density polyethylene (LDPE) sachets. The role of microbes in modulating the emission of synthetic attractants from impregnated nylon strips was also investigated. Mosquito behavioural responses were evaluated through randomised fully replicated assays. Untreated and attractant-impregnated nylon strips were re-used throughout the study while LDPE sachets were immediately replaced after depletion of individual attractant compounds. Significantly and consistently higher proportions of mosquitoes were attracted to treated nylon strips than all other treatments over a one year study period. After one year of intermittent exposure, additional volatile organic compounds and various bacterial populations were found on attractant-treated nylon strips. The most abundant bacteria were *Bacillus thuringiensis* and *Acinetobacter baumannii*. The responses of host-seeking *An. gambiae* ($P = 0.17$), *An. gambiae* sensu lato ($P = 0.26$) and *Mansonia* spp. ($P = 0.17$) to baited traps was not influenced by autoclaving attractant-treated strips prior to the start of experiments. More female *An. funestus* ($P < 0.001$) and other anopheline ($P < 0.007$) mosquitoes were attracted to autoclaved than to non-autoclaved attractant-treated nylon strips. By contrast, more female *Culex* mosquitoes were attracted to non-autoclaved compared to autoclaved nylon strips that had been impregnated with attractants ($P < 0.042$). Female *An. gambiae* s.l. and *An. funestus* trapped were predominantly unfed ($P < 0.001$). We conclude that frequent re-treatment of nylon strips with mosquito attractants does not sustain their trapping efficacy and is, therefore, not necessary. It appears that bacterial action and autoclaving affect the residual attraction of treated nylon strips to mosquitoes over time. The presence of additional VOCs and the potential role of microbes on residual activity of attractant-treated nylon strips need further investigations. In doing so, novel semiochemicals could be identified for development of improved traps for surveillance or intervention of malaria and other mosquito-borne diseases.

Introduction

Malaria parasites and their vectors rapidly develop resistance to anti-malarial drugs and insecticides, respectively, thereby threatening the prospects of controlling and eliminating this disease (Greenwood, 2009, Mendis et al., 2009). These obstacles can be overcome by developing new and improved technologies for sampling, surveillance and control of malaria mosquitoes to complement tools that are currently used (Cook et al., 2007, Logan et al., 2008, Pickett et al., 2010). In the course of the previous two decades, odour-baited trapping technologies have been tested for mass trapping, and sampling specific species of mosquito vectors (Kline, 2006, Kline, 2007). However, successful deployment of such tools is partly dependent on efficacy, cost-effectiveness, and sustainability of slow-release odour-dispensing devices. Low Density Polyethylene (LDPE) sachets have been effectively used to dispense attractants for tsetse flies and agricultural insect pests (Torr et al., 1997, Cork, 2004, Mihok et al., 2007, Shem et al., 2009). Although LDPE sachets have also been used to release mosquito attractants (Smallegange and Takken, 2010, Jawara et al., 2011, Verhulst et al., 2011a), recent findings indicate that such treated sachets attract fewer *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *An. gambiae*) mosquitoes compared to nylon strips (Okumu et al., 2010a, Mukabana et al., 2012a).

Additional evidence has shown that attractant-treated nylon strips remain consistently more attractive up to 40 consecutive nights post-treatment to host-seeking *An. gambiae* mosquitoes than LDPE sachets filled with the same attractant blend (Mukabana et al., 2012a). This suggests that attractant-treated nylon strips have a residual activity and can therefore be used to lure host-seeking *An. gambiae* mosquitoes for prolonged periods without impregnating them anew. Residual activity is important for it reduces time and labour for preparation of fresh baits as well as costs. Currently, nothing has been reported about long-term residual activity of attractant-treated strips on host-seeking *An. gambiae* mosquitoes beyond the 40 consecutive nights post-treatment (Mukabana et al., 2012a), and whether attractant chemicals applied on the strips remain intact by the end of the study period. Furthermore, although the association between skin microbiota and attraction of *An. gambiae* mosquitoes to humans is evident (Braks and Takken, 1999, Verhulst et al., 2009, Verhulst et al., 2011b), it is not known whether this relationship can explain the attraction of mosquitoes to synthetic odour baits and/or if variants of the initial attractant compounds are produced by microbial action over time.

This study was designed to (a) investigate long-lasting attraction of host-seeking *An. gambiae* mosquitoes to odour-treated nylon strips, (b) identify volatile organic compounds (VOCs) present on treated-nylon strips after being used to attract mosquitoes over one year, (c) identify microbes present on treated-nylon strips one year post-treatment, (d) evaluate

behavioural responses of host-seeking *An. gambiae* to volatiles produced by microbes isolated from treated nylon strips one year post-treatment, and (e) to test if autoclaving of treated nylon strips would affect their residual attraction for malaria mosquitoes under semi-field and field conditions.

Materials and Methods

Mosquitoes

The Mbita strain of *An. gambiae s.s.* was used for the semi-field bioassays conducted under ambient climatic conditions. Adult mosquitoes were blood-fed three times a week on a human arm, and provided with 6% (w/v) glucose solution on filter paper. Eggs were laid on wet filter paper and placed in plastic trays containing filtered water from Lake Victoria. Larvae were fed (approximately 0.5 mg/larva) thrice a day on Tetramin® baby fish food (Melle, Germany). Pupae were collected daily, placed in clean cups half filled with filtered lake water and then transferred to mesh-covered cages (30 × 30 × 30 cm). A total of 200 female mosquitoes aged 3 - 5 d and without prior access to a blood meal were randomly aspirated from the cage, and starved for 8 h before the start of semi-field experiments (20:00 - 06:30 h). The mosquitoes were only provided with water on cotton towels placed on top of mosquito holding cups. All semi-field experiments were carried out within a screen-walled greenhouse at the Thomas Odhiambo Campus (TOC) of the International Centre of Insect Physiology and Ecology (*icipe*) (00°25'S, 34°13'E, at 1240 m above sea level) located near Mbita Point Township in western Kenya. Laboratory assays were carried out at the Laboratory of Entomology, Wageningen University, The Netherlands using *An. gambiae s.s.* colony which originated from Suakoko, Liberia. The mosquitoes were reared according to the methods described previously (Qiu et al., 2006, Verhulst et al., 2011b).

Mosquito attractants

The synthetic mosquito attractant ôIfakara blend 1 (IB1)ô was used in all mosquito behavioural experiments. Blend IB1 was constituted from 11 chemical compounds (Okumu et al., 2010a, Smallegange et al., 2010, Mukabana et al., 2012a). The eleven compounds, except carbon dioxide, were released from LDPE (Audion Elektro, Weesp, The Netherlands) sachets or strips of nylon (Bata Shoe Company, Kenya). Each LDPE sachet measured 2.5 cm × 2.5 cm and was filled with one milliliter of an individual chemical constituent of IB1. The sheet thicknesses of LDPE sachets used to dispense the individual attractant compounds were 0.2 mm (99.6% propionic acid, 99.9% butanoic acid, 99% pentanoic acid, 99% 3-methylbutanoic acid, and distilled water), 0.1 mm (98% heptanoic acid, 99.9% octanoic acid), 0.03 mm (99% tetradecanoic acid and 25% ammonia solution), and 0.05 mm (L-lactic acid (85%). Distilled

water was obtained from Buyimpex Laboratory Equipment Suppliers Limited in Kisumu, Kenya, while the rest of the compounds were purchased from the Sigma-Aldrich® Corporation (SIAL:NASDAQ GS), Germany.

Individual nylon strips were separately soaked in one milliliter of each chemical constituent at optimal concentrations of 0.01% (propionic acid), 1% (butanoic acid), 0.0001% (pentanoic acid, heptanoic acid and octanoic acid), 0.000001% (3-methyl butanoic acid), 0.00025% (tetradecanoic acid), 2.5% (ammonia), 85% (lactic acid) and 100% (distilled water) (Okumu et al., 2010a, Mukabana et al., 2010b). The strips measured 26.5 cm × 1 cm and contained 90% polyamide and 10% spandex. Tetradecanoic and octanoic acids were dissolved in ethanol while propionic, butanoic, pentanoic, heptanoic, 3-methylbutanoic acid and ammonia were dissolved in distilled water. The treated nylon strips were air-dried at room temperature for 5 h before the start of the experiments.

Carbon dioxide (approximately 63 ml/min) was produced by mixing 2 L of tap or river water, 17.5 g of instant dry yeast (Angel® Company, China) and 250 g of refined sugar (Sony sugar Company Ltd, Kenya) 30 min prior to the start of each experiment (Smallegange et al., 2010, Mukabana et al., 2012a). This gas was delivered through a silicon tube (0.5 cm internal diameter) into an MM-X trap (American Biophysics, North Kingstown, RI, USA) baited with blend IB1. The LDPE sachets filled with individual attractant chemicals were weighed before and after each experiment (Torr et al., 1997, Okumu et al., 2010 b). Sachets filled with IB1 blend components were only replaced upon leakage or depletion. Although control and IB1-treated nylon strips were prepared once and re-used (without replenishment) in all experiments of this study, CO_2 was prepared anew on each experimental night (Mukabana et al., 2012a).

Long-lasting attraction of host-seeking *An. gambiae* mosquitoes to odour-treated nylon under semi-field conditions

In a previous study, it was explicitly demonstrated that IB1-impregnated nylon strips attracted *An. gambiae* mosquitoes for 40 consecutive nights post-treatment (Mukabana et al., 2012a). On this basis, a randomised 4 × 4 Latin Square experimental design was used to investigate whether this result could be reproduced at weekly intervals over a period of one year (i.e. 52 nights). We also investigated whether treated nylon strips attracted significantly higher numbers of malaria mosquitoes than similarly treated LDPE sachets over the same time period. The four treatments used were (a) control LDPE sachets (no odour), (b) control nylon strips (no odour), (c) LDPE sachets filled with blend IB1, and (d) IB1-treated nylon strips. Control LDPE sachets were filled with 1 ml of water and sealed whereas control nylon strips were soaked in 1 ml of water and air dried (Mukabana et al., 2012a). This experiment was replicated thrice. The successive replicates were staggered to commence at intervals of one

week.

Sachets or nylon strips were suspended on metal hooks and mounted inside odour outlet tubes of separate MM-X traps. The traps were suspended such that the outlet was at a height of 15 cm above the ground (Schmied et al., 2008, Jawara et al., 2009) and operated on 12 V. The MM-X traps were used to dispense odorants and collect mosquitoes attracted to each treatment. Treatments were assigned to particular traps throughout the study and alternated nightly to overcome the potential effect of site on mosquito catches (Smallegange et al., 2010, Verhulst et al., 2011b). The numbers of mosquitoes collected in individual traps at the end of each experimental night were counted and recorded. Treated and control nylon strips and LDPE sachets were separately stored in the refrigerator at 4^{o}C between experiments. After each experimental night, un-trapped mosquitoes were recaptured immediately from the screen -walled greenhouse using manual aspirators and killed. Traps were thoroughly cleaned using 70% methanol solution prior to subsequent experiments. The ambient temperature and relative humidity (RH) during the experiments were recorded using data loggers (Tinytag® Ultra, model TGU-1500, INTAB Benelux, The Netherlands).

The same odour-treated nylon strips were examined for the presence of attractant compounds and microbes at the end of this experiment. Examinations of IB1-treated nylon for the composition of VOCs released, presence of microbes, and effect of microbe-produced volatiles on attraction of *An. gambiae* were conducted at the Laboratory of Entomology, Wageningen University in The Netherlands.

Identification of attractant compounds present on nylon strips one year post-treatment

The aim of this investigation was to determine whether original and/or additional VOCs were present on IB1-treated nylon strips that had been used to attract *An. gambiae* mosquitoes once per week for a period of 52 nights after treatment (see previous section of this article). Headspace sampling was used to collect VOCs released from individual IB1-treated nylon strips (4.5 cm × 1 cm). Volatiles were entrained using purge and trap on Tenax-TA 20/35 (Alltech), from IB1-impregnated nylon strips put together in a glass container of which the lid was fitted with air in- and outlets. The nylon strips were separately suspended on a wire and placed in a cuvette. To reduce background volatiles, air was sucked into the cuvette through a standard glass cartridge containing 100 mg Tenax-TA (Verhulst et al., 2009). Headspace volatiles were entrained at a flow rate of 100 ml/min for two hours on a second cartridge containing 100 mg Tenax-TA connected to the outlet of the cuvette. The samples were analysed using thermal desorption (TD) with gas chromatography and mass spectrometry detection (GC/MS). A Thermo trace GC ultra coupled with Thermo trace DSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA) were used for separation and detection of volatile compounds from IB1-treated nylon strips, respectively. Prior to the release of volatiles, each sample was dry-purged under a flow of nitrogen (20 ml min^{-1}) for 10

min at ambient temperature in order to remove moisture. This was followed by release of volatiles thermally from the Tenax TA adsorbent material using ultra 50:50 thermal desorption unit (Markes, Llantrisant, UK) at 250^{o}C for 10 min under helium flow of 20 ml min^{-1}, while re-collecting the volatiles in a cooled solvent trap at 10^{o}C using UNITY thermal desorber (Markers). Once the desorption process was complete, volatile compounds were released from the cold trap at a fast heating rate of 40^{o}C s^{-1} to 280°C for 10 min. The volatiles were transferred to a ZB-5MSi analytical column (30 m L × 0.25 mm I.D. × 1.00 µm F.T. (Phenomenex, Torrance, CA, USA)) in splitless mode for further separation. The GC oven temperature was initially held at 40°C for 2 min and thereafter raised at 10^{o}C min^{-1} to a final temperature of 280°C and kept constant for 4 min under a helium flow of 1 ml min^{-1} in a constant flow mode. The DSQ mass spectrometer (MS) was operated in scan mode within a range of 35 - 350 amu at 5.38 scans s^{-1}. The spectra were recorded in electron impact ionisation (EI) mode at 70 eV while MS transfer line and ion source were set at 275 and 250^{o}C, respectively. Compound identification was based on comparison of mass spectra with those in the NIST 2005 and Wageningen Mass Spectral Database of Natural Products MS libraries. Experimentally calculated linear retention indices (LRI) were also used as additional measures for confirming the identity of compounds.

Identification of microbes present on attractant-treated nylon one year post-treatment

Current evidence has demonstrated the existence of an association between microbiota on the human skin and attraction of *An. gambiae* mosquitoes to humans (Verhulst et al., 2009, Verhulst et al., 2010). However, it is not known if variants of the initial attractant compounds applied on nylon strips are produced by microbial action over time. Therefore, the purpose of this investigation was to ascertain the presence of microbes on IB1-treated and control nylon strips that had been used to attract host-seeking *An. gambiae* mosquitoes once per week for a period of 52 nights after treatment.

All treated nylon strips (4.5 cm × 1 cm) were separately streaked on Trypticase Soy Agar (TSA) plates and incubated overnight at 34^{o}C. The same procedure was repeated by using strips of equal size and number cut from a piece of nylon sock worn for 12 h by a human volunteer and control nylon strips. The most abundant bacterial species derived from colonies of IB1-treated nylon strips were identified. Thus, two bacterial isolates were identified by sequencing the 16S rDNA following the protocol of Drancourt et al. (2000). The two bacterial colonies were picked from the plates using sterile pipette tips and transferred to 20 µl lysis buffer (50 mM NaOH, 0.20% SDS) in 1.5 ml Eppendorf tubes. After heating for 15 min at 95^{o}C, each tube was placed on ice followed by addition of 200 µl water. The samples were centrifuged for 5 min at 12,000 rpm before 0.5 µl of the supernatant with DNA was used for Polymerase Chain Reaction (PCR). Extracted DNA was ampliýed by PCR using the universal 16S rDNA primers fD1 and rp2 (Drancourt et al., 2000, Weisburg et al., 1991).

Ampliýcations and sequencing of ampliýed products were done as previously described (Drancourt et al., 1997) (Eurofins MWG Operon, Ebersberg, Germany). The 16S rDNA sequences were compared with those available in the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov) to reveal the species identity of the bacterial isolates. The association between presence of microbes and attraction of *An. gambiae* mosquitoes to IB1-treated nylon strips was thenceforth validated under laboratory, semi-field and field conditions.

Effect of microbe-produced volatiles on attraction of *An. gambiae* in the laboratory

The attraction of *An. gambiae* to volatiles produced by the two most abundant bacteria isolated and identified from IB1-treated nylon strips in the preceding section was evaluated. Both isolates were grown overnight in a liquid medium (15g tryptone, 5g soytone and 5g sodium chloride/1000ml H_2O) at 34^oC before testing for behavioural responses of host-seeking *An. gambiae* (Verhulst et al., 2009). The liquid medium was diluted to an optimal concentration of 263 cfu/cm^2 by plating on TSA. Attractiveness of the volatiles produced by the two bacterial isolates was tested in a dual-port olfactometer as described previously (Smallegange et al., 2005, Verhulst et al., 2009). For each test, 30 female *An. gambiae* mosquitoes aged 5 - 8 d old and which had never received a blood meal, were selected 14 h before the experiment and placed in a release cage containing tap water presented on damp cotton wool. The experiments were performed during the last 4 h of the scotophase. In each trial, test odours were released in the air stream before a group of mosquitoes was set free from a cage placed 1.60 m downwind from the two ports. After 15 min, mosquitoes that entered each of the two trapping devices were counted. Excised blocks of TSA (1.5 × 1.5 × 0.3 cm) with or without microbiota were placed on a glass slide (1.5 × 1.5 cm) and placed in each trapping device (Verhulst et al., 2009). Each treatment was tested four times over two days. The sequence of test odours was randomised on the same day and between days. Test stimuli were alternated between right and left ports of the olfactometer to rule out any positional effects.

Effect of autoclaving on attraction of *An. gambiae* to treated nylon in a semi-field setting

This experiment was designed to establish whether sterilization by autoclaving affected attraction of host-seeking *An. gambiae* mosquitoes to untreated and IB1-treated nylon strips already exposed to ambient climatic conditions once per week for 52 nights post-treatment. The study was achieved through randomised dual-choice bioassays comprising (a) non-autoclaved control versus autoclaved control nylon strips, (b) non-autoclaved control versus autoclaved IB1-treated nylon strips, (c) non-autoclaved control versus non-autoclaved IB1-treated nylon strips, (d) autoclaved control versus autoclaved IB1-treated nylon strips, (e) autoclaved control versus non-auto autoclaved nylon strips, and (f) non-autoclaved versus

autoclaved IB1-treated nylon strips. Each bioassay was conducted for four nights inside a screen-walled greenhouse. The autoclaved and non-autoclaved IB1-treated and control nylon strips for individual treatments were separately wrapped in aluminium foil and refrigerated at -4^{o}C between experimental nights. Sets of control and IB1-treated nylon strips were separately placed in a 200 ml glass bottle (Pyrex®, England), sealed and autoclaved to 121^{o}C at100 kPa (15 psi) for 30 min (Webeco Vertikal model B-C-H-stand, Germany) prior to the start of each experiment. Non-autoclaved control and IB1-treated nylon strips were retained at -4^{o}C. The attraction of wild malaria mosquitoes to the same treatments was tested thereafter.

Validating the role of microbes on attraction of wild malaria vectors to treated nylon

This investigation was aimed at establishing whether sterilization by autoclaving influences the attraction of wild malaria mosquitoes to IB1-treated nylon strips previously exposed to ambient climatic conditions. The experiments were carried out from December 2011 to January 2012 at Kigoche village situated near Ahero town in the Kano plains of Kisumu County, western Kenya. The area is situated approximately 110 km east of *icipe* -TOC campus where all the semi-field experiments had been conducted. Kigoche village is located at 00^{o}34ôS, 034^{o}65ôE and 1158 m above sea level. Irrigated rice farming, which is the main economic activity in the area provides suitable breeding habitats for malaria and other mosquito vectors. Malaria endemicity is mainly sustained by *P. falciparum* parasites (Githeko et al., 1993) transmitted by *An. gambiae, An. arabiensis* Patton and *An. funestus* Giles (Githeko et al., 1993, Mukabana et al., 2012b). Four mud-walled houses (measuring 15.8 to 22.5 m^{2}) with open eaves, corrugated iron-sheet roofs, one or two rooms but without ceiling were randomly selected for the study. The houses were spaced along a transect within a distance range of 27 to 315 m apart to maximize homogeneity in mosquito density and exclude interactions between treatments. Each study house was used and occupied routinely by 2 - 5 dwellers who slept under untreated bed nets and did not use repellents during experimental nights.

A randomised 4 × 4 Latin Square experimental design comprising (a) non-autoclaved control nylon strips, (b) autoclaved control nylon strips, (c) non-autoclaved IB1-treated nylon strips, and (d) autoclaved IB1-treated nylon strips was conducted for 20 consecutive nights. Blend IB1-treated and control nylon strips were autoclaved at 121^{o}C for 30 min. The strips were autoclaved 30 min prior to deployment of traps under the eave of village houses (18:00 ï 06:30 h). All strips were re-used throughout the study and stored at -4^{o}C between experimental nights. Individual treatments assigned to specified MM-X traps were suspended outdoors adjacent to the bedroom of selected houses. The treatments were alternated at nightly intervals among the different experimental houses. At the end of each experimental night, traps containing adult mosquitoes were placed inside a freezer at a field laboratory located at the Ahero Multi-purpose Development Training Institute (AMDTI). Freezing killed

mosquitoes and arrested gonotrophic development of all females. Mosquitoes collected from each trap were morphologically identified (Gilles and Coetzee, 1987), counted and recorded based on sex and species (*An. gambiae* sensu lato, *An. funestus*, *Culex, Mansonia* species and other anophelines). The other anophelines included species of *Anopheles* other than *An. gambiae* s.l. and *An. funestus*.

Ethical approval

This study was approved by the ethical review committee of the Kenya Medical Research Institute (KEMRI/RES/7/3/1). The aim and protocols of the study were explained to local leaders and household heads before seeking permission to conduct the study.

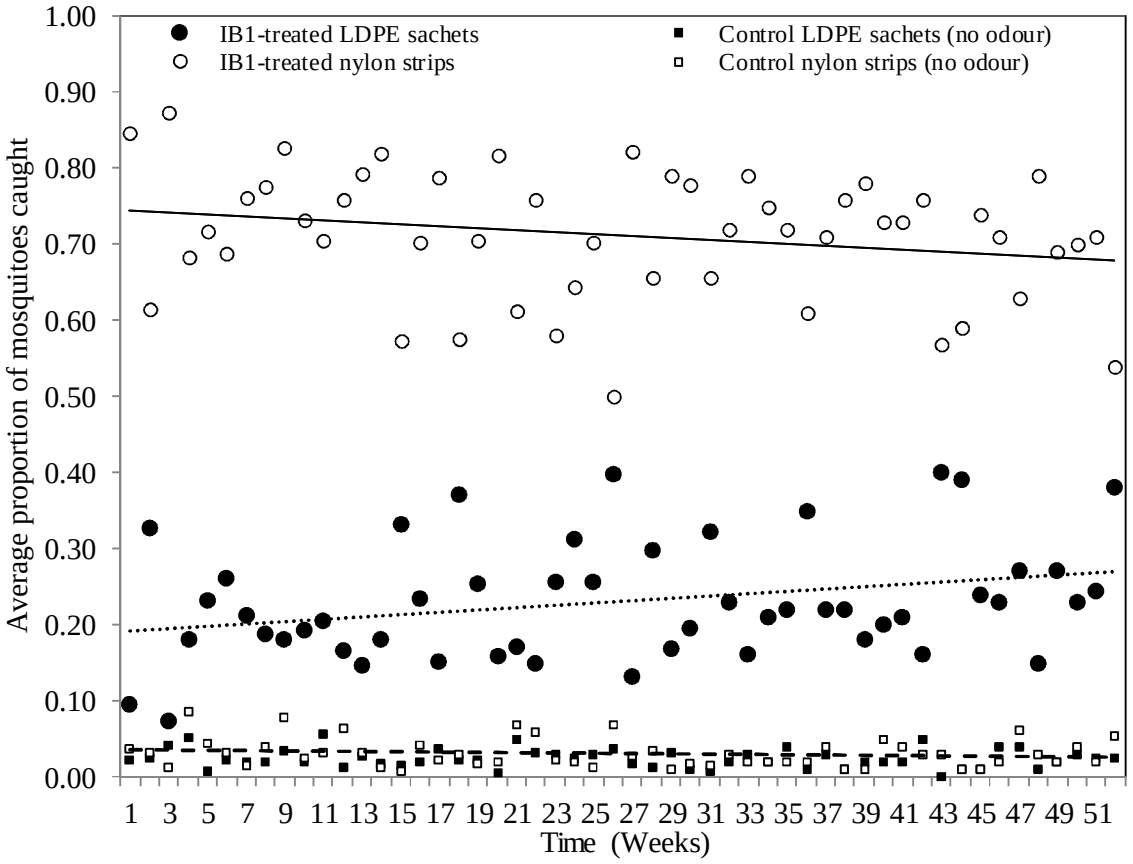
Data analysis

A baseline-category logit model was used to analyse residual activity of blend IB1 on attraction of *An. gambiae* mosquitoes (Agresti, 2002). For each two-choice test in the olfactometer and screen-walled green house, a χ^2-test was used to analyse whether the total (i.e. sum of all replicates) number of mosquitoes that was trapped in the treatment trapping device and the total number collected in the control trapping device, between both treatment or control devices differed from a 1:1 distribution ($P < 0.05$). A Generalized Linear Model (assuming a binomial distribution, a logit link function and with dispersion estimated) was used to investigate the effect of time of the day (i.e. olfactometer experiments only), treatments and day on the trap entry response. The trap entry response was defined as the number of female mosquitoes caught in both trapping devices of the olfactometer or MM-X traps within a screen-walled green house as the percentage of mosquitoes that flew out of the release cage or cup, respectively (Verhulst et al., 2009b, Qiu et al., 2006). The effect of sterilization by autoclaving on attractiveness of IB1-impregnated nylon strips to field mosquitoes trapped was evaluated by using a Generalized Linear Model (GLM) fitted with Poisson distribution and a logarithm link function (Smith, 1995, Verhulst et al., 2011). The effects of treatment and house position on mosquito catches were tested as parameters in the model. The GLM was followed by pairwise comparisons with Least Square Difference correction to test for differences in trap entry response between treatments. All analyses ($P < 0.05$) were performed using IBM SPSS statistical software, version 20.0.

Results

Long-lasting attraction of *An. gambiae* to treated nylon under semi-field conditions

A total of 15,272 (48.9%) female *An. gambiae* mosquitoes were captured during the 52 nights over which the experiment was carried out. The mosquitoes trapped responded to control LDPE sachets (n = 397, 2.6%), control nylon strips (n = 428, 2.8%), LDPE-sachets filled with IB1 (n = 3,344, 21.9%) and IB1-treated nylon strips (n = 11,103, 72.7%) (Figure 1). Significant and consistently higher proportions of mosquitoes were attracted to IB1-treated nylon strips than the other treatments over the one year study period ($P < 0.001$). Control LDPE sachets and control nylon strips caught similar proportions of mosquitoes over time ($P = 0.20$).



Fig**ure 1:** Proportions of mosquitoes caught in traps containing IB1-treated nylon strips (−--), LDPE sachets filled with blend IB1 (------), control nylon strips (ð Dž) and control LDPE sachets (Ƶ - Ƶ), over 40 consecutive nights post-treatment. Lines and symbols representing mosquito catches due to control nylon strips and control LDPE sachets are superimposed over each other. Open (IB1-treated nylon strips) and closed circles (LDPE sachets filled with IB1) represent observed values. Lines represent the Baseline-category logit model fit showing trends of proportions of mosquitoes attracted over time.

Identification of attractant compounds present on nylon strips one year post-treatment

GC-MS analysis of the IB1-treated nylon strips revealed 28 volatile organic compounds (Table1). Of the original 10 compounds present in blend IB1 except carbon dioxide, pentanoic acid and 3-methyl butanoic acid were the only intact ones detected. The most abundant volatiles were nonanal, 2-(2-methoxyethoxy) ethanol, 3-methylbutanoic acid, 2-ethyl-1-hexanol, pentanoic acid, benzyl alcohol, pseudocumene and butyl heptanoate.

Table 1: Volatile compounds detected in the headspace of IB1-impregnated nylon strips after 52 post-treatment nights of repeated exposure for collection of female *An. gambiae* at weekly intervals within a semi-field facility.

Number	Compound	CAS no	[a]RT (min)	[d]Identification	Abundance
1	2-Methoxyethanol	109-86-4	4.26	LRI, MS	+
2	1-Butanol	71-36-3	4.79	LRI, MS	+
3	Methyl methacrylate	80-62-6	5.76	LRI, MS	+
4	3-Methyl-1-butanol	123-51-3	6.17	LRI, MS	+
5	3-Penten-2-one	625-33-2	6.29	LRI, MS	+
6	(*E*)-2-Methyl-2-butenal	497-03-0	6.4	LRI, MS	(+)
7	1-Pentanol	71-41-0	6.83	LRI, MS	+
8	Ethyl lactate	97-64-3	7.76	LRI, MS	+
9	3-Methylbutanoic acid	503-74-2	8.23	LRI, MS	++++
10	3-Methyloctane	2216-33-3	8.94	LRI, MS	+
11	Pentanoic acid	109-52-4	9.05	LRI, MS	+++
12	Styrene	100-42-5	9.44	LRI, MS	+
13	2-(2-Methoxyethoxy) ethanol	111-77-3	10.13	LRI, MS	++++
14	alpha-Pinene	80-56-8	10.3	LRI, MS	+
15	Pseudocumene	95-63-6	11.38	LRI, MS	++
16	2-Ethyl-1-hexanol	104-76-7	11.79	LRI, MS	++++
17	Benzyl alcohol	100-51-6	12.01	LRI, MS	+++
18	Dihydromyrcenol	18479-58-8	12.56	LRI, MS	+
19	Nonanal	124-19-6	13.11	LRI, MS	++++
20	Isopulegol	89-79-2	14.03	LRI, MS	+
21	Camphor	76-22-2	14.1	LRI, MS	+
22	Neoiso-isopulegol	21290-09-5	14.23	LRI, MS	+
23	Ethyl octanoate	106-32-1	14.52	LRI, MS	+
24	1-Methylene-1H-Indene	2471-84-3	14.82	MS	+
25	Citronellyl formate	105-85-1	15.06	LRI, MS	+
26	2,3-Diethyl-4,5-dimethylfuran	15764-16-6	15.18	MS	+
27	Butyl heptanoate	5454-28-4	15.92	LRI, MS	++
28	Butyl octanoate	589-75-3	17.32	LRI, MS	+

CAS no refers to a standard identification number of the compound.
RT[a]: retention time of compounds in the chromatographic window.
Identification[b]: Identification (Tentatively) based on retention indices (RI) and/or mass spectra (MS).

Identification of microbes present on attractant-treated nylon one year post-treatment

Plating of IB1-treated nylon strips on TSA agar resulted in more bacterial growth than plating a worn nylon sock or control strips on the same agar. Sequencing of the bacterial cultures

showed that, although many bacterial populations were present on the plate streaked with IB1 -treated nylon strips (Figure 2), *Bacillus thuringiensis* IBL 4222 contig00573 and *Acinetobacter baumannii* ABNIH4 contig00148 were the most abundant. Amplification of the DNA from control nylon strips did not yield enough DNA for sequencing.

Effect of microbe-produced volatiles on attraction of *An. gambiae* in the laboratory

Olfactometer traps baited with *B. thuringiensis* IBL 4222 contig00573 or *A. baumannii* ABNIH4 contig00148 caught significantly more mosquitoes than the traps with agar alone (P = 0.002 and P < 0.001, respectively) (Figure 3).

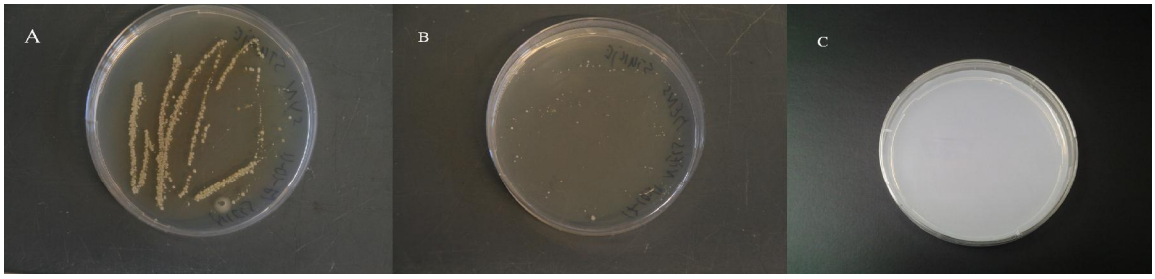


Figure 2: Bacterial culture from strips of a nylon sock worn for 12 h by a human volunteer (panel A) and IB1-treated nylon strips after 52 post-treatment nights of exposure for collection of female *An. gambiae* at weekly intervals spread out over one year (panel B).

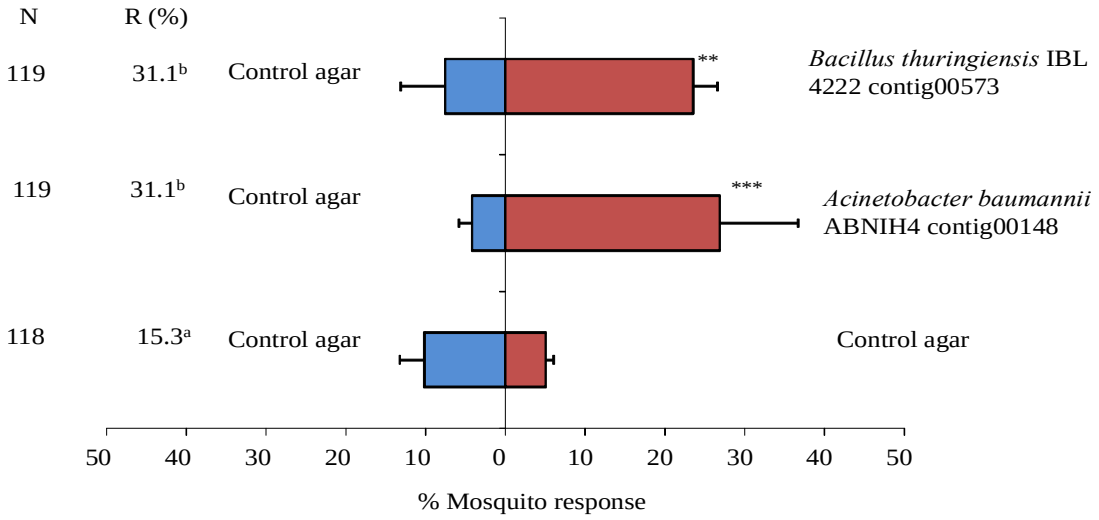


Figure 3: Mean response of female *An. gambiae* to *B. thuringie*nsis IBL 4222 contig00573 and *A. baumannii* ABNIH4 contig00148 isolates in a dual-choice olfactometer. The bacteria were isolated from IB1-treated nylon strips that had been exposed repeatedly for collection of female *An. gambiae* for 52 post-treatment nights at weekly intervals spread out over one year. Error bars represent standard errors of the mean; ***: G^2 -test P < 0.001; **: G^2 -test P < 0.01. N is the number of mosquitoes released. R is trap entry response expressed as the number of female mosquitoes caught in both trapping devices divided by the number of mosquitoes that flew out of the release cage. Response rates (%) followed by different letters differ significantly at P < 0.05 (Generalized Linear Models).

There was no significant difference between the responses of mosquitoes caught in the left and the right traps of the olfactometer when both contained agar alone (P = 0.16). Treatment had a significant effect on mosquito trap entry responses (P < 0.002).

Effect of autoclaving on attraction of *An. gambiae* to treated nylon in a semi-field setting

Significantly more *An. gambiae* were attracted to non-autoclaved IB1-impregnated nylon strips compared to non-autoclaved (P < 0.001) and autoclaved (P < 0.001) control nylon strips (Figure 4). Similarly, a considerably higher proportion of *An. gambiae* responded to autoclaved IB1-treated nylon strips than to non-autoclaved (P < 0.001) and autoclaved (P < 0.001) control nylon strips. However, mosquitoes responded equally to non-autoclaved versus autoclaved IB1-treated nylon strips (P = 0.17), and also to non-autoclaved control versus autoclaved control nylon strips (P = 0.68).

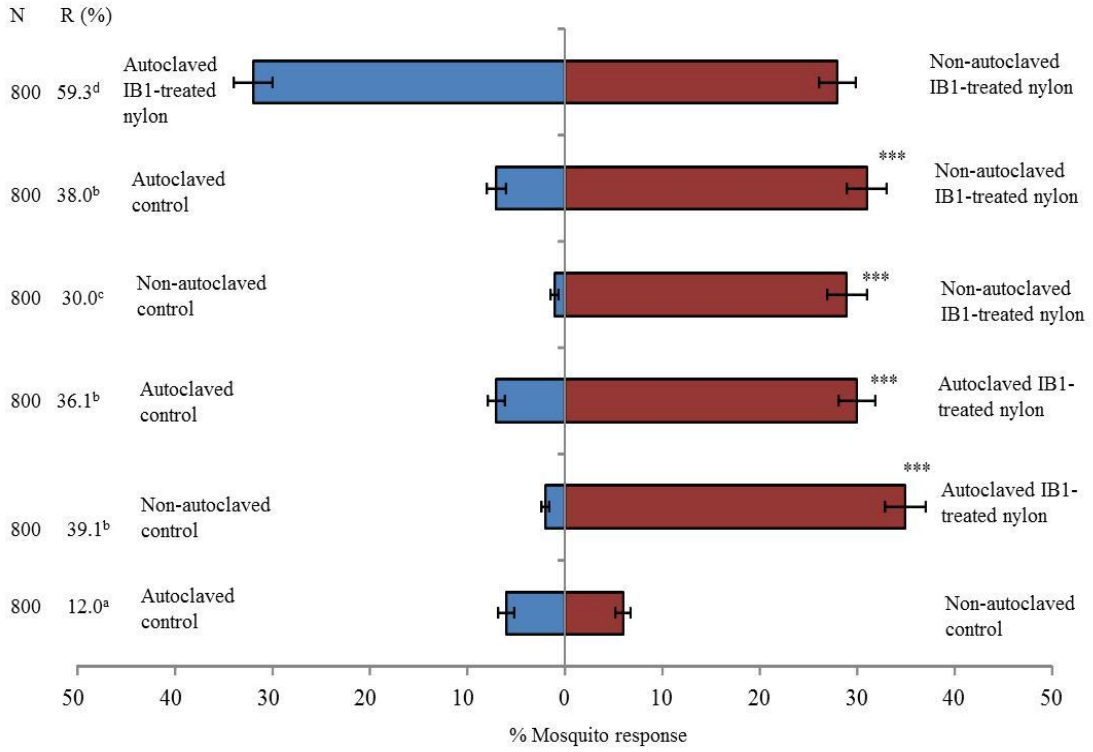


Figure 4: Effect of autoclaving on the percentage of *An. gambiae* attracted to IB1-treated nylon strips and control nylon strips (no odour) during dual-choice bioassays in a semi-field facility for four nights. The IB1-treated nylon strips and their controls had been exposed repeatedly for collection of female *An. gambiae* for 52 post-treatment nights at weekly intervals spread out over one year. Error bars represent standard errors of the mean; ***: χ^2 -test P < 0.001. N is the total number of mosquitoes released. R is trap entry response expressed as the number of female mosquitoes caught in both trapping devices divided by the number of mosquitoes that flew out of the release cup. Response rates (%) followed by different letters differ significantly at P < 0.05 (Generalized Linear Models).

Validating the role of microbes on attraction of wild malaria vectors to treated nylon

This study was conducted at an average temperature of 24.5 ± 0.8°C, 75.3 ± 9.1% RH and a total rainfall of 294.7 mm during the 20 d study period. A total of 1,547 mosquitoes were trapped, including 237 (15.3%) males and 1,310 (84.7%) females. The female mosquitoes comprised *An. gambiae* s.l. (8.2%), *An. funestus* (16.1%), *Culex* spp. (45.6%), *Mansonia* spp. (3.1%) and other anopheline spp. (dominated by *An. coustani* Laveran and *An. ziemanni* Grunberg*)* (27.0%) (Table 2).

Whereas autoclaved IB1-treated nylon strips attracted more *An. gambiae* s.l. than non-autoclaved IB1-treated nylon strips, the difference was not significant (P = 0.26). Autoclaved IB1ï treated nylon strips were more attractive to *An. funestus* (P < 0.001) and other anopheline mosquitoes (P < 0.007) compared to non-autoclaved IB1-impregnated nylon strips. Moreover, larger numbers of *Culex* spp. responded to non-autoclaved IB1-treated nylon strips than to autoclaved IB1-treated nylon strips (P < 0.042). In addition, similar numbers of *Mansonia* spp. responded to non-autoclaved and autoclaved IB1-treated nylon strips (P = 0.17).

Table 2: Mean number (±SE) of outdoor female mosquitoes attracted to autoclaved and non-autoclaved control nylon strips (without odour) or IB1-treated nylon strips at Kigoche village.

Treatment	**N**	**Mean number ± SE of mosquitoes caught**				
		***An. gambiae* s.l.**	***An. funestus***	***Culex* spp.**	***Mansonia* spp.**	**Other anophelines**
Control non-autoclaved nylon strips (no odour)	20	0.15 ± 0.09^{a}	0.65 ± 0.2^{a}	1.68 ± 0.27^{a}	0.13 ± 0.06^{a}	1.25 ± 0.22^{a}
Control autoclaved nylon strips (no odour)	20	0.60 ± 0.17^{a}	0.75 ± 0.19^{a}	3.20 ± 0.39^{b}	0.28 ± 0.12^{a}	1.18 ± 0.09^{a}
Non-autoclaved IB1-treated nylon strips	20	2.05 ± 0.32^{b}	3.30 ± 0.41^{b}	12.07 ± 0.77^{c}	0.56 ± 0.16^{b}	5.57 ± 0.52^{b}
Autoclaved IB1-treated nylon strips	20	2.60 ± 0.36^{b}	5.85 ± 0.54^{c}	10.03 ± 0.70^{d}	0.92 ± 0.22^{b}	7.56 ± 0.63^{c}

N is the number of experimental nights, SE is the standard error of the mean catch per night. Mean values within the same column having no letter in common differ significantly at P < 0.05 (Generalized Linear Models).

The 237 male mosquitoes caught comprised *An. gambiae* s.l. (26.2%), *An. funestus* (36.3%), *Culex* spp. (24.9%), *Mansonia* spp. (4.2%) and other *Anopheles* spp. (8.4%) (Table 3). Trap catches of *An. gambiae* s.l., *An. funestus* and *Culex* spp. were dependent on treatment type (P < 0.001 for all). Significantly more *An. gambiae* s.l. (P < 0.022), *An. funestus* (P < 0.004) and *Culex* spp. (P < 0.001) mosquitoes were attracted to autoclaved IB1-treated nylon strips

compared to non-autoclaved IB1-treated nylon strips. The mean numbers of *Mansonia* spp. and other anopheline mosquitoes collected between IB1-baited traps were not different (P = 0.14 and P = 1.00, respectively).

Table 3: Mean number (±SE) of outdoor male mosquitoes attracted to autoclaved and non-autoclaved control nylon strips or IB1-treated nylon strips at Kigoche village.

Treatment	N	Mean number ± SE of mosquitoes caught				
		An. gambiae s.l.	*An. funestus*	*Culex* spp.	*Mansonia* spp.	Other anophelines
Control non-autoclaved nylon strips (no odour)	20	0.25 ± 0.11^{a}	0.35 ± 0.13^{a}	0.65 ± 0.18^{a}	0.10 ± 0.07^{a}	0.15 ± 0.09^{a}
Control autoclaved nylon strips (no odour)	20	0.20 ± 0.10^{a}	0.55 ± 0.17^{a}	0.85 ± 0.21^{a}	0.10 ± 0.07^{a}	0.05 ± 0.05^{a}
Non-autoclaved IB1-treated nylon strips	20	0.90 ± 0.21^{b}	1.10 ± 0.23^{b}	0.25 ± 0.11^{b}	0.05 ± 0.05^{a}	0.40 ± 0.14^{a}
Autoclaved IB1-treated nylon strips	20	1.75 ± 0.30^{c}	2.30 ± 0.34^{c}	1.20 ± 0.24^{a}	0.25 ± 0.11^{a}	0.40 ± 0.14^{a}

N is the number of experimental nights, SE is the standard error of the mean catch per night. Mean values within the same column having no letter in common differ significantly at $P < 0.05$ (Generalized Linear Models).

Discussion

This study indicates that consistently more female *An. gambiae* mosquitoes were attracted to IB1-treated nylon strips than LDPE sachets and controls at weekly intervals during 52 repeated exposures spread out over one year post-treatment in a semi-field setting. After 52 nights of repeated exposures, additional volatile compounds and microbes were found on IB1-treated nylon strips. The most abundant bacteria were *B. thuringiensis* and *A. baumannii*. Volatile compounds released by each of the two bacteria grown on agar attracted significantly more female *An. gambiae* mosquitoes than agar alone in the olfactometer. Subsequent experiments demonstrated that both autoclaved and non-autoclaved IB1-treated nylon strips were equally attractive to female *An. gambiae* in a screened-walled greenhouse and also to wild *An. gambiae* s.l. and *Mansonia* spp. in a rice growing village of western Kenya. Whereas autoclaving enhanced the attractiveness of the IB1-treated nylon strips for female *An. funestus* and other anopheline mosquitoes, the majority of female *Culex* spp. responded to non-autoclaved IB1-treated nylon strips as opposed to autoclaved IB1-treated ones. The majority of male *An. gambiae* s.l., *An. funestus* and *Culex* spp. were caught in a trap baited with autoclaved IB1-treated nylon strips. There was insufficient DNA extracted from untreated

nylon strips as a quantitative measure for the amount of microbes on the original strips and therefore, further investigations are ongoing in a study that is not part of this thesis.

These findings confirmed that nylon strips provide a more suitable matrix for the dispensing of attractant odorants than LDPE sachets (Okumu et al., 2010a, Mukabana et al., 2012a). A semi-field study reported by Mukabana et al. (2012a) found that IB1-treated nylon strips were consistently more attractive to host-seeking mosquitoes than LDPE sachets filled with IB1 up to 40 consecutive nights of post-treatment. However, the results reported in this chapter also demonstrated that treated nylon strips sustain the release of individual components of IB1 for attraction of female *An. gambiae* over a one year post-treatment period when deployed at weekly intervals and stored at 4^{o}C between experimental nights. Thus, residual activity of attractant-treated nylon strips as shown in this study is an indicator of improved sustainability and robustness for the use of odour-baited tools during field operations. The findings agree with observations made by Pates et al. (2001) and Qiu et al. (2004) concerning long-term attraction of female *An. gambiae* to human emanations collected on nylon stocking. Nonetheless, the ultimate temporal effect of IB1-compounds and their derivatives on nylon strips has to be established as part of an evaluation process about efficient release systems for both insect repellents and attractants in a push-pull strategy (Cook et al., 2007, Takken, 2010).

Determination of whether original attractants were still available on treated nylon strips that had been used weekly over 52 nights post-treatment confirmed the presence of pentanoic acid and 3-methyl butanoic acid as the only components originally present in the IB1 blend. Some of the 26 additional compounds including 1-butanol, 3-methylbutanoic acid and 3-methyl-1-butanol are known to be produced by skin bacteria or found in skin sweat (Meijerink et al., 2000, Verhulst et al., 2009, Verhulst et al., 2010), while (*E*)-2-methyl-2-butenal, pentanoic acid and styrene are produced on human skin (Bernier et al., 2000, Wood and Kelly, 2010). Whereas 1-pentanol is known to be found in human sweat and also produced by yeast (Meijerink et al., 2000, Smallegange et al., 2010b), benzyl alcohol is a constituent of hand odour as well as a product of bacterial activity (Schulz and Dickschat, 2007).

These observations suggest that IB1-treated nylon strips may have become contaminated with additional chemicals. The finding of bacteria on the nylon strips after one year suggests that some of these chemicals may be of bacterial origin (Verhulst et al, 2010). For example, 1-butanol and 3-methyl-1-butanol are bacterial break-down products of tetradecanoic acid on human skin (Verhulst et al., 2009, Verhulst et al., 2010). Indeed, a novel synthetic odour blend of 3-methyl-1-butanol, L-lactic acid, ammonia and tetradecanoic acid has been shown to be more attractive to host-seeking *An. gambiae* s.l. and *An. funestus* compared to blend IB1 under semi-field and field conditions (Verhulst et al., 2011b, Mukabana et al., 2012b). Furthermore, nonanal detected in the headspace of IB1-treated nylon strips is one of the human-derived volatile compounds that play an integral role in differential attractiveness of human volunteers to mosquito vectors (Logan et al., 2008). Individuals that release higher

quantities of three aldehydes (octanal, nonanal and decanal) and two ketones (geranylacetone and 6-methyl-5-hepten-2-one) have been shown to be ónattractiveô to *Aedes aegypti* Linnaeus and *An. gambiae* mosquitoes, compared to those emitting lower quantities of these compounds that are óattractiveô(Logan et al., 2008, Logan, 2008).

Host-seeking behaviour of *An. gambiae* and other dipterans is mediated by VOCs produced from either skin glands or skin microflora, or both (Braks et al., 1999, Takken and Knols, 1999). Verhulst et al. (2010, 2011a). Subsequent evidence has shown that VOCs produced by skin microbiota affect differential attractiveness of humans through stimulation or inhibition of host-seeking responses of mosquito vectors whereas some are neutral (Verhulst et al., 2010, Verhulst et al., 2011a). For example, the attractiveness of a standard blend (ammonia, L -lactic acid and tetradecanoic acid) to *An. gambiae* mosquitoes was reduced by addition of 2-phenylethanol but enhanced when combined with 3-methyl-1-butanol (Verhulst et al., 2011a). Exploitation of the association between human skin microbiota, and the production of odorous compounds that function as kairomones for host-seeking mosquitoes has been reported (Verhulst et al., 2009, Verhulst et al., 2010, Verhulst et al., 2011b).

It has also been proposed that human attractiveness to *An. gambiae* and other mosquitoes is affected by species composition, density, and metabolic activity of the skin microbiota (Verhulst et al., 2009, Verhulst et al., 2010). However, there is no information about the type and role of microbes that colonize attractant-treated nylon strips. Indeed, this potential was demonstrated by higher attraction of female *An. gambiae* mosquitoes to volatiles produced by *B. thuringiensis* and *A. baumannii* bacteria than to agar alone in the olfactometer. The findings suggest that residual attractiveness of IB1-treated nylon strips over time is likely to be affected by microbes that establish on strips over time and it could be in part be ascribed to the emission of microbe-produced volatiles. Additionally, plating of IB1-treated nylon strips resulted in more bacterial growth than plating of a nylon sock that had been worn for only 12 h, possibly because treated nylon strips had been exposed for a longer time (i.e. 52 nights) and this may have resulted in increased bacterial activity (Braks and Takken, 1999).

In a subsequent experiment, sterilisation of IB1-treated nylon strips by autoclaving prior to deployment did not cause any appreciable effect on the attractiveness of IB1-treated nylon strips to host-seeking *An. gambiae* s.l. and *Mansonia* mosquitoes. However, the influence was more profound on natural populations of female *An. funestus, Culex* spp., other anophelines, male *An. gambiae* s.l *An. funestus* and *Culex* spp. It is likely that, upon deployment, microbes may have colonised IB1-treated nylon strips and produced kairomones, repellent or neutral compounds. The compounds may have interacted with original attractants that were applied to the nylon strips thereby modulating the host-seeking responses of mosquitoes. Autoclaving is often used to kill bacteria in a medium or agar plates, leaving behind VOCs already produced. Heating may also have helped to volatilize attractant compounds or changed the composition of the nylon and/or synthetic components thereby influencing the composition and

concentration of the odour plume encountered by the mosquitoes. Moreover, destruction of the micro-organisms by autoclaving may have resulted in release of attractive volatiles from the microbial cells and adsorption to the nylon. These tentative suggestions require evidence-based explanations hence the need for further investigations. Alternatively, sterilisation by use of UV radiation or sterile treatment of the strips with chemicals could be considered in future experiments. Because the effect of microbial volatiles on the behavioural response of *An. gambiae* has been studied (Verhulst et al., 2009, Verhulst et al., 2010), but not of other mosquito species including *An. funestus*, *Culex* spp and other *Anopheles* species, it is difficult to conclude how microbes may affect such mosquitoes. This can be shown by examining their responses to volatile compounds produced when similar bacteria are grown on agar.

It is expected that autoclaving of IB1-treated nylon strips results in the loss of volatile compounds that were on the strips thereby reducing their attractiveness to host-seeking mosquitoes. However, this was not the case for *An. gambiae s.s, An. gambiae* s.l., *An. funestus, Mansonia* and other anopheline species except *Culex* spp. It is likely that, in spite of continued loss of volatile compounds from treated nylon strips over time as a result of repeated exposure or autoclaving, mosquitoes still had the capacity to respond to traces of the original compounds that were left behind. Perhaps, traces of original compounds found on treated nylon strips were below the detection rate of GC-MS. Recent evidence has shown the responses of *An. gambiae* to attractant blends containing very low dilutions of 3-methylbutanoic acid (Okumu et al., 2010a), 3-methyl-1-butanol (Mukabana et al., 2012b) and 1-butylamine (Chapter 7). At the moment, experiments aimed at unravelling the effect of VOCs produced by microbes on long-term residual attractiveness of IB1-treated nylon strips to host-seeking mosquitoes are in progress. The findings may facilitate kairomone identification and storage of odorant treated-fabrics prior for field operations and the optimal post-treatment period of effectiveness for the attraction of malaria vectors.

The collection of mosquitoes during a dry season coincided with maturing of the rice crop near Kigoche village, and therefore prevailing conditions seem to have favoured a higher abundance of *An. funestus* compared to *An. gambiae* s.l. (Chandler et al., 1975; Imbahale et al., 2011). This is because *An. funestus* breeds in slow-moving water containing emergent vegetation whereas *An. gambiae* and *An. arabiensis* prefer temporary, shallow and sunlit water bodies (Imbahale et al., 2011). Similar to previous studies, the odour bait used was more selective for female than male mosquito populations because only females engage in blood feeding (Okumu et al., 2010a, Jawara et al., 2011, Mukabana et al., 2012b). The odour blend was also associated with human host odorants to which host-seeking female mosquitoes respond. Thus, the blend may be deployed effectively for sampling, surveillance as well as intervention of mosquito vectors through mass trapping, mating disruption and lure and kill strategies (Kline and Lemire, 1998, Kline, 2007).

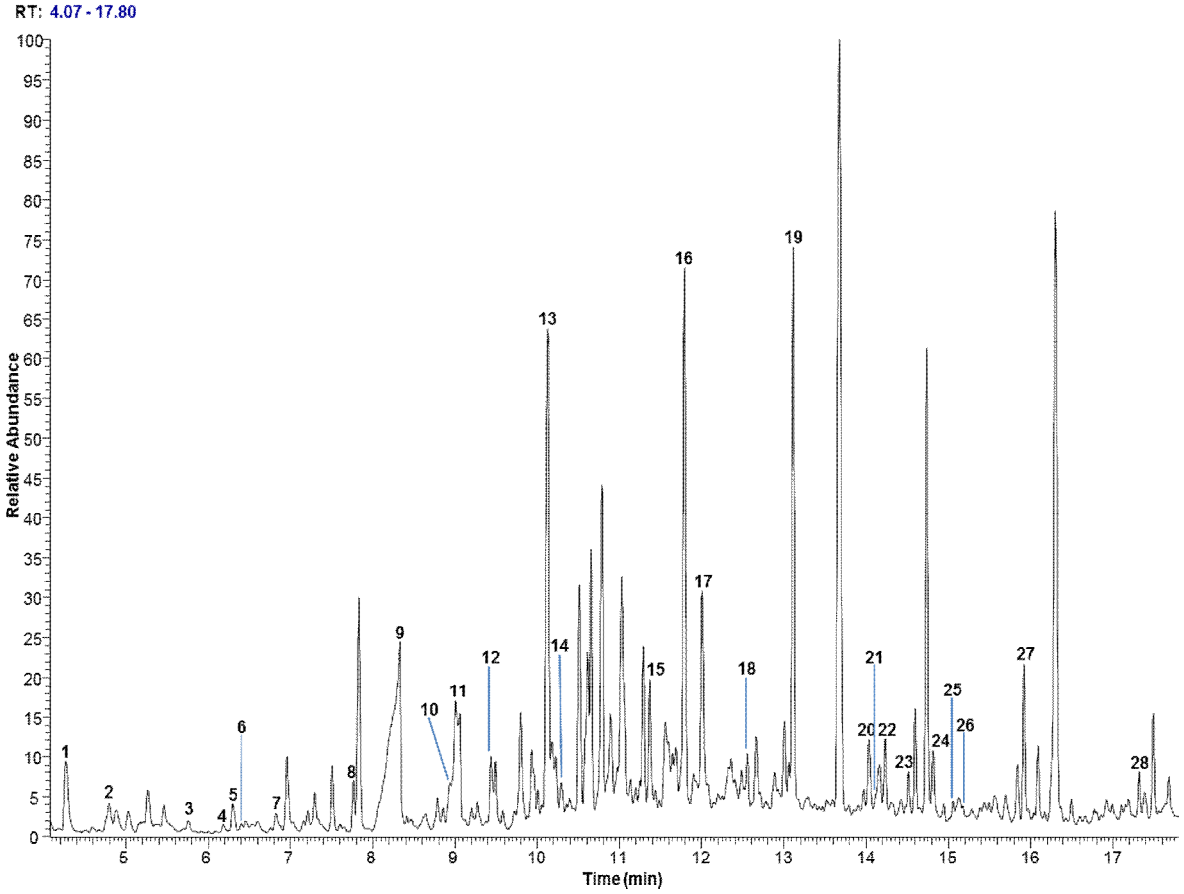
Conclusion

This study has demonstrated the feasibility and sustainability of odour-baited technologies for sampling and surveillance of malaria and other mosquito vectors. However, preliminary results on the presence of additional VOCs and the potential role of microbes on residual activity of attractant-treated nylon strips require further investigations.

Acknowledgements

We thank David Alila and Andrew Brian Abuya for rearing mosquitoes used in our semi-field experiments, Marjolein de Rijk for analysis of VOCs released from IB1-treated nylon strips and Frans Jacobs for sequencing bacterial isolates obtained from treated nylon strips. We acknowledge Fred Kisanya and Susan Imbahale for hosting our laboratory activities at the AMDTI. Our thanks also go to the inhabitants of Kigoche Village including Charles Oketch for allowing us to work in their homesteads. This study was funded by a grant obtained from the Foundation of National Institutes of Health (FNIH) through the Grand Challenges in Global Health Initiative (GCGH#121) and a sandwich Ph.D. scholarship of Wageningen University and Research Centre, The Netherlands to CKM. CKM was hosted by ICIPE ï MPR&TC as a dissertation research internship scholar.

Appendix



Appendix 1: Chromatogram of volatile compounds detected in the headspace of IB1-impregnated nylon strips after 52 post-treatment nights of exposure for collection of female *An. gambiae* at weekly intervals. Numbers indicated next to each peak correspond with those in Table 1. The identification number of each of the 28 compounds listed in Table 1 is shown next to corresponding peaks in this chromatogram. Peaks without a number represent contaminants derived from other breakdown products of the Tenax adsorbent.

Chapter 5

Long-lasting attraction of malaria vectors to synthetic odour baits placed indoors and outdoors in western Kenya

To be submitted in a slightly modified form as: Mweresa, C.K., Omusula, P., Otieno, B., van Loon, J.J.A., Takken, W. and Mukabana, R. W. Long-lasting attraction of malaria vectors to synthetic odour baits placed indoors and outdoors in western Kenya

Abstract

The development of effective odour-baited trapping tools for monitoring and control of mosquito vectors requires sustained release of behaviourally-active semiochemicals. The present study was designed to investigate if attractant-treated nylon strips and low density polyethylene (LDPE) sachets used at weekly intervals remain attractive to indoor and outdoor host-seeking malaria vectors up to one year post-treatment. Behavioural responses of the main vectors of malaria and other mosquito species to attractant-treated devices were evaluated through a randomised Latin square experimental design in western Kenya. Responses of natural populations of mosquitoes to synthetic odour baits were compared with natural human emanations using CDC traps. The study was simultaneously conducted inside and outside traditional village houses at weekly intervals for 48 nights spread over one year.

Attractant-treated nylon strips and LDPE sachets were consistently attractive to indoor and outdoor host-seeking *Anopheles gambiae* sensu lato and *An. funestus* malaria vectors up to one year post-treatment. Whereas dispensing of synthetic blend IB1 from both nylon strips and LDPE sachets had no effect on outdoor trap catches of *An. gambiae* s.l. ($P = 0.47$), the latter was significantly more attractive to *An. funestus* ($P < 0.003$). Humans and IB1-treated nylon strips resulted in similar catches of indoor-biting *An. gambiae* s.l. ($P = 0.91$) and each of them was significantly more attractive than IB1 dispensed from LDPE sachets ($P < 0.002$ and $P < 0.001$, respectively). Humans lured significantly more *An. funestus* into houses compared to blend IB1 released from nylon strips ($P < 0.013$) or LDPE sachets ($P < 0.001$). IB1-treated nylon strips were significantly more attractive to indoor-biting *An. funestus* compared to IB1 contained in LDPE sachets ($P < 0.001$). The majority of trapped malaria vectors was unfed. *Anopheles gambiae* s.l. dominated outdoor collections, fed either on bovines (62.5%) or on humans (37.5%) and comprised *An. gambiae s.s.* (2.1%) and *An. arabiensis* (97.9%). However, *An. funestus* were more common in indoor catches, and had fed more often on humans (66.7%) than on cattle (33.3%). One *An. funestus* was tested positive for *P. falciparum* sporozoites. This study demonstrates the long-lasting residual activity of nylon strips treated with attractive synthetic odour bait on malaria vectors. As the odour-baited traps caught high numbers of mosquitoes, they may be considered for routine monitoring and even control of malaria vectors.

Introduction

Substantial reductions in malaria morbidity and mortality have been realized in the recent past due to intensification of mass drug administration, use of indoor residual spraying and long-lasting insecticidal bed nets (Mendis et al., 2009). However, mosquito vector species continue to sustain high levels of malaria transmission in endemic areas where some bite outdoors, hence the need for developing novel monitoring and intervention tools (Alonso et al., 2011, Govella and Ferguson, 2012). Such tools should ideally target different behavioural phases of the vector including outdoor feeding and resting, oviposition, mating, sugar feeding and host seeking. Semiochemicals play a prominent role in all these behavioural phases (Takken and Knols, 1999), and exploitation of such cues for behavioural interference may provide novel strategies for mosquito control (Zwiebel and Takken, 2004, Logan and Birkett, 2007).

Recently, the utilization of kairomones for manipulation of host-seeking behaviour of the malaria vector *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *An. gambiae*) was successfully demonstrated (Mukabana et al., 2010, Smallegange and Takken, 2010). The advantage of this technology is that odour-baited traps provide a complementary tool for targeting both outdoor and indoor biting malaria vectors (Kline, 2007, Alonso et al., 2011). However, consistently high trap catches of such vectors require efficient substrates which allow sustained release of odorant compounds over long periods of time (Torr et al., 1997, Cork, 2004). Previous studies have demonstrated that low density polyethylene (LDPE) sachets serve this purpose (Cork, 2004). Indeed, LDPE sachets have been effectively deployed to dispense synthetic attractants of host-seeking malaria mosquitoes and other mosquito vectors under field conditions (Jawara et al., 2011, Verhulst et al., 2011, Mukabana et al., 2012a). Tsetse flies are routinely controlled using LDPE sachets for the release of odorant attractants, where the flies were found to respond to the odorant cues from several tens of meter distance (Torr et al., 1997). Recent field studies indicated that traps baited with attractants contained in LDPE sachets collected fewer indoor biting *An. gambiae* s.l. and *An. funestus* Giles compared to similarly-baited nylon strips (Okumu et al., 2010, Mukabana et al., 2012a). Additional semi-field studies have also demonstrated that attractant-treated nylon strips do lure consistently and significantly more host-seeking *An. gambiae* mosquitoes into traps than similarly baited LDPE sachets in the short-term for 40 consecutive nights of post-treatment (Mukabana et al., 2012b).

Residual activity is important because it reduces time and labour for preparation of fresh baits as well as costs of odorants and nylon strips used. Semi-field studies indicated that, because of residual activity, treated nylon strips sustained attraction of more *An. gambiae* at weekly intervals for 52 nights post-treatment than LDPE sachets filled with similar attractants (Chapter 3). However, nothing has been reported about the long-term residual activity of

attractant-treated nylon strips on host-seeking *An. gambiae* and other important malaria vectors under field conditions. Therefore, longitudinal experiments were performed in a rice agro-ecosystem village in western Kenya. The specific objectives of the present study were to: (a) investigate if attractant-treated nylon strips and LDPE sachets remain attractive to outdoor- and indoor-biting host-seeking malaria vectors up to one year post-treatment, (b) evaluate the effect of long-term residual activity of attractant-treated materials on indoor collections of malaria vectors of different abdominal status, (c) determine the blood meal source of blood-fed malaria vectors collected, and (d) estimate the *Plasmodium falciparum* infection rate of *An. gambiae* s.l. and *An. funestus* collected in the traps.

Materials and Methods

Field study site

The present study was conducted between July 2011 and June 2012 at Kigoche village near Ahero Market in Kisumu County, western Kenya. The village is located in the flood plain of River Nyando at 00°34ôS, 034°65ô E at 1158 m above sea level. Annual rainfall ranges between 1000 - 1800 mm, temperature between 17.0 - 32.0°C and relative humidity (RH) is approximately 65.0%. Malaria endemicity is mainly sustained by *P. falciparum* parasites transmitted by *An. gambiae sensu stricto*, *An. arabiensis* Patton and *An. funestus* (Githeko et al., 1993, Mukabana et al., 2012a). The predominant breeding sites of mosquitoes include small water pools, hoof and foot prints, rice paddies, shaded and unshaded irrigation water channels and streams. Irrigated rice farming is the main economic activity. Supplementary traditional farming of maize, millet, bananas, sweet potatoes, beans, cassava, sorghum and rearing of indigenous cattle, goats, sheep and poultry is also practiced. At night cattle, goats and sheep are tethered outside, adjacent to houses where owners dwell. A majority of the residents live in mud-walled houses, roofed with corrugated iron sheets, without ceiling, with one or two rooms, one door and open eaves.

Ethical approval

The present study was approved by the scientific and ethical 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 resident volunteers before permission to carry out the study was sought. Written informed consent was obtained from three male volunteers aged between 22 and 25 years old. These volunteers slept under the untreated bed nets.

Selection and characteristics of study houses

Three village houses were randomly selected for trapping outdoor biting mosquitoes in their surroundings whereas four were utilised for collections of indoor biting mosquitoes. Selected houses measuring between 15.8 and 22.5 m^2, had not been treated with indoor residual sprays. The houses located at a distance of 28 - 631 m apart (Sumaye et al., 2012, Mukabana et al., 2012a) were aligned along a transect on the edge of a rice growing field. Each house was located 10 - 20 m away from a cowshed and within a range of 100 m away from irrigation water channels and rice paddies. The exact location of individual houses was determined by a hand-held global positioning system (GPS) receiver (Trex HC series, Garmin International, Inc, USA). House numbers were indicated on the door for random assignment and systematic rotation of treatments.

Meteorological conditions

Indoor and outdoor temperature and RH of study houses were simultaneously recorded at an interval of 30 min using a data logger (Tinytag® Ultra, model TGU-1500, INTAB Benelux, The Netherlands). During the same period, monthly data of outdoor conditions of air temperature, RH and rainfall data were obtained from a weather station located at the Ahero Irrigation Research Station (AIRS), 800 m away from the study village.

Preparation and dispensing of a synthetic mosquito odour bait

A synthetic mosquito attractant referred to as the Ifakara blend 1 (IB1) was used (Okumu et al., 2010). Optimal dilutions of individual attractant compounds were released using two types of matrix: nylon strips and LDPE sachets (Okumu et al., 2010, Mukabana et al., 2012a). Carbon dioxide was supplied through silicon tubing (0.5 cm internal diameter). The nylon strips and LDPE sachets containing blend IB1 were separately suspended inside the odour plume tube of individual MM-X counter flow traps (American Biophysics, North Kingstown, RI, USA) (Okumu et al., 2010). Carbon dioxide produced by mixing 250 g of refined sugar, 17.5 g of dry instant yeast (Smallegange et al., 2010) and 2 L of river water (Mukabana et al 2012a) was delivered into both IB1-baited traps at a flow rate of ~63 ml/min through a silicon tubing. A single MM-X trap without odour stimulus was also included in outdoor experiments to serve as negative control.

During experiments (18:30 ï 06:30 h), the odour outlet tube of individual traps was positioned 15 cm off the ground level (Schmied et al., 2008, Jawara et al., 2009) or mattress on a bed. All MM-X traps were operated on a 12 V car battery. The IB1-impregnated nylon strips were used once per week for 48 weeks post-treatment spread over one year. During the study, the treatments were used one night per week and then stored at 4^oC between

experimental nights. Whereas IB1-treated nylon strips were used repeatedly throughout the entire period, blend IB1-containing LDPE sachets were only replaced upon leakage or depletion (Mukabana et al., 2012b). Thus, all LDPE sachets containing blend IB1 were weighed before and after single experimental nights to determine the need for refilling depleted attractant compounds. The sugar-yeast mixture, which served as a source of CO_2 was prepared 30 min prior to the start of every experimental night. All traps were marked and baited with the same treatment (attractant-treated or control nylon strips or LDPE sachets) throughout the study. Treatments were systematically and simultaneously alternated on a weekly basis among houses used for either indoor or outdoor experiments to overcome competition for mosquitoes, site and house effect.

Reference monitoring of outdoor biting mosquitoes

For comparison of mosquito diversity during the experimental period, one standard Centre for Disease Control (CDC) miniature trap with light (Model 512; John W. Hock Company, Gainesville Fl. USA) was used. The trap was operated on a 6 V battery (Gaston Battery Industry Ltd, China) and placed outdoors next to a human-occupied house, approximately 100 m away from houses where indoor and outdoor mosquito collections were made (Qiu et al., 2007, Jawara et al., 2011).

Long-term activity of attractant-treated materials on outdoor biting malaria vectors

Long-lasting attraction of outdoor biting malaria and other mosquitoes to blend IB1 released from nylon strips and LDPE sachets was evaluated at weekly intervals up to 48 weeks post-treatment over one year. This was achieved through a randomised 3 × 3 Latin Square experimental design comprising three treatments namely (a) empty MM-X trap without odour (control), (b) MM-X trap baited with LDPE sachets filled with IB1, and (c) MM-X trap containing IB1-impregnated nylon strips. Individual MM-X traps for each treatment were suspended outside the bedroom but under eaves of separate village houses occupied and used routinely by 1 to 4 dwellers. In between the weekly experiments the nylon strips were stored in a refrigerator.

Long-term activity of attractant-treated materials on indoor catches of malaria vectors

The efficacy of blend IB1 dispensed from nylon strips and LDPE sachets to attract malaria vectors and other mosquitoes into village houses was compared to the attractiveness of three human volunteers aged 22 - 25 years. This was carried out in a randomised 4 × 4 Latin Square design comprising (a) no stimulus (negative control), (b) IB1-contained in LDPE sachets, (c) IB1-impregnated nylon strips, and (d) a human volunteer. House-entering mosquitoes were collected in unlit CDC traps placed next to an untreated bed net from which the host stimuli

and blend IB1 were released (Mboera et al 1998; Okumu et al 2010) (Figure 1). In the control house, the bed was covered by a bed net without a host or synthetic odour stimulus. All bed-nets were new and had not been treated with insecticides or repellents.

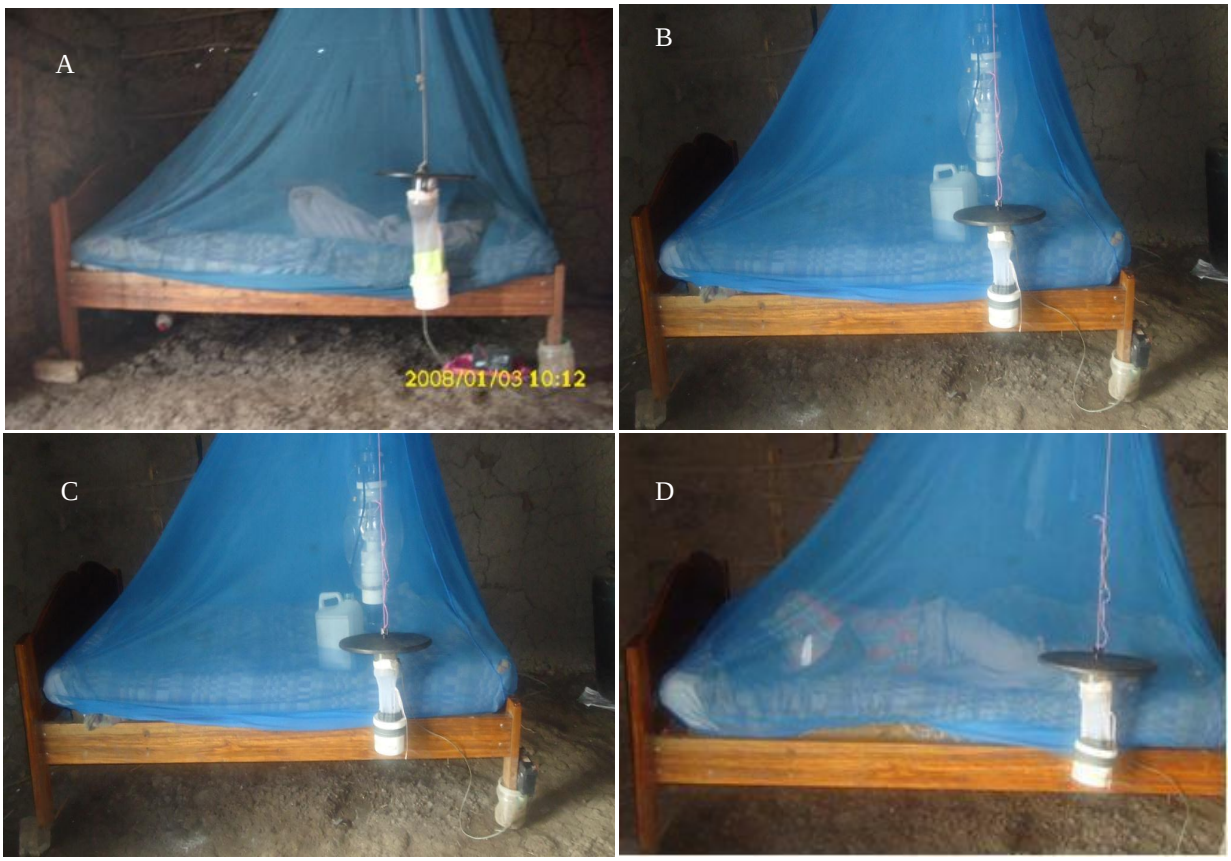


Figure 1: Picture showing indoor collection of mosquitoes in a village house containing a bed without a bait (panel A), a MM-X trap baited with blend IB1 dispensed from LDPE sachets (panel B) or IB1-treated nylon strips (panel C) and, a human volunteer (Panel D) under a bed net without insecticide treatment. House-entering mosquitoes were collected in unlit CDC traps placed next to the bed nets. The experiments were conducted in Kigoche village at weekly intervals for 48 nights (18:30 ï 06:30 h) spread over one year (July 2011 ï June 2012) post-treatment.

The human subjects were non-smokers, did not consume alcohol, and were free from malaria and chronic illness. Volunteers were also tested for malaria in the morning before experiments and those confirmed positive were treated with artemether-lumefantrine, and exempted from participating in the experiments for one week. Houses selected for this particular experiment were not used for any other purpose over the study period while neighbouring houses remained under normal occupation and routine utilization by their owners.

Each study house had a mattress placed on a bed on which a volunteer slept or over which a MM-X trap baited with IB1 dispensed from nylon strips or LDPE sachets was suspended. The odour outlet of the MM-X trap was 15 cm above the mattress. The suction fan of the MM-X

trap was disabled for it to dispense an odour plume without trapping attracted mosquitoes (Okumu et al., 2010). Each bed net was completely tucked under the mattress to prevent mosquitoes from accessing human volunteers or IB1-baited traps. Only one out of the four houses was occupied by a human volunteer during each experimental night. The three volunteers slept alternately in all experimental houses.

Collection and identification of mosquitoes trapped indoors and outdoors

At the end of each experimental night, the fans of all traps were turned off. Both MM-X and CDC traps used to collect indoor and outdoor biting mosquitoes, respectively, were transported to a field laboratory at the Ahero Multipurpose Development Training Institute (AMDTI) and frozen at -4^{o}C for 30 min. The immobilized mosquitoes were emptied into labelled Petri dishes and identified based on morphological features as male and female *An. gambiae* s.l., *An. funestus*, *Culex* spp., *Mansonia* spp., and other anopheline mosquitoes (all anopheline spp. caught except *An. gambiae* s.l. and *An. funestus*) (Gilles and Coetzee, 1987), counted and recorded. The gonotrophic status of female samples of *An. gambiae* s.l. and *An. funestus* was classified morphologically as unfed, blood-fed (both partially and fully fed) or gravid and preserved in 2 ml Eppendorf tubes containing silica gel covered with a thin layer of cotton wool. The preserved samples were analysed for (a) blood meal source, (b) *Plasmodium* sporozoite infections and (c), identity of sibling species within the *An. gambiae* species complex using the rDNA-polymerase chain reaction (PCR) assay (Scott et al., 1993). A random selection of 100 female *An. gambiae* s.l. from all indoor and outdoor collections was used for identification of sibling species. All analyses were carried out at the Centre for Global Health Research, Kenya Medical Research Institute (KEMRI), Kisumu, Kenya.

Determination of blood meal source

The abdomens of 24 blood-fed *An. gambiae* s.l. and 12 *An. funestus* mosquitoes trapped during the study were cut off transversely at the joint with the thorax. Individual posterior abdominal portions containing blood-meals were placed in separate vials, labelled and identified by a direct enzyme-linked immunosorbent assay (ELISA) (Beier et al., 1988). Each sample was screened simultaneously for a human or bovine source of blood meal.

Estimation of *Plasmodium* infection rate of *An. gambiae* s.l. and *An. funestus*

One hundred females of *An. gambiae* s.l. and 100 *An. funestus* were randomly selected from each treatment for sporozoite analysis. The head and thorax of individual *An. gambiae* s.l. and *An. funestus* were tested singly for expression of circumsporozoite proteins (CSP) of *P. falciparum* by an enzyme-linked immunosorbent (CSP-ELISA) assay (Beier et al., 1990.). A follow-up malaria prevalence survey was conducted by using malaria clinical cases recorded

monthly at 10 medical facilities adjacent to the study area over a two-year period (i.e. 2011 ï 2012) and those confirmed by microscopy.

Data analysis

The effect of climatic variables on monthly catches of *An. gambiae* s.l. and *An. funestus* observed from different treatments was determined by multiple backward regression (Minakawa et al., 2004). A Generalized Linear Model with Poisson distribution linked to a log function (dispersion estimated) was used to investigate the effect of residual activity of attractant-treated materials on capture rates of *An. gambiae* s.l. and *An. funestus* over time. The same model was used to determine the influence of treatment, house and day on different physiological conditions of both malaria mosquitoes (Smith, 1995, Verhulst et al., 2011). All analyses were carried out using IBM SPSS statistical software, version 19 ($P < 0.05$).

Results

Meteorological conditions and mosquito diversity

A mean temperature of 24.2 ± 0.2^{o}C, 60.2 ± 1.2 % RH and total rainfall of 1,114 mm were recorded outdoors over the entire study period (July 2011 ï June 2012). At the same time, an average temperature of 22.3 ± 0.3^{o}C and 78.2 ± 2.2 % RH prevailed indoors. Rainfall showed a significant positive fluctuation with both outdoor and indoor RH ($P < 0.011$ for both). Both indoor ($P = 0.94$) and outdoor ($P = 0.29$) temperatures remained consistently stable over time. Heavy rainfall associated with floods affected mosquito collections for one week (August 2011 and April 2012), and two weeks (in May 2012). This is why weekly mosquito collections were conducted for 48 nights instead of 52. In contrast, rainfall was very low in January 2012. Partial regression coefficients indicated no significant effects of monthly rainfall, mean RH or temperature on monthly catches of *An. gambiae* s.l. or *An. funestus* attracted to individual traps over time ($P > 0.05$) (Figure 2).

The diversity of malaria vectors caught in a CDC light trap deployed outdoors for monitoring mosquitoes abundance during the study was compared with unbaited and IB1-baited MM-X trap collections. A total of 969 femlae *An. gambiae* s.l. were caught outdoors in a MM-X trap without abait (1.4%), baited with blend IB1 dispensed from LDPE sachets (23.5%), nylon strips (28.7%) and alit CDC trap (46.3%) used for monitoring. Of the 389 outdoor biting female *An. funestus*, 3.3% were collected in unbaited MM-X trap while the rest were attracted to IB1-filled LDPE sachets (22.4%), IB1-impregnated nylon strips (55.3%) or alit CDC trap (21.3%).

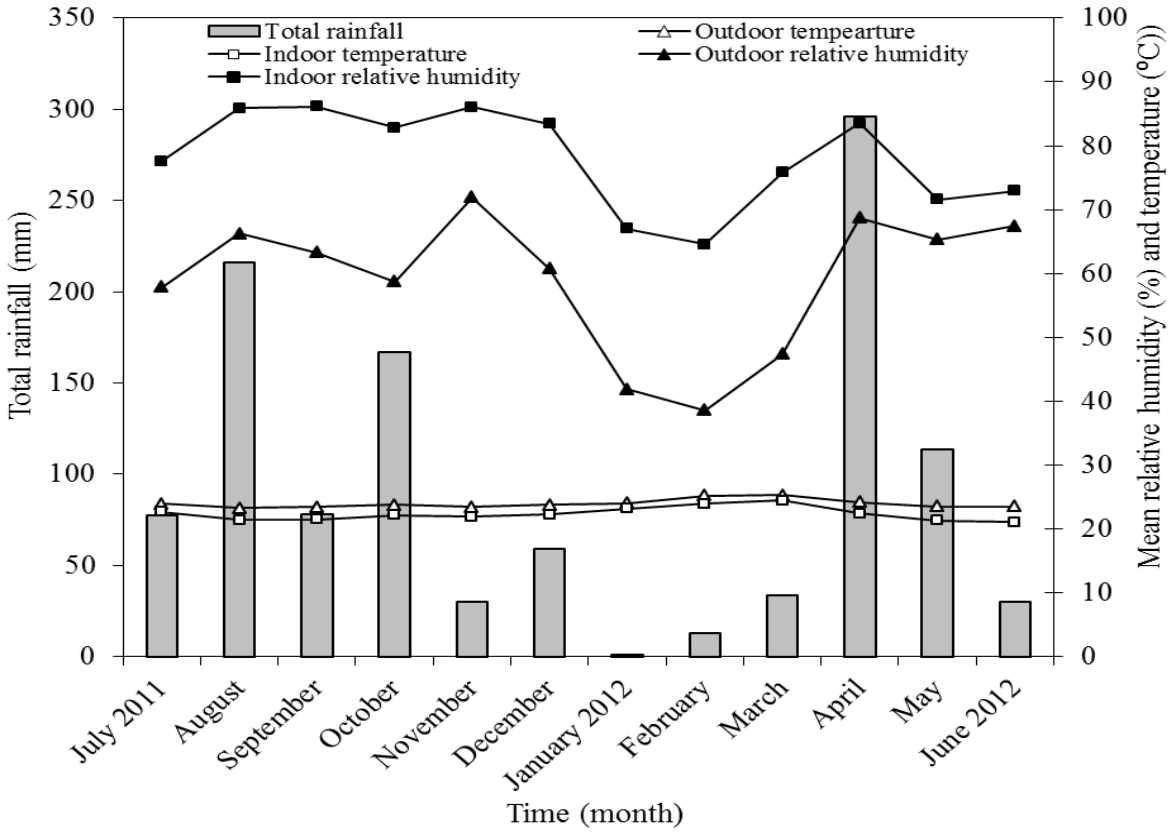


Figure 2: Average monthly values of ambient outdoor and indoor climatic conditions recorded during a one year (July 2011 ï 2012) study period in Kigoche village. Climatic variables are indicated in the legend.

Outdoor biting malaria vectors

More female mosquitoes (n = 2,486, 86.7%) were caught outdoors than males (n = 382, 13.3%) during 48 nights of weekly collections over one year post-treatment period of nylon strips. Female mosquito catches comprised *An. gambiae* s.l. (n = 520, 20.9%), *An. funestus* (n = 315, 12.7%), *Culex* spp. (n = 1,225, 49.43%), *Mansonia* spp. (n = 254, 10.2%) and other anopheline spp. (n = 172, 6.9%).

There was no difference in the attraction of female *An. gambiae* s.l. to IB1-filled LDPE sachets (P = 0.70) and IB1-treated nylon strips (P = 0.62) over time. The 520 *An. gambiae* s.l. caught during the study were found in a trap without a bait (5.0%), baited with IB1 dispensed from LDPE sachets (43.1%), and IB1-treated nylon strips (51.9%) (Figure 3A). The responses of *An. gambiae* s.l. to IB1 applied on nylon strips and dispensed from LDPE sachets were not statistically different (P = 0.47). IB1-baited traps attracted more *An. gambiae* s.l. than unbaited traps (P < 0.001). Trap collections of female *An. gambiae* s.l. increased in September 2011 and May 2012, preceding months of heavy rainfall and floods. In general, the 520 *Anopheles gambiae* s.l. caught in three traps over the 48 nights post-treatment period were unfed (82.9%), blood fed (3.1%) and gravid (14.0%).

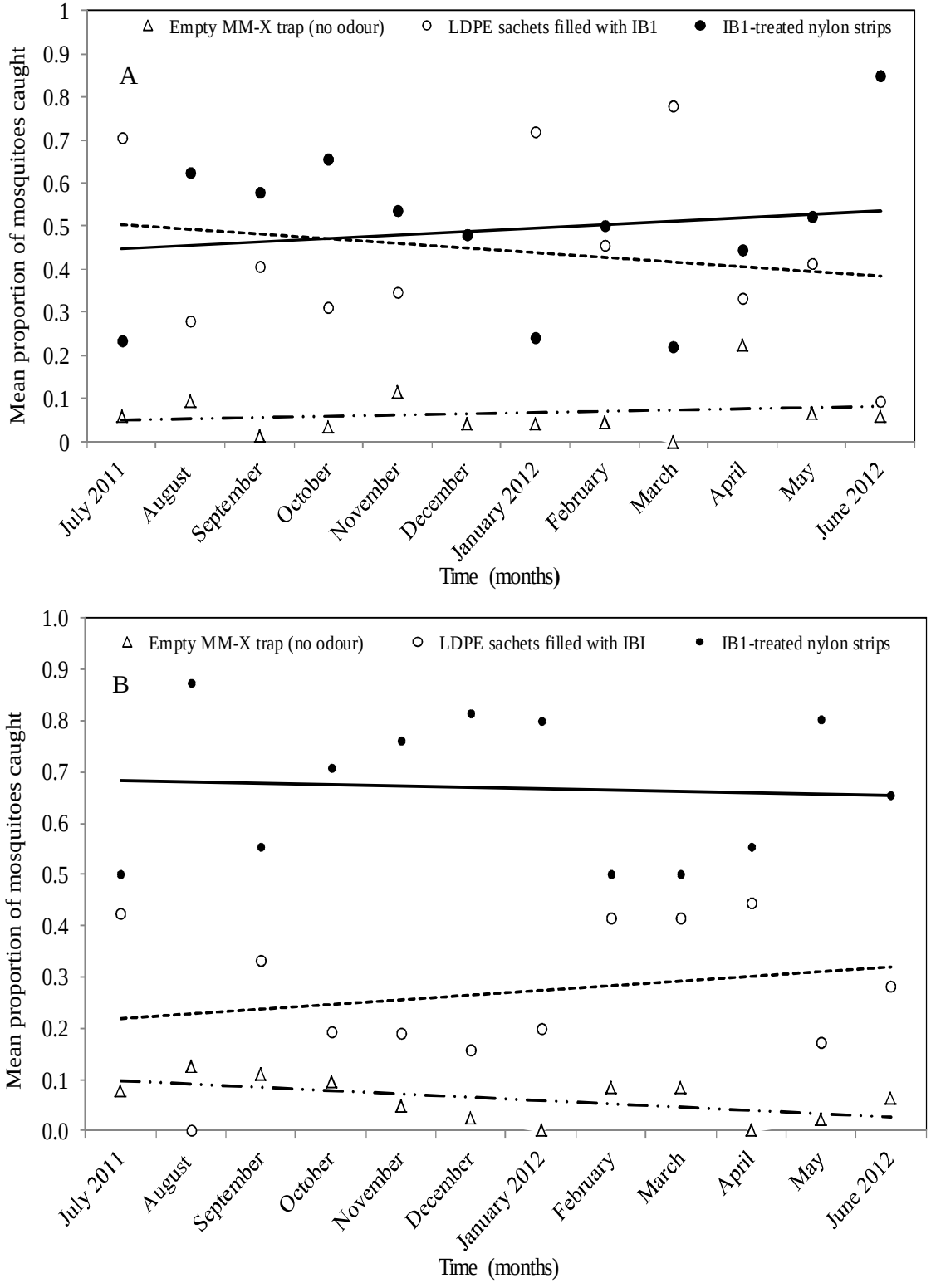


Figure 3: Mean monthly proportions of wild female *An. gambiae* s.l. (panel A) and *An. funestus* (panel B) caught outside village houses in an unbaited MM-X trap (no odour) (Ƶ - - Ƶ), a MM-X trap baited with IB1-contained in LDPE sachets (--------) and, a MM-X trap baited with IB1-treated nylon strips (Ƶ) at weekly intervals for 48 nights spread out over one year post-treatment. Legends in both panels indicate observed mean proportions of mosquitoes caught monthly in each treatment. Lines represent trends of monthly proportions of mosquitoes attracted by outdoor traps over time in Kigoche village.

The responses of female *An. funestus* to blend IB1 dispensed from LDPE sachets (P = 0.13) and IB1-treated nylon strips (P = 0.53) were consistent over time. The 315 *An. funestus* mosquitoes caught in the course of the study were found in a trap without a bait (6.0%), baited with LDPE sachets filled with blend IB1 (26.7%) and IB1-treated nylon strips (67.3%) (Figure 3B). Blend IB1 was significantly more attractive to *An. funestus* when released from nylon strips than from LDPE sachets (P < 0.003). Generally, the 315 *An. funestus* mosquitoes collected at weekly intervals over 48 nights post-treatment included unfed (90.5%), blood-fed (2.2%) and gravid (7.3%) physiological stages.

Indoor biting malaria vectors

More female mosquitoes (n = 1,035, 86.0%) than males (n = 169, 14.0%) were trapped over the entire study period. Female mosquitoes comprised *An. gambiae* s.l. (24.6%), *An. funestus* (39.2%), *Culex* spp. (30.4%), *Mansonia* spp. (2.5%) and other *Anopheles* spp. (3.2%). Trap collections of female *An. gambiae* s.l. (n = 255, 38.6%) were lower compared to *An. funestus* (n = 406, 61.4%).

There was no change in the attraction of female *An. gambiae* s.l. into houses occupied by a human (P = 0.28), baited with blend IBI released from LDPE sachets (P = 0.22) and nylon strips (P = 0.39) over time (the 48 nightly collections spread over one year). The 255 *An. gambiae* s.l. collected were unfed (85.9%), blood-fed (3.1%) or gravid (11%) (Table 1). Human odour and IB1-impregnated nylon strips were equally attractive to unfed *An. gambiae* s.l. (P = 0.55), and each of them was significantly more attractive compared to blend IB1 dispensed through LDPE sachets (P < 0.001 for both). Indoor collections of blood-fed and gravid *An. gambiae* were not different among treatments (P = 0.48 and P = 0.06, respectively).

Trap collections of female *An. funestus* mosquitoes into houses occupied by a human (P = 0.48), IB1 released from LDPE sachets (P = 0.74) and IB1-treated nylon strips (P = 0.83) were consistent over the entire post-treatment period. The 406 *An. funestus* mosquitoes trapped were unfed (95.1%), blood-fed (1.2%) and gravid (3.7%) (Table 1). Humans attracted significantly more unfed *An. funestus* than IB1 dispensed from nylon strips (P < 0.002) or LDPE sachets (P < 0.001). However, blend IB1 was significantly more attractive to unfed *An. funestus* when released from nylon strips than LDPE sachets (P < 0.001). The responses of blood-fed and gravid *An. funestus* were not dependent on treatment and the catches were also very low for meaningful statistical analysis.

Table 1: Abdominal conditions of female *An. gambiae* s.l. and *An. funestus* caught inside village houses when exposed to an unlit CDC trap (without a bait), blend IB1 released from LDPE sachets, the IB1 blend dispensed from nylon strips and a human sleeping under a bed net. Mosquito catches are expressed as mean (± S.E) number per night. The study was conducted in Kigoche village for 48 nights at weekly intervals spread out over one year post-treatment.

Treatment	N	Mean number ± SE of mosquitoes caught					
		An. gambiae s.l.			*An. funestus*		
		Unfed	Blood-fed	Gravid	Unfed	Blood-fed	Gravid
Unlit CDC trap (without a bait)	48	0.15 ± 0.05^{a}	0.06 ± 0.04^{a}	0.02 ± 0.02^{a}	0.27 ± 0.26^{a}	_	_
LDPE sachets filled with IB1	48	0.75 ± 0.13^{b}	_	0.10 ± 0.05^{a}	1.33 ± 0.17^{b}	_	_
IB1-treated nylon strips	48	1.92 ± 0.20^{c}	0.10 ± 0.05^{a}	0.21 ± 0.07^{a}	2.81 ± 0.24^{c}	0.10 ± 0.4	0.04 ± 0.03^{a}
Human	48	1.75 ± 0.19^{c}	_	0.25 ± 0.07^{a}	3.62 ± 0.28^{d}	_	0.27 ± 0.08^{a}

N is the number of nightly collections, dash (_) shows no mosquito was caught, whereas SE is the standard error of the mean number of catches per night. Mean ± SE mosquito catches within the same column assigned different letter superscripts are significantly different at $P < 0.05$ (GLM).

Blood meal source

Outdoors blood-fed malaria vectors responded to traps baited with blend IB1 dispensed from LDPE sachets or nylon strips whilst those caught indoors were found in a trap without bait or baited with IB1-treated nylon strips. A total of 24 female *An. gambiae* s.l. and 12 *An. funestus* were analysed for their blood meal source. The 24 *An. gambiae* s.l., had fed either on bovines (62.5%) or humans (37.5). However, 66.7% of *An. funestus* had fed on humans compared to bovines (33.3%) (Figure 4).

Sporozoite rates and identity of sibling species of *An. gambiae* complex

Of the 100 *An. gambiae* s.l. and 100 *An. funestus* mosquitoes tested for *P. falciparum* CSP antigen, only one *An. funestus* female was infective. The mosquito was lured into the trap dispensing the IB1 blend from nylon strips in October 2011. Furthermore, PCR indicated that *An. gambiae* s.l. of Kigoche village comprise mainly *An. arabiensis* (n = 92, 97.9%) and only few *An. gambiae s.s.* (n = 2, 2.1%), whereas four samples were not amplified because of poor storage. A subsequent investigation (January 2011 - December 2012) confirmed a 25% malaria prevalence by microscopy based on 71893 clinical malaria cases observed at 10 medical facilities adjacent to the study area.

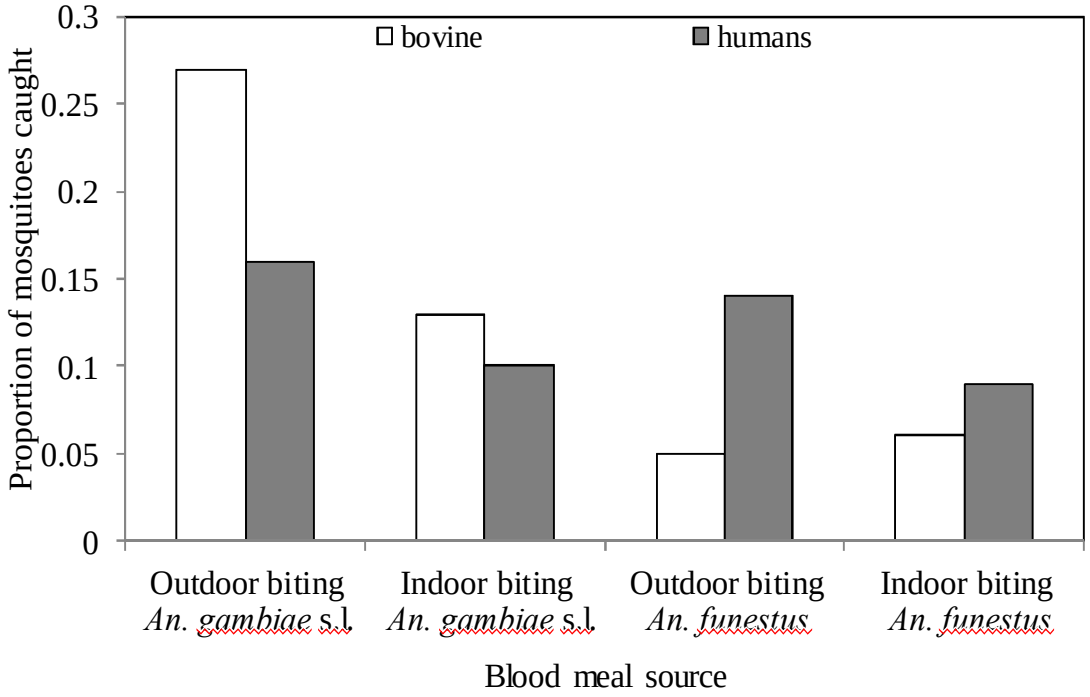


Figure 4: Proportions of outdoor and indoor *An. gambiae* s.l. and *An. funestus* having bitten humans or bovines as blood meal hosts.

Discussion

This study demonstrated that the IB1 blend dispensed from LDPE sachets and nylon strips remained attractive to natural populations of female *An. gambiae* s.l. and *An. funestus* indoors and outdoors when used at weekly intervals for 48 nights of one year post-treatment. Although catches varied depending on the rainy season, overall mosquito responses to each treatment over the 48 weeks of sampling did not change. Dispensing of blend IB1 from nylon strips resulted in similar outdoor trap collections of *An. gambiae* s.l. as LDPE sachets, however, the IBI-treated nylon strips were significantly more attractive to *An. funestus* than LDPE sachets filled with similar attractants. When collecting indoors, both humans and IB1-treated nylon strips attracted similar catches of *An. gambiae* s.l. and each of them was more attractive compared to IB1 dispensed from LDPE sachets. Humans lured significantly more *An. funestus* into houses compared to blend IB1, however, IB1-treated nylon strips attracted significantly more *An. funestus* than IB1 dispensed from LDPE sachets. The majority of individuals of both mosquito species were unfed. *Anopheles gambiae* s.l. dominated outdoor collections, fed either on bovines (62.5%) or on humans (37.5%) and, comprised *An. gambiae s.s.* (2.1%) and *An. arabiensis* (97.9%). By contrast, *An. funestus* was more abundant in indoor catches, fed more on humans (66.7%) than on cattle (33.3%), and was tested positive for *P. falciparum* sporozoites.

Low density polyethylene sachets are commonly used as slow-release dispensers for semiochemicals applied to monitor and disrupt agricultural insect pests and haematophagous insects including tsetse flies and malaria vectors, as they allow a predictable and constant release of odorants (Okumu et al., 2010, Heuskin et al., 2011). In a study conducted in Tanzania, LDPE sachets demonstrated a higher potential as a slow-release material of blend IB1 to malaria vectors than open glass vials, but nylon strips performed better than LDPE sachets (Okumu et al., 2010). However, the residual activity of the strips and the LDPE sachets was not investigated. Therefore, the current study has shown that both nylon and LDPE sachets have potential as long-lasting release matrices for synthetic odorant cues (Torr et al., 1997, Cork, 2004) under ambient conditions in a rice agro-ecosystem.

There was no significant variation in the responses of *An. gambiae* s.l. and *An. funestus* to odour-baited traps and human emanations over a prolonged post-treatment period regardless of the changes in temperature, relative humidity or rainfall. This study demonstrates how application of long-lasting residual activity may enhance sustainability and convenience of odour-based technology for area-wide sampling and control of indoor and outdoor host-seeking malaria vectors. The present results are consistent with our recent semi-field studies where IB1-treated nylon strips attracted more female *An. gambiae* than LDPE sachets for a period of 40 consecutive nights (Mukabana et al., 2012b) and, weekly for 52 nights post-treatment (Chapter 4). Whereas attraction of outdoor biting *An. gambiae* s.l. by baited traps did not change over the entire post-treatment time, responses of this malaria vector to blend IB1 were not affected by the use of LDPE sachets or nylon strips as an odour-dispensing matrix. These results are in contrast to a previously published semi-field study (Mukabana et al., 2012b), but they concur with outdoor responses of *An. gambiae* s.l. to IB1-treated nylon strips that were prepared daily for each experimental night (Mukabana et al., 2012a). Unlike in studies reported by both Okumu et al. (2010) and Mukabana et al. (2012b), the nylon strips utilised in the current work were treated once with blend IB1 and deployed repeatedly at one-week intervals for 48 nights over one year post-treatment period. Thus, synthetic odour blends dispensed from nylon have a long active residual life span, which is of great advantage for surveillance purposes or even for removal trapping of disease vectors like malaria mosquitoes. Furthermore, our results imply that the attractiveness of blend IBI to mosquitoes over time was dependent on the type of slow-release system used, as well as type and feeding behaviour of the malaria vectors.

The prolonged attractiveness of IB1-treated nylon strips to local malaria vectors may be partly attributed to attractants produced by colonization of microbes upon deployment (Chapter 4). This is based on our understanding that some of the volatile compounds produced by human skin microbiota affect host-seeking behaviour of *An. gambiae* (Verhulst et al. 2009, Smallegange and Takken, 2010). Such compounds have been identified, isolated and evaluated under semi-field and field conditions for luring host-seeking malaria vectors into

traps (Verhulst et al., 2010, Verhulst et al., 2011). Therefore, IB1-treated nylon strips may have been contaminated by establishment of microbes from the environment over time. The microbes are likely to have produced subsequent attractive volatiles to the mosquitoes as the original IB1 blend. Recent preliminary headspace analysis of IB1-treated nylon strips utilised at weekly intervals for 52 nights post-treatment in semi-field conditions demonstrated the presence of 26 extra volatile compounds (Chapter 4). Some of the 26 additional volatile compounds are known to be produced by skin bacteria, found in skin sweat (Meijerink 2000, Verhulst et al., 2009, 2010), produced on human skin (Bernier 2000) while others were break-down products of bacterial activity of long chain fatty acids (Verhulst et al., 2010, Verhulst et al., 2011). Based on this evidence, the effect of microbes present on attractant-treated fabrics and associated volatiles needs to be researched further as this may facilitate kairomone identification and storage of odorant-impregnated fabrics between experimental nights.

Practically, attractant-treated nylon may be utilised reliably and repeatedly to collect both outdoor and indoor biting African malaria mosquitoes in the presence or absence of humans (Mukabana et al., 2012a; Jawara et al., 2011). Like in a study conducted in The Gambia, both IB1-baited traps recorded equally high mosquito diversity as a CDC light trap deployed outdoors and indoors in the same village (Jawara et al., 2009). Our results also concurred with CDC trap collections which showed that, of the outdoor populations of malaria vectors, *An. gambiae* s.l. was more abundant than *An. funestus.* Thus, a combination of long-lasting residual effect and the release of novel enhanced synthetic attractant blends may be exploited as a tool for replacement of the human landing catches (Dia et al., 2005, Mboera, 2005).

Associations between malaria transmission and climatic variables have been explored in many studies and revealed inconsistencies at local levels (Chaves and Koenraadt, 2010, Stresman, 2010). It was observed that rainfall, temperature and RH had no significant association with mean collections of *An. gambiae* s.l. and *An. funestus* attracted to blend IB1 irrespective of the odour-dispensing material of choice over the entire study period. Therefore, host-seeking malaria vectors may have evolved to maximize chances of encountering a blood host in nature where all a biotic factors interact. Wind, temperature, RH humidity along with other factors are important to most insect vectors because they affect host-odour plume structure, strength of the olfactory signal and subsequent flight orientation to the odour source (Gibson and Torr, 1999, Day 2005, Cooperband and Cardé, 2006).

Regardless of fluctuations in climatic variables, mosquito collections were generally stable over the entire study period because breeding occurred throughout the year in adjacent rice paddies irrigated through open channels (Minakawa et al., 2002, Diuk-Wasser et al., 2006, Mwangangi et al., 2008). Moreover, prevailing rainfall, temperature and RH allowed high mosquito abundance, survival and malaria transmission (Afrane et al., 2005, Stresman, 2010). For *An. funestus,* trap catches were not always positively correlated with rainfall as they breed

in more permanent habitats including irrigation channels and along rivers compared to *An. gambiae* s.l. which breeds in temporal, shallow, small and sunlit habitats (Gilles and Coetze, 1987, Gimnig et al., 2001, Imbahale et al., 2011). Since village houses were adjacent to breeding sites, newly emerged or adult mosquitoes with limited dispersal ability may have contributed to high catches of unfed host-seeking mosquitoes (Nyaguara et al., 2012). The synthetic mosquito attractants used served as an appropriate trap lure for unfed malaria vectors, thereby suggesting that, a novel odour bait is needed for collection of malaria vectors in blood-fed and gravid conditions. Thus, deployment of effective odour-baited tools may be used for disruption of malaria transmission by reducing survival rates of adult mosquitoes through mass-trapping, lure and kill or push-pull strategies (Kline, 2007, Takken, 2010).

Whereas host-seeking malaria vectors are mainly guided by host odour cues (Takken and Knols, 1999, Verhulst et al., 2010), feeding preferences are strongly influenced by innate species-specific properties, nutritional requirements, and availability of particular hosts (Killeen et al., 2001). From our results, *An. funestus* was confirmed as the most important local vector of malaria as it was more abundant in indoor collections, fed more often on human blood than *An. gambiae* s.l., and was positive for *Plasmodium* sporozoites (Githeko et al., 1993, Githeko et al., 1996, Bøgh et al., 2001). A combination of such characteristics with relatively higher survival rate and innate susceptibility to *Plasmodium* infection presents *An. gambiae* s.l. and *An. funestus* as the most important vectors of malaria in Africa (Charlwood et al, 1997, Takken and Knols, 1999). However, these findings disagree with a previous conclusion that malaria risk is reduced in irrigation schemes due to displacement of the highly anthropophilic *An. funestus* by the opportunistic *An. arabiensis* that thrives better in rice fields (Ijumba and Lindsay, 2001). Thus, indoor and outdoor deployment of odour-baited tools may disrupt malaria transmission by both vectors.

Both indoor and outdoor collections of *An. gambiae* s.l. were predominantly *An. arabiensis* that had fed more on bovines than humans. This may suggest that, subject to variation in geographical location and species karyotype, herding of cows as a supplementary activity reduces risk of malaria transmission by *An. arabiensis* (Killeen et al., 2001). Contrary to this suggestion, in the Gambia *An. arabiensis* fed more readily on domestic animals/cattle than *An. gambiae s.s*, but there was no difference in their sporozoite rates (Bøgh et al., 2001). Recent studies conducted in East Africa have also indicated that the presence of large numbers of cattle does not confer effective zooprophylaxis against malaria transmitted by *An. arabiensis* or *An. pharoensis* (Tirados et al., 2011). In view of both studies, application of long-term residual activity of highly attractive odorants identified from human skin is likely to lure both *An. gambiae* s.l. (i.e. *An. gambiae s.s*. and *An. arabiensis*) and *An. funestus* into traps regardless of the presence of bovines.

Conclusion

This study reports progress made in the development of an odour-baited tool for sampling of indoor and outdoor host-seeking malaria vectors. The residual activity of IB1-treated nylon strips to *An. gambiae* s.l. was similar to that of human emanations *in vivo*, suggesting that use of this synthetic bait can replace the widely-used CDC + human-under-a-bed-net method. However, the lower attractiveness of IB1-treated nylon strips compared to the attractiveness of humans to *An. funestus* implies that the attractiveness of the synthetic blend and/or the release matrix needs to be improved for this important malaria vector species. With the availability of these novel tools, the possibility of area-wide application could be considered in diverse epidemiological situations as these devices affect a wide range of malaria and other disease vectors.

Acknowledgements

We are thankful to household owners of Kigoche village for allowing us to conduct this study in their homesteads. Nicholas Mulaya is appreciated for analyses of blood meals, sporozoites and identification of sibling species of *An. gambiae* complex in our samples and, George Opetu for providing us with meteorological data. We are grateful to Fred Kisanya and Susan Imbahale for housing us at the AMDTI facility. Finally, I thank Elisha Okwatch, Charles Oketch and John Okello as they volunteered to sleep in our experimental houses over the study period. This study was supported by a grant from the Foundation for the National Institutes of Health (FNIH) through the Grand Challenges in Global Health initiative (GCGH #121) and a sandwich Ph.D. scholarship of the Wageningen University and Research Centre, The Netherlands (MCK). MCK was hosted by ICIPEï TRO campus as a Dissertation Research Internship Program (DRIP) scholar.

Chapter 6

Comparison of light and odours as stimuli for sampling malaria and other mosquitoes

To be submitted in a slightly modified form as: Mweresa, C.K., Omusula, P., Otieno, B., van Loon, J.J.A., Takken, W. and Mukabana, R. W. Comparison of light and odours as stimuli for sampling malaria and other mosquitoes

Abstract

Systems used for sampling of adult Afrotropical malaria vectors are mainly baited with light, odour or both. However, reliability and efficacy of these systems vary; hence there is a need for a more standardized tool. This study evaluated the suitability of a synthetic odour blend, light and humans as baits for sampling host-seeking malaria vectors in a rice agro-ecosystem in western Kenya. The attractiveness of a synthetic odour blend referred to as the Ifakara blend 1 (IB1), light, a human, a human + light, or a house without any stimulus attractive for malaria and other mosquitoes was assessed in five village houses. Experiments were conducted once per week for 30 nights through a randomised 5 × 5 Latin square design. Trapped mosquitoes were predominantly females (83.1%). The responses of indoor-biting *Anopheles gambiae* sensu lato to a human and IB1-treated nylon strips were similar ($P = 0.42$), but light alone and a human + light were significantly more attractive than IB1 or a human only ($P < 0.001$, for all). Attraction of indoor biting *An. funestus* mosquitoes to IB1-treated nylon strips or light were not different ($P = 0.54$). A human + light attracted the highest mean numbers of female *An. gambiae* s.l., and *An. funestus* compared to all other treatments ($P < 0.001$, for all comparisons). No blood-fed *An. gambiae* s.l. and *An. funestus* were trapped in houses occupied by a human alone. *Anopheles funestus* fed more frequently on humans than bovines while *An. gambiae* s.l. displayed a higher feeding preference for bovines than humans. This study has demonstrated that synthetic odour baits can be used to replace a human in mosquito sampling devices. Visual and olfactory cues are both useful in modulating mosquito host-seeking behaviour, and interaction between the two cues may provide a more robust and reliable tool for sampling malaria and other mosquito vectors.

Introduction

Sampling of malaria vectors for the estimation of transmission intensity and surveillance is achieved through different techniques. The choice of technique to use depends on objectives of the study, environment and access to resources (Le Goff et al., 1993). The most commonly used methods include light traps, humans under a bed net + light trap, pyrethrum spray catches, and human-landing catches (HLC) (Dia et al., 2005, Mboera, 2005). Each of these methods varies in terms of reliability and efficacy; hence there is a need for a standardised sampling tool (Kelly-Hope and McKenzie, 2009). For example, light traps capture mosquitoes with higher sporozoite rates compared to HLC thus leading to an overestimation of the entomological inoculation rate (EIR). Moreover, light traps are not species-specific and mosquito specimens caught are not always in good condition for studies requiring dissection (Le Goff et al., 1993). Light traps have generally been found to underestimate the abundance of host-seeking anophelines and outdoor-biting malaria vectors (Mbogo et al., 1993, Costantini et al. 1998a, Govella et al., 2011, Overgaard et al., 2012). Currently, light traps set beside bed nets occupied by a human have been proposed as a comparable and unbiased alternative to HLC in sampling host-seeking mosquitoes possibly because malaria vectors are attracted to both odour and light (Lines et al., 1991, Constantini et al., 1998a, Ndiath et al., 2011). However, the estimation of EIR by use of both stimuli is influenced by trap position and variation in individual attractiveness of the human bait (Mboera et al., 1998, Mboera, 2005, Verhulst et al., 2011).

The HLC technique is the most direct, reliable and frequently used for sampling both indoor and/or outdoor feeding mosquitoes, and is considered as the reference method for determining the frequency of contact between humans and malaria vectors (Ndiath et al., 2011a, Le Goff et al., 1993). Nonetheless, collectors are exposed to potential mosquito-borne infections and hence it is unethical, results depend on skills and experience of the operators as well as their differential attractiveness to malaria vectors (Wanji et al., 2003, Mboera, 2005); in addition, blood-fed mosquitoes are rarely caught (Le Goff et al., 1997). Human landing catches may also overestimate mosquito densities because collectors avail their forearms and limbs to host-seeking mosquitoes, yet this may not represent what happens naturally. To eliminate the effects of differential attractiveness during collections with HLC, and the light trap / human-under-a bed-net combination, the same volunteers are required throughout the study, which reduces the possibility of area-wide sampling (Wanji et al., 2003). Pyrethrum spray catches (PSC) are also used for the collection of indoor resting and blood-fed female mosquitoes, however, catches are usually dependent on feeding-resting behaviour of the local vectors and may not represent the true biting fraction of the population as blood-fed mosquitoes sometimes enter a house as resting site, following a blood meal that was taken elsewhere (Githeko et al., 1996, Duchemin et al., 2001, Ndiath et al., 2011). In view of these

shortcomings, standardisation of all the sampling techniques is needed for validation of entomological studies related to malaria (Kelly-Hope and McKenzie, 2009). This presents an opportunity for integration of odour-baited trapping of malaria vectors. Odour-based trapping methods have been rigorously tested for sampling crop (Cook et al., 2007) and animal (Kline, 2007, Pickett et al., 2010) pests.

Based on ample evidence that host-seeking in malaria vectors is mainly mediated by host odours (Takken, 1991, Takken and Knols, 1999), several candidate behaviourally-disruptive organic compounds (cBDOCs) have been identified, assayed and constituted into synthetic mosquito baits (Smallegange et al., 2005, Smallegange et al., 2009, Okumu et al., 2010a, Verhulst et al., 2011a, Mukabana et al., 2012a). Recent experiments have yielded promising results about the suitability of using such novel synthetic odour baits for sampling and control (Kline, 2007) of malaria vectors under field conditions (Qiu et al., 2007, Jawara et al., 2009, Okumu et al., 2010a, Verhulst et al., 2011b, Mukabana et al., 2012a). The findings of these studies indicate that synthetic odour baits dispensed from mosquito traps can be used reliably to collect selective and alive samples of both indoor- and outdoor-biting malaria and other mosquito vectors as an alternative to HLC and other common methods. The goal of this study was to investigated whether a synthetic odour bait may be used in place of a light trap, a human or light + a human-under-a bed-net to sample indoor biting malaria and other mosquito vectors in a rice growing village in western Kenya. To achieve this goal, the responses of mosquitoes including local malaria vectors in various physiological states to light, a human and a synthetic odour blend were evaluated in village houses. In addition, blood meal source and *Plasmodium falciparum* infection rates of *Anopheles gambiae* sensu lato and *An. funestus* Giles collected were determined.

Materials and Methods

Field study site

This study was conducted between November 2011 and June 2012 in five houses located in Kigoche village, near Ahero Market in Kisumu County, western Kenya. The village is located at 00°34ôS, 034°65ôE and 1158 m above sea level. Ahero is a rice agro-ecosystem located in the flood plain of River Nyando. Ahero experiences an approximate rainfall ranging from 1000 to 1800 mm, temperature of 17 ï 32°C and 65% relative humidity (RH) throughout the year. *Plasmodium falciparum* is the primary cause of malaria in the area (Githeko et al., 1993). The parasite is transmitted throughout the year mainly by *An. gambiae* Giles *sensu stricto*, *An. arabiensis* Patton and *An. funestus* (Atieli et al., 2009, Bukhari et al., 2011, Mukabana et al., 2012a). The vectors breed predominantly in small water pools, hoof and foot prints, rice paddies and shaded and unshaded irrigation water channels. Irrigated rice farming

is the main economic activity. Supplementary traditional farming of maize, millet, bananas, sweet potatoes, beans, cassava, sorghum and rearing of indigenous cattle, goats, sheep and poultry is also practiced. At night cattle, goats and sheep are tethered outside, adjacent to houses where owners dwell.

Ethical approval

This study was approved by the ethical committee of the Kenya Medical Research Institute (KEMRI/RES/7/3/1). The purpose and protocol of the study were explained to local leaders, household heads and resident volunteers before permission to carry out the study was sought. Written informed consent was obtained from three male volunteers aged between 22 and 25 years old. The human subjects were non-smokers, did not consume alcohol, and were free of chronic illness including malaria. On the day of experiments, volunteers were screened for the presence of *P. falciparum* parasites using a rapid malaria diagnostic test (SCIMEDX Corporation, Deville, New Jersey, USA) as well as light microscopy at the Ahero Sub-district hospital located 5 km away. One of the volunteers confirmed positive was treated with artemether-lumefantrine and exempted from experiments on that particular night.

Selection of study houses

A total of five houses measuring 15.8 - 22.5 m^2 were randomly selected. The houses were mud-walled, roofed with corrugated iron sheets, had either one or two rooms, one door, open eaves, without a ceiling and were spaced within a range of 28 - 481 m apart (Hill et al., 2007, Mukabana et al., 2012a, Sumaye et al., 2012). Each house was located 10 - 20 m away from cowsheds and within a maximum distance of 100 m away from irrigation water channels and rice paddies. The houses had not been treated with indoor residual insecticidal sprays and were free from human occupation for the whole study period. A hand-held global positioning system (GPS) receiver (Trex HC series, Garmin International, St Olathe, KS, USA) was used to determine the exact location of all selected houses. House numbers were indicated on the main entry door for systematic rotation of treatments after each experimental night to overcome house effect on mosquito catches.

Meteorological conditions

The prevailing temperature and RH in the experimental houses were recorded at intervals of 30 min using a data logger (Tinytag® Ultra, model TGU-1500, INTAB Benelux, The Netherlands). Monthly averages of outdoor environmental conditions, *viz.* air temperature, wind speed, RH and rainfall data were calculated based on values recorded at the Ahero Irrigation Research Station (AIRS) located approximately 800 m away from the study houses.

Preparation and dispensing of synthetic mosquito odour bait

A synthetic mosquito attractant referred to as the Ifakara blend 1 (IB1) was used. The blend consisted of optimal dilutions of ammonia (2.5%), L-lactic acid (85%), tetradecanoic acid (0.00025%), 3-methyl-1-butanoic acid (0.000001%), distilled water (100%), propionic acid (0.01%), butanoic acid (1%), pentanoic acid (0.0001%), heptanoic acid (0.0001%) and octanoic acid (0.0001%) impregnated on individual nylon strips as described by Okumu et al. (2010a) and sugar-produced carbon dioxide (Smallegange et al., 2010). All attractant-impregnated nylon strips were hooked at one end and suspended inside the odour plume tube of the MM-X counter-flow trap (American Biophysics, North Kingstown, RI, USA) (Okumu et al., 2010b). The carbon dioxide was produced by mixing 250 g of refined sugar, 17.5 g dry instant yeast and 2 L of river water, and delivered into an MM-X trap baited with IB1-treated nylon strips through silicon tubing (0.5 cm diameter). The ingredients used for carbon dioxide production were mixed in a plastic container placed next to an IB1-baited trap. During experiments (18:30 ï 06:30 h), the odour outlet tube of the trap was suspended over a bed at 15 cm above the mattress level and operated on a 12 V car battery. The IB1-treated nylon strips were prepared once and used repeatedly over the entire study period while the sugar-yeast mixture was prepared daily as CO_2 production ceased after one experimental night.

Reference monitoring of outdoor biting mosquitoes

For comparison of mosquito diversity during the experimental period, one standard Centre for Disease Control (CDC) miniature trap with light only (Model 512; John W. Hock Company, Gainesville FL, USA) was used. The trap was operated on a 6 V battery (Gaston Battery Industry Ltd, China) and placed outdoors under the eave of a human-occupied house, approximately 100 m away from houses where indoor mosquito collections were simultaneously made (Qiu et al., 2007, Jawara et al., 2011). No odour bait was added to the CDC light trap as the reference trap.

Attraction of malaria and other mosquitoes to humans, light and synthetic odorants

A randomized 5 × 5 Latin square design was used to investigate the attraction of malaria vectors to a synthetic odour bait, light, a human, and light/human combination at weekly intervals for 30 nights. The treatments included (a) unlit CDC miniature light trap (without light, odour or a human), (b) one MM-X trap baited with IB1, (c) one human volunteer, (d) one lit CDC light trap without an additional stimulus, and (e) one human volunteer + one lit CDC light trap. All houses in the homesteads except those selected for experiments were occupied and used routinely by dwellers throughout the study period.

House-entering mosquitoes were collected in unlit CDC traps placed next to a bed covered with an insecticide-free bed net from which the stimuli were released (Figure 1) (Mboera et al., 1998a; Okumu et al., 2010). In the control house, the bed net contained an unused bed without any stimulus. The odour outlet of the MM-X trap was elevated at 15 cm from the mattress level. The suction fan of the MM-X trap was disabled for it to dispense an IB1 odour plume without trapping attracted mosquitoes (Okumu et al., 2010). Each bed net was tucked under the mattress to prevent mosquitoes from accessing human volunteers or the IB1-baited trap (Figure 1). The three volunteers slept alternately in each experimental house such that two of them participated in the study on single nights in treatments (c) and (e). Vaseline petroleum jelly was applied around the carbon dioxide delivery tubing, and on suspension and power cables to prevent ants from accessing and preying on mosquitoes caught inside CDC light traps.

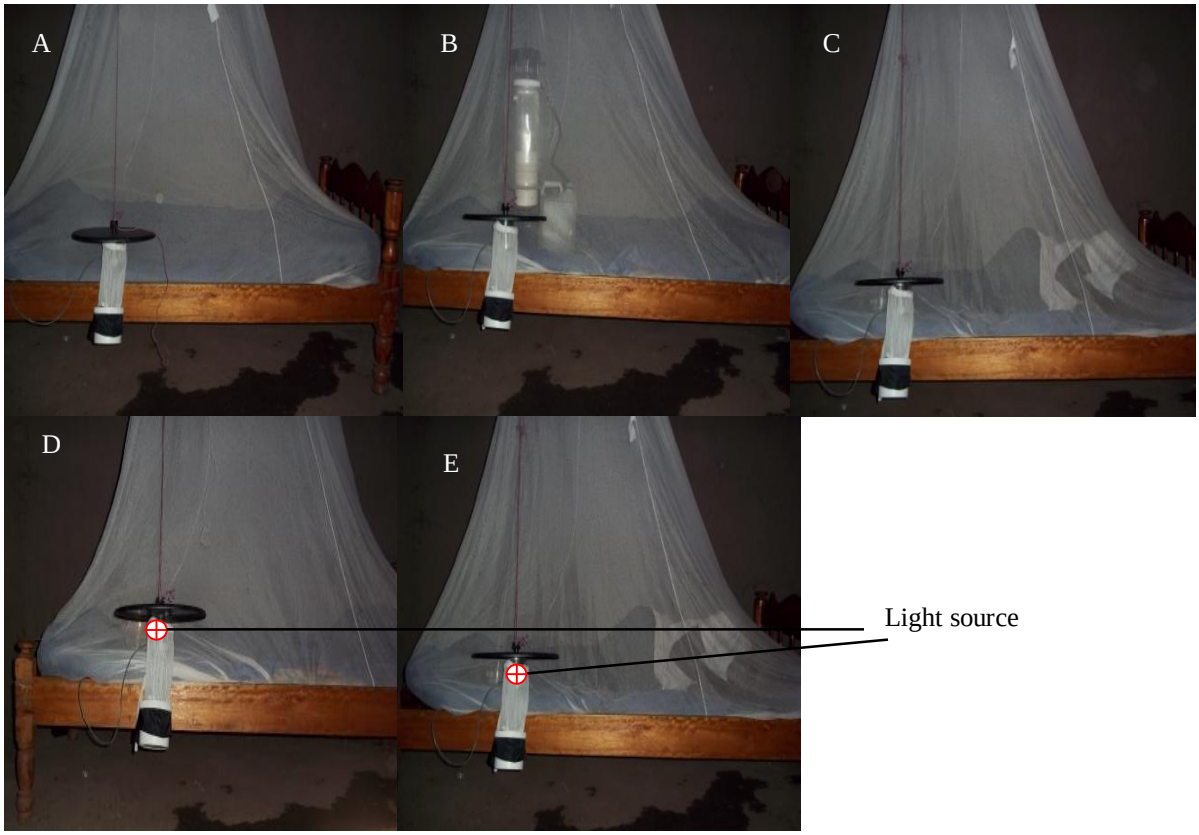


Figure 1: Picture showing indoor collection of mosquitoes in a village house containing a bed without a bait (panel A), MM-X trap baited with IB1-treated nylon strips (panel B), a human volunteer (Panel C), light only (panel D) from a CDC miniature trap suspended outside the bed net, and a human volunteer under a bed net without insecticide treatment + light from a CDC miniature trap placed besides the bed net (panel E). House-entering mosquitoes were collected either in unlit CDC traps placed next to the bed nets from which blend IB1 and host stimuli were released or CDC trap with light suspended besides a bed net without any other stimulus or with host stimuli. The experiments were conducted in Kigoche village at weekly intervals for 30 nights (18:30 ï 06:30 h) post-treatment.

Collection and identification of trapped mosquitoes

At the end of each experimental night, traps were disconnected from batteries and transported to a field laboratory at the Ahero Multipurpose Development Training Institute (AMDTI) for freezing of collected mosquitoes for 30 min. The frozen adult mosquitoes were emptied into labeled Petridishes and identified based on morphological features as male and female *An. gambiae* s.l., *An. funestus, Culex* spp., *Mansonia* spp., and other anophelines (all collected *Anopheles* species except *An. gambiae* s.l. *and An. arabiensis*) mosquitoes (Gilles and Coetzee, 1987), counted and recorded. Thereafter, females of *An. gambiae* s.l. and *An. funestus* were classified morphologically according to their gonotrophic status as unfed, blood -fed (partially and fully fed) or gravid and preserved in 2 ml Eppendorf tubes containing silica gel covered with a thin layer of cotton wool. The preserved samples were analyzed for blood meal sources and presence of *Plasmodium* sporozoites at the Centre for Global Health Research, Kenya Medical Research Institute (KEMRI), Kisumu, western Kenya.

Determination of blood meal source of *An. gambiae* s.l. and *An. funestus*

The abdomen of all 25 samples of blood-fed *An. gambiae* s.l. and *An. funestus* mosquitoes trapped during the study were cut off transversely at the attachment point to the thorax. Individual posterior abdominal portions containing a blood-meal were placed in separate vials and labeled for subsequent identification by a direct enzyme-linked immunosorbent assay (ELISA). Each blood meal sample was screened simultaneously for both human and bovine antibodies (Beier et al., 1988).

Estimation of *P. falciparum* infection rate of *An. gambiae* s.l. and *An. Funestus*

A total of 120 females of *An. gambiae* s.l. and 120 *An. funestus* were randomly selected from each treatment for sporozoite analysis. The head and thorax of individual *An. gambiae* s.l. and *An. funestus* were tested singly for the presence of *P. falciparum* circumsporozoite proteins (CSP) using ELISA (CSP-ELISA) (Beier et al., 1990).

Data analysis

The responses of mosquitoes to different treatments were evaluated using a Generalized Linear Model (GLM) fitted with Poisson distribution and logarithm link function (Smith, 1995, Verhulst et al., 2011b). The effects of treatment and house position on mosquito catches were tested as parameters in the model. Pairwise comparisons between mean numbers of mosquitoes caught between different treatments were determined by using least significant differences. Blood meal source and sporozoite rate were calculated as a proportion of *An.*

gambiae s.l. and *An. funestus* mosquitoes tested. All analyses (Ŭ= 0.05) were performed using IBM SPSS statistical software, release 19.

Results

Meteorological conditions and mosquito diversity

An average temperature of 22.5 ± 1.5^{o}C and 74.4 ± 17.4% RH prevailed indoors over the entire study period (November 2011 - June 2012). During the same period, outdoor conditions were characterized by an average temperature of 24.1 ± 8.5^{o}C, RH of 57.8 ± 13.1%, and a total rainfall of 576 mm. The highest amount of rainfall occurred in April 2012 and caused floods for five days.

A CDC light trap placed elsewhere in the same village to monitor outdoor mosquito diversity and abundance attracted fewer male mosquitoes (n = 89, 3.4%) than females (n = 2537, 96.6%). The female mosquitoes included *An. gambiae* s.l. (7.1%), *An. funestus* (1.1%), *Culex* spp. (45.1%), *Mansonia* spp. (18.6%) and other anopheline mosquitoes (27.5%).

Responses of female mosquitoes to a synthetic odour blend, light and humans

Fewer males (n = 306, 16.9%) were collected than females (n = 1,509, 83.1%). Indoor collections of female mosquitoes comprised of *An. gambiae* s.l. (n = 343, 22.7%), *An. funestus* (n = 579, 38.4%), *Culex* spp. (n = 382, 25.3%), *Mansonia* spp. (n = 130, 8.6%) and other anopheline spp. (n = 75, 5.0%) (Table 1).

Whereas humans and IB1-impregnated nylon strips attracted similar mean numbers of female *An. gambiae* s.l. (P = 0.42), light was significantly more attractive compared to each of the two stimuli (P < 0.001 for both). In general, significantly more *An. gambiae* s.l., *An. funestus* and *Culex* mosquitoes responded to a human + light than to a human or light alone (P < 0.001 for all). There was no difference between the responses of *An. funestus* to light alone and IB1-treated nylon strips (P = 0.54). Blend IB1 was significantly less attractive to *An. funestus* compared to a human (P < 0.001) or a human + light (P < 0.001). Although blend IB1 attracted more *Culex* spp. than a human (P < 0.001), each of the two catches was significantly lower compared to light or a human + light (P < 0.001 for both). The mean numbers of *Mansonia* spp. caught by light alone and by light + a human were similar (P = 0.12), though

each of them was significantly more attractive when compared to a human (P < 0.001 for both) or blend IB1 (P < 0.001 for both). Humans and IB1-baited traps were equally attractive to *Mansonia* spp (P = 0.56) and other anopheline mosquitoes (P = 0.05). Significantly higher

catches of other anopheline spp. were found in houses containing light than in houses with a human ($P < 0.002$), or a human + light ($P < 0.039$).

Responses of male mosquitoes to a synthetic odour blend, light and humans

Table 1: Mean number (± SE) of female mosquitoes caught per night in village houses without a bait, with IB1-treated nylon strips, a human, light, or a human + light. The study was conducted in Kigoche village at weekly intervals for 30 nights.

Treatment	N	Mean ± SE number of female mosquitoes caught				
		An. gambiae s.l.	*An. funestus*	*Culex* spp.	*Mansonia* spp.	Other anophelines
Unlit CDC trap (without a bait)	30	0.23 ± 0.09^{a}	0.20 ± 0.08^{a}	0.06 ± 0.04^{a}	_	_
IB1-treated nylon strips	30	1.50 ± 0.22^{b}	3.23 ± 0.32^{b}	2.25 ± 0.27^{b}	0.13 ± 0.06^{a}	0.15 ± 0.07^{a}
Human	30	1.77 ± 0.24^{b}	4.90 ± 0.40^{c}	1.11 ± 0.19^{c}	0.19 ± 0.08^{a}	0.42 ± 0.12^{a}
Lit CDC light trap	30	3.10 ± 0.32^{c}	2.90 ± 0.31^{b}	3.72 ± 0.35^{d}	1.87 ± 0.25^{b}	1.12 ± 0.20^{b}
Human + lit CDC light trap	30	4.83 ± 0.40^{d}	8.07 ± 0.52^{d}	4.32 ± 0.38^{d}	1.28 ± 0.20^{b}	0.57 ± 0.14^{a}

N is the number of experimental nights, SE is the standard error of the mean number of catches per night whereas a dash implies that no mosquito was caught. Mean ± SE mosquito catches within the same column assigned different letter superscripts are significantly different at $P < 0.05$ (Generalized Linear Models).

The 306 male mosquitoes caught included *An. gambiae* s.l. (24.2%), *An. funestus* (43.5%), *Culex* spp. (20.6%), *Mansonia* spp. (4.9%) and other anopheline spp. (6.9%) (Table 2). In general, male mosquitoes were collected in houses without a bait (2.2%), occupied by a human (5.2%), IB1-treated nylon strips (6.2%), light (42.8%) or a human + light (43.5%).

There was no difference between the responses of *An. gambiae* s.l. to light and light + a human ($P = 0.35$), but each of the two treatments was more attractive compared to a human ($P < 0.001$, for both) or blend IB1 alone ($P < 0.001$, for both). Similarly, light and light + a human attracted similar mean numbers of male *An. funestus* ($P = 1.00$), however, each treatment was more attractive when compared to a human, blend IB1 or control ($P < 0.001$ for all). The responses of *Culex* spp. to a human + light and light alone were not different ($P = 0.35$), but higher than a human or control ($P < 0.001$ for all). Furthermore, attractiveness of IB1-treated nylon to *Culex* spp. was similar to light alone ($P = 0.08$), though lower than a combination between human and light ($P < 0.034$). The collections of male *Mansonia* and other anopheline mosquitoes were not affected by the type of bait used ($P = 0.09$ and $P = 0.75$, respectively).

Table 2: Mean number (±SE) of male mosquitoes caught per night in village houses without a bait, with IB1-treated nylon strips, a human, light, or a human + light. The study was conducted in Kigoche village at weekly intervals for 30 nights.

Treatment	N	Mean ± SE catches of male mosquitoes caught				
		An. gambiae s.l.	*An. funestus*	*Culex* spp.	*Mansonia* spp.	Other anophelines
Unlit CDC trap (without a bait)	30	–	0.13 ± 0.07^{a}	0.02 ± 0.03^{a}	0.03 ± 0.03^{a}	0.02 ± 0.03^{a}
IB1-treated nylon	30	0.13 ± 0.07^{a}	0.13 ± 0.07^{a}	0.22 ± 0.08^{ab}	–	0.05 ± 0.04^{a}
Human	30	0.17 ± 0.65^{a}	0.10 ± 0.06^{a}	0.05 ± 0.04^{a}	0.03 ± 0.03^{a}	0.12 ± 0.06^{a}
Lit CDC light trap	30	1.20 ± 0.20^{b}	2.03 ± 0.26^{b}	0.39 ± 0.11^{bc}	0.28 ± 0.10^{a}	0.20 ± 0.08^{a}
Human + lit CDC light trap	30	0.97 ± 0.27^{b}	2.03 ± 0.26^{b}	0.85 ± 0.18^{c}	0.08 ± 0.05^{a}	0.12 ± 0.06^{a}

N is the number of experimental nights, SE is the standard error of the mean number of catches per night whereas a dash means that no mosquito was caught. Mean ± SE mosquito catches within the same column assigned different letter superscripts are significantly different at $P < 0.05$ (Generalized Linear Models).

Responses of malaria vectors of different physiological status to a synthetic odour blend, light and humans

More female *An. funestus* (n = 579, 62.8%) were collected than *An. gambiae* s.l. (n = 343, 37.2%) (Table 3). The catches of both vectors varied greatly in terms of abdominal status ($P < 0.001$ for both). The majority of *An. gambiae* s.l. were unfed (76.4%) while the rest were either blood-fed (4.3%) or gravid (19.2%).

Whereas treatment had a significant influence on the catches of unfed ($P < 0.001$) and gravid ($P < 0.001$) *An. gambiae* s.l., collections of blood-fed mosquitoes were not different ($P = 0.87$). The 262 unfed *An. gambiae* s.l. were trapped in houses without a bait (1.5%), with IB1-baited nylon strips (13.7%), occupied with a human (16.4%), light (26.7%) and a human + light (41.6%). During the same period, the 66 gravid *An. gambiae* s.l. were attracted to IB1-impregnated nylon strips (7.6%), a human (15.2%), light (30.3%) and a human + light (45%).

The majority of *An. funestus* collected were unfed (94.3%) while the rest were either gravid (4.0%) or blood-fed (1.7%). The catches of 546 unfed *An. funestus* varied significantly among treatments ($P < 0.001$). Thus, unfed *An. gambiae* s.l. were trapped in houses without a bait (1.1%), with light (15.4%), IB1-treated nylon strips (16.7%), a human (24.9%) and a human + light (41.9%). Trap catches of blood-fed and gravid *An. funestus* was not influenced by treatment ($P = 0.34$ and $P = 0.052$, respectively) possibly because of low collections. Catches made in human-occupied houses did not contain blood-fed malaria vectors.

Table 3: Mean number (±SE) of *An. gambiae* s.l. and *An. funestus* of different abdominal status caught per night in village houses without a bait, with IB1-treated nylon strips, a human, light, or a human + light. The study was conducted in Kigoche village at weekly intervals for 30 nights.

Treatment	N	Mean ± SE number of malaria mosquitoes in different abdominal conditions					
		An. gambiae s.l.			*An. funestus*		
		Unfed	Blood-fed	Gravid	Unfed	Blood-fed	Gravid
Unlit CDC trap (no bait)	30	0.13 ± 0.07[a]	0.10 ± 0.06[a]	_	0.20 ± 0.08[a]	_	_
IBI-treated nylon strips	30	1.20 ± 0.20[b]	0.13 ± 0.07[a]	0.17 ± 0.07[a]	3.03 ±0.32[b]	0.17 ± 0.08[a]	0.03 ± 0.03[a]
Human	30	1.43 ± 0.22[b]	_	0.33 ± 0.11[a]	4.53 ± 0.40[c]	_ð _	0.37 ± 0.11[a]
Lit CDC light trap	30	2.33 ± 0.28[c]	0.10 ± 0.06[a]	0.67 ± 0.2[ab]	2.80 ± 0.31[b]	0.03 ± 0.03[a]	0.07 ± 0.05[a]
Human + lit CDC light trap	30	3.63 ± 0.35[d]	0.17 ± 0.08[a]	1.03 ± 0.19[b]	7.63 ± 0.50[d]	0.13 ± 0.07[a]	0.30 ± 0.10[a]

N is the number of experimental nights, SE is the standard error of the mean number of catches per night whereas a dash implies that no mosquito was caught. Mean ± SE mosquito catches within the same column assigned different letter superscripts are significantly different at $P < 0.05$ (Generalized Linear Models).

Sources of blood meal

A total of 15 blood-fed *An. gambiae* s.l. and 10 *An. funestus* were caught from four baits except the human stimuli alone. The 25 blood-fed mosquitoes were trapped in houses without a bait (12%), light (16%), a human + light (36%) and IB1-treated nylon strips (36%). Overall, 28.6% of *An. gambiae* s.l. blood meals were of human origin while 71.4% were from bovines. In contrast, fewer *An. funestus* had fed on bovines (30%) than humans (70%).

Estimation of *Plasmodium* infection rate of *An. gambiae* s.l. and *An. Funestus*

A sub-sample of 120 female *An. gambiae* s.l. and 120 *An. funestus* mosquitoes were tested for *P. falciparum* sporozoites. One *An. funestus* (0.8%) was *Plasmodium* positive while all *An. gambiae* s.l. turned out to be negative.

Discussion

This study has demonstrated that mosquitoes respond differently to the odour, light, and light + odour stimuli. The majority of all trapped mosquitoes were females. The attractiveness of a human and IB1 dispensed from nylon strips to female *An. gambiae* s.l. were similar. A combination of human and light attracted the highest mean numbers of female *An. gambiae* s.l., *An. funestus* and *Culex* spp. (Costantini et al., 1998a). For *An. funestus,* human stimuli were more attractive than the IB1 blend, while light attracted a similar number of mosquitoes as IB1. Blood-fed *An. gambiae* s.l. and *An. funestus* were not trapped in houses occupied by a human alone suggesting that mosquitoes that had fed on blood elsewhere did not enter such houses or that blood-fed mosquitoes do not respond to human odours. *Anopheles funestus* was the most abundant species and preferred humans to bovines. In contrast, *An. gambiae* s.l. was more dependent on bovines than humans as source of blood meal. All male mosquitoes except *Culex* spp. demonstrated a preference for light compared to the other stimuli.

All treatments including an outdoor CDC miniature light trap placed elsewhere in the same village attracted similar mosquito populations, implying that synthetic odour cues can reliably be incorporated into mosquito sampling tools (Le Goff et al., 1993, Costantini et al., 1998a). The higher collection of *An. funestus* indoors compared to outdoors suggests that this malaria vector is more endophilic/endophagic than *An. gambiae* s.l. As approximately 98% of *An. gambiae* s.l. collected in Kigoche village was *An. arabiensis*, this result is not surprising as the species is much more exophagic than *An. funestus* (Russell et al., 2011, Govella and Ferguson, 2012).

Humans and IB1-treated nylon strips were equally attractive to female *An. gambiae* s.l. suggesting that the IB1 blend may provide an alternative stimulus for sampling such vectors instead of HLC (Okumu et al., 2010a). *Anopheles funestus* was less attracted to IB1 than to human stimuli, which was different from *An. gambiae* s.l.. These observations are contrary to the findings by Okumu et al. (2010a) and Mukabana et al. (2012a) where *An. funestus* and *An. gambiae* s.l. were equally attractive to blend IB1. The different results from the present study are possibly attributed to (a) post-impregnation period over which IB1-treated nylon strips were used, (b) time interval between experimental nights, (c) a seasonal effect (Minakawa et al., 2002, Mwangangi et al., 2008, Koenraadt et al., 2004) or (d) source of carbon dioxide. According to Okumu et al. (2010a) and Mukabana et al. (2012a), IB1-treated nylon strips were prepared and used for one experimental night, and all experiments were performed over consecutive nights. In the current study, IB1-treated nylon strips were prepared once and deployed repeatedly at weekly intervals for 30 nights between November 2011 and June 2012 alongside a light stimulus and a human volunteer. Nevertheless, the reasons for the lower attractiveness of blend IB1 than a human to host-seeking *An. funestus* in Kigoche village are

currently not known and require additional studies.

Exposure of both female *An. gambiae* s.l. and *An. funestus* to a combination of a light source and a human-under-a bed net provided the most attractive and robust combination of cues for both vectors (Lines et al., 1990, Costantini et al., 1998a, Mboera et al., 1998b), probably because multimodal stimuli are involved. This explains why a CDC light trap + a human under a bed net are widely used for sampling malaria and other mosquito vectors (Costantini et al., 1998a). Light also seems to cause a higher tendency of female mosquitoes attracted to human odorants to remain and fly around the net thereby increasing the probability of passing nearby the trap. Thus, light + a human as attractive stimuli would be more suitable in studies where a wide diversity and high abundance of *Anopheles* species are required including males as they were more attracted to light than to host odour cues (Takken, 1991).

Whereas odour is an important cue for host-seeking malaria vectors, mosquitoes are also attracted to light sources and vision plays a role in directing close-range responses (Takken and Knols, 1999, Gibson and Torr, 1999). Therefore, host volatile chemicals including carbon dioxide are added to light traps to increase the number of mosquitoes collected (Service, 1993, Kline, 2007). Wavelength and intensity of light have been reported to have a great influence on the responses of different mosquito species in various physiological conditions, especially, those that bite at night including *Culex, Mansonia* and *Anopheles* species (Service, 1993, Quarles, 2003, Bentley et al., 2009, Govella et al., 2011). It is, therefore, likely that the potential of attractive synthetic odour baits to sample malaria vectors and prevent human-vector contact can be further improved by addition of suitable light cues (Lines et al., 1990, Costantini et al., 1998a, Gibson and Torr, 1999). As odour-baited trapping systems are operated on electric power, it is possible that the addition of light to such a system will allow for simultaneous and large scale sampling of mosquitoes without a confounding effect of differential attractiveness of human subjects. Although blend IB1 and humans attracted similar numbers of female *An. gambiae* s.l. that were considerably lower than those attracted to either light or light + a human, a follow-up study should be conducted in houses with different light sources and intensities. The research should also include a synthetic odour bait + light as this combination of cues was not tested in the current study. Such findings will provide more insight in the robustness of odour-based technology when applied in village houses with different light conditions and how this affects the risk of malaria transmission.

Both *An. gambiae* s.l. and *An. funestus* showed differential host preferences for blood meal sources even though the numbers of blood-fed samples were not large enough for meaningful statistical inferences (Githeko et al., 1994, Costantini et al., 1998b, Pates et al., 2001, Dekker et al., 2002). Analysis of the blood meal source confirmed that, unlike *An. funestus*, the majority of *An. gambiae* s.l. preferred bovine sources to humans hence reducing their exposure to *Plasmodium* infection (Chandler et al., 1975, Githeko et al., 1994, Mukiama and

Mwangi, 1989). This was consistent with previous findings because the *An. gambiae* s.l. collected were largely *An. arabiensis* (Mathenge et al., 2005, Atieli et al., 2009, Mukabana et al., 2012a), a species that feeds more often on bovines than *An. funestus* (Takken and Verhulst, 2013). The need for some of the indoor-resting *An. arabiensis* to seek alternative hosts outdoors may have also been prompted by inaccessibility of ñhostsò as odorant cues released from human baits and synthetic odour blends were enclosed and protected by bed-nets. *Anopheles funestus* seems to be a more important vector of malaria in Kigoche village as it was the most abundant species among indoor collections (Githeko et al., 1993, Githeko et al., 1994) and had a higher preference to feed on humans (Chapter 5, White 1974). Based on breeding ecology, *An. funestus* holds a special position in malaria epidemiology of a rice agro -ecosystem because it proliferates throughout the year and bridges transmission during dry seasons (Highton et al., 1979, Bousema et al., 2010).

The high abundance of indoor-biting *An. funestus* coupled with mixed feeding and resting behaviour of *An. arabiensis* exposes the local residents to indoor and outdoor malaria transmission (Alonso et al., 2011). The transmissions may be reduced by using odour-baited devices to supplement and enhance the effectiveness of long-lasting insecticide-treated nets and indoor residual spraying (Alonso et al., 2011, Hiscox et al., 2012). Moreover, deployment of odour-baited traps outside houses could be used to sample and intercept malaria vectors as they exit or enter houses to seek for blood meals and resting sites (Kline, 2007, Okumu et al., 2010c).

Conclusion

Visual and olfactory cues affect the response of mosquitoes to baited traps, and both cues interact to enhance trap collections of female mosquitoes. The IB1 and human stimuli attracted female *An. gambiae* s.l., *An. funestus* and *Culex* spp., but the catches were greatly increased by addition of light. *Anopheles funestus* was more abundant and anthropophagic, and may be a better vector of malaria than *An. arabiensis* in Kigoche village. As the IB1 blend was an attractive stimulus for a range of mosquito species, utilization of highly attractive novel synthetic odour baits in a more effective system can provide a robust and reliable tool for sampling mosquitoes, especially if these are supplemented with a light source.

Acknowledgements

We are grateful to inhabitants of Kigoche village for allowing us to conduct this study in their homesteads. Nicholas Mulaya is appreciated for blood meal source and sporozoite analyses of our samples and George Opetu for allowing us to access meteorological data. Our final

gratitude goes to Elisha Okwatch, Charles Oketch and John Okello as they volunteered to sleep in our experimental houses over the whole study period. This investigation was supported by a grant from the Foundations for the National Institutes of Health (FNIH) through the Grand Challenges in Global Health initiative (GCGH #121) and a sandwich Ph.D. scholarship of Wageningen University and Research Centre, The Netherlands to CKM. CKM was hosted by *icipe*-TRO campus as a dissertation research internship scholar.

Chapter 7

Enhanced attraction of African malaria vectors to a synthetic odour blend

To be submitted in a slightly modified form as: Mweresa, C.K., Mukabana, W. R., Omusula, P., Otieno, B., van Loon, J.J.A. and Takken, W. Enhanced attraction of African malaria vectors to a synthetic odour blend

Abstract

The deployment of odour-baited tools for sampling and control of malaria vectors is limited by the lack of potent synthetic mosquito attractants. Recently, a synthetic mixture of mosquito attractants referred to as ñthe Mbita blend,ò was shown to attract as many host-seeking malaria mosquitoes as were attracted to human subjects. We hypothesized that the attractive effect of this blend could be enhanced by addition of one or more compounds shown to cause behavioral responses in these mosquitoes during high through-put laboratory screening bioassays. We tested the capacity of the Mbita blend (ammonia + L-lactic acid + tetradecanoic acid + 3-methyl-1-butanol + carbon dioxide) to attract host-seeking malaria mosquitoes by addition of selected dilutions of butyl-2-methylbutanoate (1:10,000), 2-pentadecanone (1:100), 1-dodecanol (1:10,000) and 1-butylamine (1:10,000,000). The experiments were conducted in semi-field enclosures and in a village in western Kenya. In semi-field enclosures, the attraction of *Anopheles gambiae sensu stricto* females to the Mbita blend-baited traps was not affected by addition of butyl-2-methylbutanoate. There was, however, a significant increase in the proportion of *An. gambiae* caught in traps containing the Mbita blend augmented with the selected dilutions of 1-butylamine, 2-pentadecanone and 1-dodecanol. When tested in the village, addition of 1-butylamine on the Mbita blend enhanced collection of female *An. gambiae* sensu lato, *An. funestus* and *Culex* mosquitoes. Although 1-dodecanol increased attraction of *An. gambiae* s.l. to the Mbita blend, addition of 2-pentadecanone improved trap catches of *An. funestus* and *Culex* mosquitoes. The majority of female *An. gambiae* s.l. were either unfed or gravid as *An. funestus* were predominantly unfed. This study demonstrates the possibility of enhancing synthetic mosquito attractant blends for indoor collections of both unfed and gravid *An. gambiae* s.l. Our findings provide promising results for the optimization and utilization of synthetic attractants for sampling and control of major malaria vectors in unfed and gravid conditions.

Introduction

The selection of human hosts by host-seeking malaria mosquitoes is mainly mediated by olfactory cues, with heat and moisture playing a minor role (Costantini et al., 1996, Takken and Knols, 1999). This implies that identification and optimization of synthetic analogs of human odour provide an opportunity for the development of odour-baited tools for trapping of host-seeking *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *An. gambiae*) (Takken and Knols, 1999, Dekker et al., 2002, Smallegange et al., 2005, Verhulst et al., 2011). Such tools can be used for surveillance and control of malaria mosquitoes through mass trapping, lure and kill as well as push-pull strategies (Kline, 2007, Pickett et al., 2010). However, developing more potent synthetic mosquito attractants than are currently available is a major challenge in increasing the scale at which odour-baited tools for malaria control are deployed. Moreover, novel synthetic attractant blends are required for different populations of malaria mosquitoes as variations in host preference cause differences in the response to host-derived volatiles (Kweka and Mahande, 2009, Okumu et al., 2010, Mukabana et al., 2012a).

Previous field studies by Qiu et al. (2007) and Jawara et al. (2009) demonstrated that whereas *An. gambiae* was attracted to traps baited with human foot odour on worn nylon or cotton socks, other mosquito vectors were caught as well. Presently, a number of host-seeking cues of *An. gambiae* have been identified and they include ammonia, L-lactic acid, carboxylic acids and volatiles associated with human skin bacteria (Smallegange and Takken, 2010, Verhulst et al., 2010). Mixtures of these compounds have been tested for behavioral responses of host-seeking malaria vectors in different systems. A field study conducted in Tanzania showed that a synthetic blend called ñIfakara Blend 1ò (IB1) was more attractive to *An. gambiae* sensu lato mosquitoes than humans (Okumu et al., 2010). In laboratory and semi-field studies, addition of 3-methyl-1-butanol to the standard blend of ammonia, L-lactic acid and tetradecanoic acid led to a threefold increase in the catches of *An. gambiae* (Verhulst et al., 2011b). Follow-up experiments conducted in western Kenya with a similar odour blend resulted in the formulation of the Mbita blend (Mukabana et al., 2012a). The Mbita blend attracted as many host-seeking malaria mosquitoes as human subjects, and was more potent than IB1 (Okumu et al., 2010). These findings offer opportunities for the development of synthetic mosquito blends that attract significantly more malaria mosquitoes than humans.

Recently, high through-put laboratory screening bioassays have shown that 1-dodecanol, 2-pentadecanone, 1-butylamine and butyl-2-methylbutanoate are remarkably attractive to *An. gambiae* at certain dilutions (Verhulst et al., 2010, Qiu et al., 2011, Smallegange et al., 2012). We hypothesized that the potential of the Mbita blend to sample and control malaria mosquitoes can be enhanced by addition of selected dilutions of either of these novel attractant compounds. The objectives of the present study were to (a) determine suitable

dilutions at which candidate compounds combined with the Mbita blend attract *An. gambiae*, (b) evaluate the effect of selected dilution of each candidate compound on the attractiveness of the Mbita blend to *An. gambiae,* and (c) evaluate the effect of selected dilution of each candidate compound on the attractiveness of the Mbita blend to indoor biting malaria and other mosquito vectors.

Materials and Methods

Mosquitoes

A laboratory-reared colony of the Mbita strain of *An. gambiae* mosquitoes was used for semi-field assays (March to July 2011). The assays were conducted at the Thomas Odhiambo Campus (TOC) of the International Centre of Insect Physiology and Ecology (*icipe*) (00°25'S, 34°13'E, at 1240 m above sea level) near Mbita Point in western Kenya. Larvae were raised within a screen-walled greenhouse while adult mosquitoes were reared in a holding room under ambient climatic conditions. Adult female mosquitoes were fed on a human arm for 10 min. to allow egg maturation. The eggs were laid on moist filter paper and dispensed into plastic trays containing filtered water from Lake Victoria. After hatching, larvae were transferred into plastic basins filled with approximately 5 L of lake water, and fed on Tetramin® baby fish food (Melle, Germany) until pupation. Larval water was replaced at an interval of two days. Before emergence, pupae were collected daily and placed in plastic cups within cages (30 cm × 30 cm × 30 cm) covered by mosquito netting. Adult mosquitoes were maintained under a photo: scotophase of 12 : 12 h and fed on 6% glucose solution supplied on a filter paper wick. Relative humidity (RH) was maintained by moist cotton wool placed on top of cages. A total of 200 female adults aged 3 - 5 d old without prior access to a blood meal were randomly collected from cages, placed in a release cup (diameter = 13 cm, height = 13.5 cm), starved for 8 h and released at the centre of a screen-walled greenhouse for each experiment (20:00 - 06:30 h) (Verhulst et al., 2011b).

Field study site

Field studies were conducted from October to November 2011 at Kigoche village (00°34ðS, 034°65ô E, and 1158 m above sea level), approximately 110 km east of the *icipe* TOC campus. The village is situated near Ahero town in the Kano plains of Kisumu County, western Kenya. On average, Ahero experiences an approximate rainfall range of 1000 - 1800 mm, temperature of 17 - 32°C and 44 - 80% RH throughout the year. The long rainy season occurs between March and August while short rains are common in October-November. Irrigated rice farming is the mainstream economic activity. Supplementary traditional farming of maize, millet, bananas, sweet potatoes, beans, cassava, sorghum and rearing of indigenous

cattle, goats, sheep and poultry is also practiced. During the night, cattle, sheep and goats are tethered outdoors adjacent to houses occupied by dwellers. Most houses consist of mud walls, open eaves, corrugated iron-sheet roofs, without ceiling, and have either one or two rooms. The major vectors of malaria are *An. gambiae s.s., An. arabiensis* Patton and *An. funestus* Giles (Mathenge et al., 2005, Bukhari et al., 2011, Mukabana et al., 2012a). The vectors breed predominantly in rice paddies, shaded and unshaded irrigation water channels throughout the year. Our baseline data revealed that a total of 5,854 out of 19,327 patients received at the Ahero sub-district health facility in October 2011 had signs of clinical malaria, with 26% of cases confirmed by microscopy.

House characteristics and selection

Five village houses were randomly selected for indoor evaluation of candidate synthetic mosquito attractant odour blends. The selected houses were mud-walled, roofed with corrugated iron sheets, without ceiling, with either one or two rooms, free from indoor residual spays, had one door, open eaves, located within a range of 100 m from irrigation water channels and rice paddies and 28 - 481 m apart (Mukabana et al., 2012a). The houses were free from human occupation during the entire study period, situated 10 - 20 m away from cowsheds and had a total area ranging from 15.8 to 22.5 m^2. Exact location of all houses was determined by using a hand-held global positioning system receiver (Trex HC series, Garmin International Inc, USA). A random identification number used for alternation of treatments among houses at an interval of one experimental night was assigned on the door. Daily conditions of air temperature, wind speed, RH and rainfall data were obtained from the Ahero Irrigation Research Station (AIRS), located approximately 800 m away from study houses. In addition, a data logger (Tinytag® Ultra, model TGU-1500, INTAB Benelux, The Netherlands) was used to record indoor temperature and RH of the greenhouse and the village houses.

Ethical approval

Scientific and ethical clearance of the present study was granted by the Kenya Medical Research Institute (KEMRI/RES/7/3/1). Inclusion consent of houses into the study was obtained from household heads and local administration.

Odour stimuli

Four potent candidate compounds were selected from recent experiments where relative attractiveness of various chemical compounds to *An. gambiae* was investigated in high-throughput laboratory olfactometers (Verhulst et al., 2010, Qiu et al., 2011, Smallegange et al., 2012). The compounds included 1-dodecanol (> 95%), 2-pentadecanone (> 98.5%), 1-

butylamine (> 99%) and butyl-2-methylbutanoate (> 98%) purchased from Fluka (Buchs, Germany). Each compound was diluted (1:100, 1:1000, 1:10,000) in paraffin oil (Merck, Germany) (Verhulst et al., 2010). Individual dilutions of each test compound were dispensed singly along with the components of the Mbita blend (Mukabana et al., 2012a) from nylon strips (Okumu et al., 2010b, Mukabana et al., 2012b) except carbon dioxide (Smallegange et al., 2010). The Mbita blend was prepared by separately impregnating solutions of ammonia (2.5%), L-lactic acid (85%), tetradecanoic acid (0.00025%) and 3-methyl-1-butanol (0.000001%) on nylon strips (26.5 cm × 1 cm) cut from nylon socks (90% polyamide and 10% spandex). The socks were purchased from Bata Shoe Company in Kenya. During preparation, ammonia, 3-methyl-1-butanol and tetradecanoic acid were diluted in distilled water whereas L-lactic acid was used in its pure form. Carbon dioxide (approximately 63 ml/ min) was produced by mixing 2 L of tap water, 17.5 g of instant dry yeast (Angel® Company, China) and 250 g of refined sugar (Sony Sugar Company Ltd, Kenya) in a plastic container.

A bundle of five nylon strips impregnated separately with each of the four components of the Mbita blend and a single dilution of one test compound was hooked on a wire ring and suspended in the odour-dispensing tube of a counterflow (MM-X) trap (American Biophysics, North Kingstown, RI, USA) to dispense the odorants and attract mosquitoes (Schmied et al., 2008, Okumu et al., 2010b). Carbon dioxide production was started 30 min. prior to the onset of the experiments and supplied through 60 cm long silicon tubing (0.5 cm diameter) into four MM-X traps baited with the Mbita blend alone or the Mbita blend augmented with either of the three test dilutions of novel compounds. An extra MM-X trap containing five untreated nylon strips soaked in 1ml of water and dried was included for control and was not supplied with carbon dioxide (i.e. no odour). During experiments, trap outlets were positioned at a height of 15 cm off ground level (Schmied et al., 2008, Jawara et al., 2009). All traps were operated on 12 V. Although control and attractant-treated nylon strips for single sets of experiments were re-used throughout without replenishment, CO_2 was prepared anew for each experimental night (Mukabana et al., 2012b). Traps assigned to particular treatments were marked and operated with the same chemical blend for the entire study. The traps were randomly assigned to different positions and alternated at an interval of one night to minimize potential confounding effects of trap position or house characteristics.

Determination of suitable dilutions of candidate compounds combined with the Mbita blend

This preliminary study was aimed at determining suitable dilutions at which individual test compounds attract the highest proportion of *An. gambiae* when added singly to the Mbita blend. To achieve this, behavioral responses of *An. gambiae* to the Mbita blend augmented separately with single concentrations (dilution 1:100, 1:1000, and 1:10,000) of each candidate compound versus untreated nylon strips (no odour) were evaluated in a randomised 4 × 4

Latin square design for 12 nights. The best dilution at which each test compound elicited the highest behavioral responses of mosquitoes to the Mbita blend was selected for subsequent experiments.

Effect of selected dilutions of candidate compounds on attractiveness of the Mbita blend

Further investigations were carried out to ascertain whether the proportion of mosquitoes attracted to the Mbita blend supplemented with a selected dilution of each test compound (derived from the preceding experiment) was significantly higher compared to that responding to the Mbita blend alone. As a result, behavioural responses of *An. gambiae* to the Mbita blend versus the Mbita blend augmented with (a) butyl-2-methylbutanoate (1:10,000) (b) 2-pentadecanone (1:100), (c) 1-dodecanol (1:10,000) and (d), 1-butylamine (1:100) were evaluated in a dual-choice bioassay. Individual bioassays were repeated over four nights within a screen-walled greenhouse (Smallegange et al., 2010, Verhulst et al., 2011b). Prior to this study, a four-night experiment was performed to determine the symmetry of the bioassay by evaluating the responses of *An. gambiae* to two MM-X traps containing untreated nylon strips. In a previous laboratory screening study, we showed that 1-butylamine was more attractive to *An. gambiae* at a dilution of 1: 10,000,000 than our candidate dilutions when released in conjunction with the standard blend. Therefore, we also tested whether the same dilution of 1-butylamine alone would have an effect on the Mbita blend as well. Test compounds with a significant effect on the attractiveness of the Mbita blend to *An. gambiae* were selected for further study at Kigoche village.

Routine procedures

At the end of each experimental night, trapped mosquitoes were rapidly immobilised at -4^{o}C for 20 min. Immobilised mosquitoes were collected, counted, recorded, labelled and used to estimate the relative attraction and suitability of different dilutions of test compounds presented with the Mbita blend to *An. gambiae* and other mosquito species. Latex gloves were worn during preparation of CO_2, nylon strips, application of attractants on nylon strips and baiting of traps to avoid contamination with human volatiles or other odorant compounds. The traps were thoroughly cleaned using 70% methanol and dried in preparation for the next night of experiments. Vaseline pure petroleum jelly was applied on suspension wire bars and electrical cables to prevent ants from preying on mosquitoes caught in the MM-X trap. Mosquitoes that had not been trapped were recaptured from the screen house using a manual aspirator and killed. The sandy floor of the greenhouse was moistened with water daily to prevent dust, reduce temperature and increase humidity. Attractant-treated nylon strips were stored at 4^{o}C in between experiments.

Effect of selected dilutions of candidate compounds on attractiveness of the Mbita blend to indoor-biting malaria and other mosquito vectors in a village

The effect of selected dilutions of 2-pentadecanone, 1-dodecanol and 1-butylamine on the attractiveness of the Mbita blend to indoor-biting malaria and other mosquitoes was tested in five village houses. This was achieved through a randomised 5 × 5 Latin Square experimental design for 25 nights (18:30 - 06:30 h). The treatments included (a) untreated nylon strips (no odour), (b) the Mbita blend alone, (c) the Mbita blend + 2-pentadecanone (1:100), (d) the Mbita blend + 1-dodecanol (1:10,000) and (e) the Mbita blend + 1-butylamine (1:10, 000,000). Attractant-treated and untreated nylon strips were re-used for the entire study period (Mukabana et al., 2012b). The traps were suspended at the center of an empty village house under the overhanging roof (Jawara et al., 2009). At the end of each experiment, traps containing mosquitoes were transported from the study houses to a field laboratory located at the Ahero Multipurpose Development Training Institute (AMDTI) and immobilised. Thereafter, collected mosquitoes were identified morphologically (Gillies and Coetzee, 1987), counted, recorded and labelled according to (i) gender as male or female *An. gambiae* s.l., *An. funestus*, *Culex, Mansonia* spp., and other anopheline mosquitoes (all collected *Anopheles* spp. except *An. gambiae* s.l. and *An. funestus*) and (ii) external abdominal appearance as unfed, blood-fed (fully and partially fed), or gravid female of *An. funestus*, and *An. gambiae* s.l. (WHO, 1975). All females of *An. funestus* and *An. gambiae* s.l were preserved in 2 ml Eppendorf tubes containing silica gel.

Statistical analysis

The effect of different behavioral stimuli (untreated nylon and prototypes of the Mbita blend impregnated on nylon strips) on mosquito attraction was estimated from the proportions of mosquitoes collected from each treatment. Proportion was expressed as the number of mosquitoes caught in one of the traps divided by the total number of mosquitoes trapped in all traps during each experimental night (Qiu et al., 2007). Individual dual-choice tests conducted during semi-field experiments were analysed by using Chi-square test. The Chi-square test was used to determine whether the distribution of mosquitoes caught in both MM-X traps differed from a 1:1 ratio. The total numbers of mosquitoes caught in each trap tested in the 4 × 4 or 5 × 5 Latin square design were analysed by using a Generalized Linear Model (GLM) assuming a Poisson distribution and logarithmic link function (Verhulst et al., 2011). Significant effects of treatment, trap position or house on mosquito catches were tested and fitted as parameters in the model. Effects were considered significant at $P < 0.05$. All analyses were performed using IBM SPSS statistical software, release 16.

Results

Suitable dilutions of candidate compounds combined with the Mbita blend

All semi-field experiments (March to July 2011) were conducted within a temperature range of 22.8 - 23.2^{o}C and 78.5 - 82.6% RH. Odour-baited traps caught significantly more *An. gambiae* mosquitoes compared to those without odour ($P < 0.001$ for all) (Table 1). The response of mosquitoes to the candidate compounds released in combination with the Mbita blend depended on dilution ($P < 0.001$). Each candidate attractant compound enhanced the response of mosquitoes to the Mbita blend at a specific dilution. These dilutions were 1:100 for 1-butylamine and 2-pentadecanone, and 1:10,000 for 1-dodecanol and butyl-2-methylbutanoate.

Table 1: Behavioral responses of *Anopheles gambiae* to three dilutions of candidate attractant compounds dispensed from nylon strips in combination with the Mbita blend. Studies were conducted in a large outdoor screened cage.

Treatment	N	Mean number ± SE of mosquitoes caught			
		Control	1:100	1:1,000	1:10,000
Mbita blend + butyl-2-methylbutanoate	12	1.7 ± 0.4^{a}	44.3 ± 1.9^{b}	44.8 ± 1.9^{b}	54.3 ± 2.1^{c}
Mbita blend + 2-pentadecanone	12	0.8 ± 0.3^{a}	57.9 ± 2.2^{b}	51.33 ± 2.1^{c}	37.3 ± 1.8^{d}
Mbita blend + 1-dodecanol	12	1.2 ± 0.3^{a}	53.9 ± 2.1^{b}	46.8 ± 2.0^{c}	66.4 ±2.4^{d}
Mbita blend + 1-butylamine	12	1.3 ± 0.4^{a}	60.9 ± 2.3^{b}	53.7 ± 2.2^{c}	48.9 ± 2.0^{c}

N is the number of experimental nights whereas SE is the standard error of the mean catch per night. Two hundred female *An. gambiae* were released per night. Mean catches in the same row that have no superscript letters in common differ significantly at $P < 0.05$ (Generalized Linear Models).

Effect of selected dilutions on attractiveness of the Mbita blend

Dual-choice bioassays were used to investigate whether the attractiveness of the Mbita blend to mosquitoes was greatly enhanced by addition of selected dilutions of each test compound (Table 2). There was no significant difference in the proportion of mosquitoes caught between unbaited traps ($P = 0.35$) implying that the choice test was symmetrical. The responses of mosquitoes to the Mbita blend augmented with 1-dodecanol (1:10,000) or 2-pentadecanone (1:100) was significantly higher compared to the Mbita blend alone ($P < 0.001$ for each). Whereas a combination of the Mbita blend with 1-butylamine (1:100) or butyl-2-methylbutanoate (1:10,000) attracted more mosquitoes, the difference was not significant from the Mbita blend alone ($P = 0.30$ and $P = 0.16$, respectively). Further experiments

confirmed that the attractiveness of the Mbita blend for mosquitoes was greatly improved by addition of 1-butylamine at 1:10,000,000 ($P < 0.020$). The enhanced potential of the supplemented Mbita blend to attract malaria and other mosquitoes indoors was subsequently evaluated in Kigoche village.

Table 2: Behavioral responses of *An. gambiae* to traps baited with the Mbita blend alone versus the Mbita blend supplemented with selected dilutions of test compounds released from nylon strips. Studies were conducted in a large outdoor screened cage.

Novel Blend (Mbita blend + optimal dilution of test compound)	N	Mean number ± SE of mosquitoes caught		
		Novel blend	Mbita blend alone	P-value
Mbita blend + butyl-2-methylbutanoate (1:10,000)	4	54. 0 ± 3.7	48.8 ± 3.5	0.30
Mbita blend + 2-pentadecanone (1:100)	4	70.8 ± 4.2	44.8 ± 3.3	0.001
Mbita blend + 1-dodecanol (1:10,000)	4	71.3 ± 4.2	47.3 ± 3.4	0.001
Mbita blend + 1-butylamine (1:100)	4	49.3 ± 3.5	42.5 ± 3.6	0.16
Mbita blend + 1-butylamine (1:10,000,000)	4	71.8 ± 4.2	58.5 ± 3.8	0.020

N is the number of experimental nights whereas SE is the standard error of the mean catch per night. A total of 200 female *An. gambiae* were released per night. Pairwise comparisons of mosquitoes caught differ significantly at $P < 0.05$ (Chi-square test).

Effect of selected dilutions on attractiveness of the Mbita blend to indoor-biting malaria and other mosquito vectors

Effect on female mosquitoes

During the 25 nights of field studies (October to November 2011), temperatures ranging from 18.3 - 32.9^{o}C and RH of 78.6 - 96.3% were recorded inside village houses. At the same time, outdoor conditions were characterised by a total of 231.6 mm of rainfall, 60.0% RH, 16.8 - 30.8^{o}C temperature and an average wind speed of 3.2 ± 0.1 km/h. Of the 1,154 mosquitoes collected indoors, 956 were females and 198 were males. The females comprised *An. gambiae* s.l. (41.0%), *An. funestus* (30.5%), *Culex* spp. (23.0%), *Mansonia* spp. (0.31%) and other anopheline spp. (5.1%) (Figure 1).

The attraction of *An. gambiae* s.l. to the Mbita blend was significantly increased by addition of 1-butylamine (1:10,000,000) or 1-dodecanol (1:10,000) ($P < 0.010$ for both), but not of 2-

pentadecanone (1:100) ($P < 0.38$). However, responses of *An. funestus* to the Mbita blend were greatly enhanced by addition of either 2-pentadecanone (1:100) or 1-butylamine (1:10,000,000) ($P < 0.049$ and $P < 0.001$, respectively). Similarly, attractiveness of the Mbita blend to *Culex* mosquitoes was improved when supplemented with 2-pentadecanone (1:100) ($P < 0.034$) or 1-butylamine ($P < 0.038$). By contrast, a combination of the Mbita blend and 1-dodecanol (1:10,000) had no effect on trap collections of *An. funestus* ($P = 0.15$) and *Culex* ($P = 1.00$). Moreover, responses of *Mansonia* spp. ($P = 0.57$) and other species of *Anopheles* ($P = 0.13$) to the Mbita blend were not affected by type of treatment.

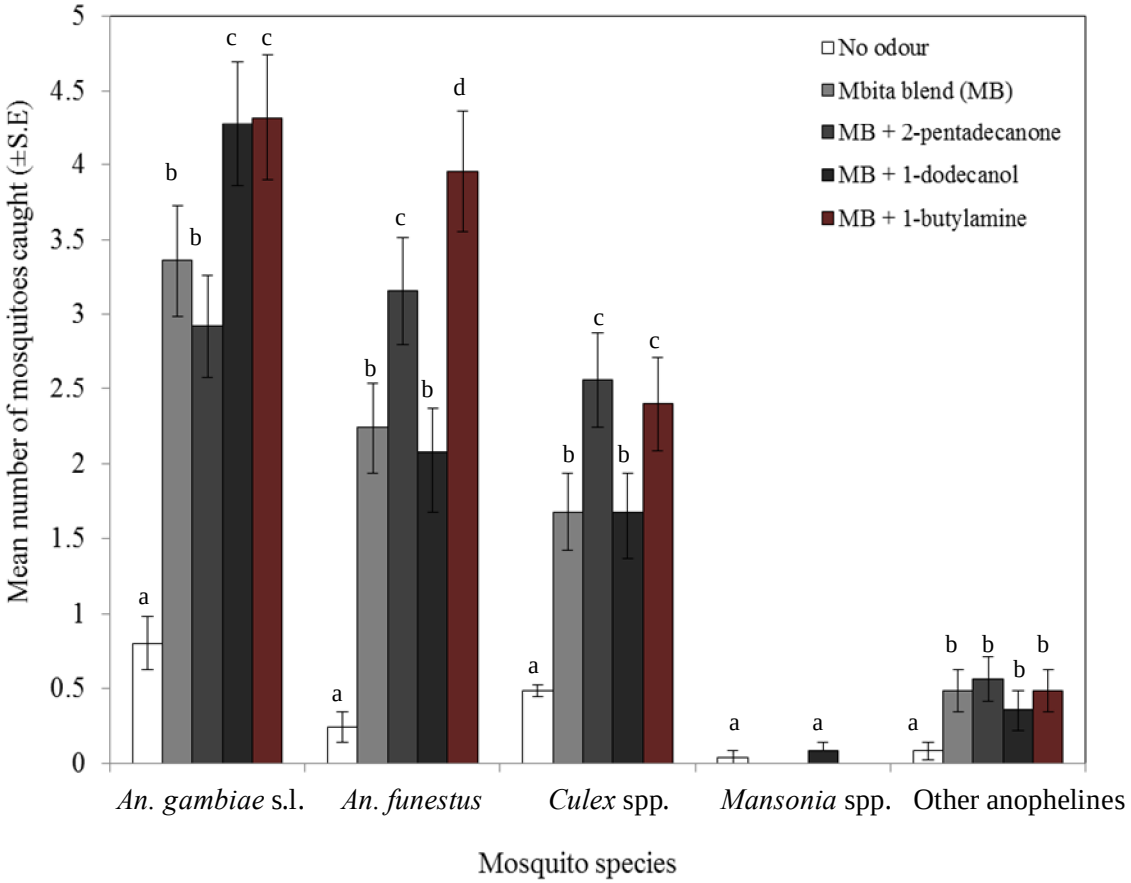


Figure 1: Mean ± SE number of female mosquitoes collected overnight in an indoor trap without odour, baited with the Mbita blend (MB) alone, the Mbita blend augmented with either 2-pentadecanone (1:100), 1-dodecanol (1:10,000) or 1-butylamine (1:10,000,000). The study was conducted in Kigoche village for 25 nights. Treatments are shown in the legend. Mean values within the same mosquito type having no letter in common differ significantly at $P < 0.05$.

Effect on malaria vectors in different physiological status

The abdominal conditions of *An. gambiae* s.l. collected were dependent on treatment ($P < 0.001$) (Figure 2A). The 392 *An. gambiae* s.l. caught were unfed (48.4%), gravid (50.4%) or blood-fed (0.8%).

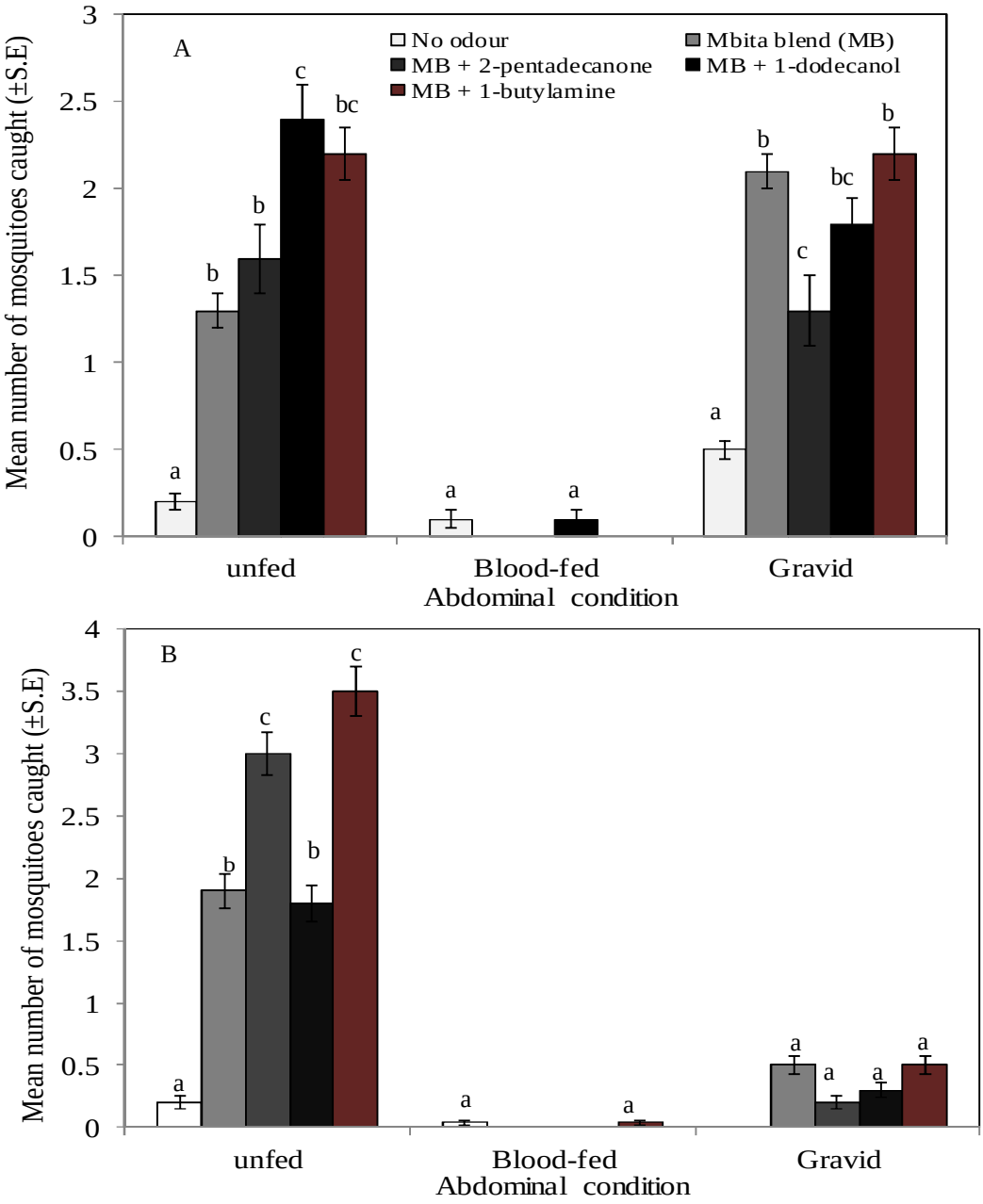


Figure 2: Mean ± SE number of *An. gambiae* s.l. (panel A) and *An. funestus* (panel B) in different abdominal conditions (unfed, blood-fed and gravid) collected overnight in an indoor trap without odour, baited with the Mbita blend (MB) alone, the Mbita blend augmented with either 2-pentadecanone (1:100), 1-dodecanol (1:10,000) or 1-butylamine (1:10,000,000). The study was conducted in Kigoche village for 25 nights. Treatments are shown in the legend of panel A. Mean values within the same abdominal condition having no letter in common differ significantly at $P < 0.05$.

The attraction of unfed *An. gambiae* s.l. to the Mbita blend was significantly increased by addition of 1-butylamine ($P < 0.024$) or 1-dodecanol ($P < 0.01$) but not so with 2-pentadecanone ($P = 0.41$). Moreover, the responses of gravid *An. gambiae* s.l. to the Mbita blend alone were not affected by addition of 1-butylamine ($P = 0.77$) or 1-dodecanol ($P = 0.48$), and they were significantly reduced in the presence of 2-pentadecanone ($P < 0.001$).

Although low catches of blood-fed *An. gambiae* s.l. were recorded in a trap without odour or with the Mbita blend supplemented with 1-dodecanol, the catches were not statistically different (P = 0.99).

Similarly, abdominal conditions of *An. funestus* collected indoors were influenced by treatment (P < 0.001) (Figure 2B). The 292 *An. funestus* caught were unfed (87.0%), blood-fed (0.7%) or gravid (12.3%). A significantly higher number of unfed *An. funestus* responded to the Mbita blend augmented with 1-butylamine (P < 0.001) or 2-pentadecanone (P < 0.012) than to the Mbita blend alone. However, the catches of unfed *An. funestus* were not affected by addition of 1-dodecanol to the Mbita blend (P = 0.83). Trap collections of both blood-fed (P = 0.87) and gravid *An. funestus* (P = 0.69) were not influenced by treatment.

Discussion

The present study demonstrated that the degree of attraction of mosquitoes to candidate compounds released in combination with the Mbita blend was concentration dependent. Addition of 1-butylamine to the Mbita blend increased the catches of female *An. gambiae* s.l., *An. funestus* and *Culex* mosquitoes. Whereas 1-dodecanol was important in increasing attraction of *An. gambiae* s.l. to the Mbita blend, addition of 2-pentadecanone improved trap catches of *An. funestus* and *Culex* mosquitoes but not of *An. gambiae* s.l.. The majority of female *An. gambiae* s.l. collected was either unfed or gravid whereas *An. funestus* were predominantly unfed.

The findings of this study are consistent with differential attractiveness of human odours to host-seeking malaria mosquitoes (Takken and Knols, 1999,Verhulst et al., 2011b). The specific responses of mosquitoes to the Mbita blend supplemented with selected dilutions of candidate compounds confirmed the findings of previous olfactometer bioassays (Verhulst et al., 2010, Smallegange et al., 2012). Unlike in olfactometer bioassays, however, 1-dodecanol and 1-butylamine enhanced the attractiveness of the Mbita blend to mosquitoes at relatively lower concentrations whereas 2-pentadecanone was effective at a higher concentration (Verhulst et al., 2010, Qiu et al., 2011, Smallegange et al., 2012). The variations may mainly be attributed to the different types of odour-dispensing devices used, and composition of the standard attractant blend to which candidate compounds were added. In the present study, all attractants except CO_2 were dispensed from nylon strips instead of LDPE sachets. In addition to ammonia, L-lactic acid and tetradecanoic acid, the Mbita blend contained 3-methyl-1-butanol as an additional component (Verhulst et al., 2011b, Mukabana et al., 2012a). These results indicate that mosquito vectors and other haematophagous insects distinguish odour blends based on quality and quantity of received stimuli (Carey and Carlson, 2011). For

examples suggest that a single bait that fits the requirements for different populations of haematophagous dipterans is currently not available (Kline et al., 1990, Kline, 2007).

Whereas 1-butylamine was consistently effective at a much lower concentration (1:10,000,000) compared to the other candidate compounds, this compound also improved the attractiveness of the Mbita blend to multiple mosquito vectors including *An. gambiae* s.l., *An. funestus* and culicines. Furthermore, a synergistic effect between 2-pentadecanone and the Mbita blend provided a potent trap lure for *An. funestus* and culicines whereas addition of 1-dodecanol increased the response of *An. gambiae* s.l. to the Mbita blend. Similar effects of synthetic odour baits to multiple mosquito vectors have also been reported in other parts of Africa. In The Gambia, a synthetic odour blend composed of ammonia, L-lactic acid, 3-methylbutanoic acid and CO_2 was highly attractive to field populations of *Anopheles*, *Culex* and *Mansonia* spp. (Qiu et al., 2007). But in East Africa, a blend of ammonia, L-lactic acid, CO_2, and seven aliphatic carboxylic acids formulated for malaria vectors attracted *Mansonia, Culex* and *Aedes* spp. as well (Mukabana et al., 2010, Okumu et al., 2010).

Human landing catches (HLC) used to sample malaria mosquitoes may be substituted by traps baited with the Mbita blend augmented with either 1-butylamine, 1-dodecanol or 2-pentadecanone (Mathenge et al., 2005, Dia et al., 2005). A recent study in the same area demonstrated that, although the Mbita blend attracted as many indoor-biting *An. gambiae* s.l. as were attracted to human subjects, each of them was significantly more attractive than the standard blend and blend IB1 (Mukabana et al., 2012a). In addition, the Mbita blend attracted more female *An. funestus* compared to the human subjects, standard blend or blend IB1. These findings suggest that replacement of HLC with odour-baited tools would be essential as the former technique is labour intensive, cumbersome, hazardous, and requires intense supervision that is difficult to sustain on a large scale (Mboera, 2005, Ndiath et al., 2011).

The high catches of unfed females of *An. gambiae* s.l., *An. funestus* and *Culex* mosquitoes also portray the possibility of deploying odour-baited tools for malaria reduction by mass trapping (Vale and Torr, 2004, Kline, 2007, Okumu et al., 2010c). However, the attractiveness of the Mbita blend prototypes should be compared with human volunteers in future studies. This may present a supplementary solution to current increase in outdoor transmission of malaria, particularly in areas where insecticide-treated bed nets and indoor residual spraying are intensively used (Bayoh et al., 2010, Russell et al., 2011). Like in agricultural systems, availability of suitable release systems and spatial repellents would allow skilful manipulation of both attractants and repellents in a push-pull technology to control malaria vectors (Pickett et al., 2010, Takken, 2010). Our novel odour baits formulated from the Mbita blend may be used to ópullô mosquitoes into traps whereas potential repellents includ-

example, natural mosquito, *Culicoides*, and tabanid populations responded differently to various combinations of butanone, CO_2, honey, 1-octen-3-ol, lactic acid and phenols (Kline et al., 1990). In Scotland, the responses of *Culicoides* species to a standard bait (CO_2 + 1-octen-3-ol) was dependent on the source and concentration of the host odour extract added (Mands et al., 2004).

A comparative study in Canada and East Africa also indicated that the attractiveness of 1-octen-3-ol, acetone, 4-methylphenol, 3-n-propylphenol, and other potential lures (human urine, stable fly faeces) to tabanids and tsetse flies was influenced by geographical location and composition of the blends used (Mihok et al., 2007). These Oviposition semiochemicals have been tested for monitoring and control of *Aedes* and *Culex* mosquito populations (Mboera et al., 2000, Trexler et al., 2003, Navarro-Silva et al., 2009, Seenivasagan and Vijayaraghavan, 2010), but have not been appropriately investigated for other vector species including anopheline mosquitoes (Pickett et al., 2010). Interestingly, our findings demonstrated that host-seeking attractants can also be deployed to lure gravid *An. arabienis* as observed with cattle urine (Kweka et al., 2011).

Unlike gravid *An. funestus*, gravid *An. gambiae* s.l. were highly and equally attracted to the Mbita blend alone, and the Mbita blend augmented with either 1-dodecanone, or 1-butylamine than with 2-pentadecanone. Nonetheless, it is not clear how synthetic odour baits formulated for host-seeking processes may have up-regulated the sensitivity of odour receptors of gravid mosquitoes. Therefore, these results should be investigated further as HLC and CDC-light trap collections of African anophelines rarely contain gravid mosquitoes as usually most females are unfed (Qiu and van Loon, 2010).

The search for a synthetic odour blend that attracts mosquito vectors in the three physiological conditions (i.e. unfed, blood-fed and gravid) would be essential for analysis of feeding behaviour, egg-laying cycle, age structure, survival rate, abundance and estimation of entomological inoculation rate (Wanji et al., 2003, Charlwood et al., 2012, Scott and Takken, 2012). In addition, low collections of male mosquitoes and high catches of female *An. gambiae* s.l. and *An. funestus* demonstrated the selective potential of using odour-baited tools to control malaria mosquitoes as an alternative solution to broad-spectrum and toxic insecticides.

Conclusion

The current study demonstrates improved attractiveness of odorant blends for malaria mosquitoes and the importance of concentrations in achieving this. The attractiveness of the Mbita blend to malaria vectors and *Culex* mosquitoes was improved by addition of selected

dilutions of 1-butylamine, 2-pentadecanone, or 1-dodecanol. This is the first study reporting that mosquito-attractant blends deployed in village houses collected both unfed and gravid *An. gambiae* s.l. mosquitoes. Our findings provide promising results on the improvement and utilization of odour-baited tools for surveillance, sampling and control of major malaria and other mosquito vectors in an African setting (Kline, 2006, Kline, 2007). Such tools can be used to augment indoor residual spraying, insecticide treated nets and artemisinin-combination therapies for malaria control and eradication (Mendis et al., 2009).

Acknowledgements

We thank David Odhiambo Alila for rearing mosquitoes used for our semi-field experiments. We also appreciate Andrew Brian Abuya for aspirating mosquitoes, Charles A. Oketch for working with Philemon during field experiments and George Odhiambo Opetu for providing us with field meteorological data. This study was funded by a grant from the Foundation of National Institutes of Health (FNIH) through the Grand Challenges in Global Health initiative (GCGH #121) and sandwich Ph.D scholarship of the Wageningen University and Research Centre, The Netherlands (CKM). CKM was hosted by ICIPE ï MPR&TC as a dissertation research internship scholar.

Chapter 8

Molasses as a source of carbon dioxide for the attraction of malaria mosquitoes *Anopheles gambiae* and *An. funestus*

Published as: Mweresa, C.K., Omusula, P., Otieno, B., van Loon, J.J.A., Takken, W. and Mukabana, W. R. Molasses as a source of carbon dioxide for the malaria mosquitoes *Anopheles gambiae* and *An. funestus.* Malaria Journal, 13: 160

Abstract

Most odour baits for haematophagous arthropods contain carbon dioxide (CO_2). The CO_2 is sourced artificially from the fermentation of refined sugar (sucrose), dry ice, pressurized gas cylinders or propane. These sources of CO_2 are neither cost-effective nor sustainable for use in remote areas of sub-Saharan Africa. In this study, molasses was evaluated as a potential substrate for producing CO_2 used as bait for malaria mosquitoes. The attraction of laboratory-reared and wild *Anopheles gambiae* complex mosquitoes to CO_2 generated from yeast-fermentation of molasses was assessed under semi-field and field conditions in western Kenya. In the field, responses of wild *Anopheles funestus* were also assessed. Attraction of the mosquitoes to a synthetic mosquito attractant, Mbita blend (comprising ammonia, L-lactic acid, tetradecanoic acid and 3-methyl-1-butanol) when augmented with CO_2 generated from yeast fermentation of either molasses or sucrose was also investigated. In semi-field, the release rate of CO_2 and proportion of *An. gambiae* mosquitoes attracted increased in tandem with an increase in the quantity of yeast-fermented molasses up to an optimal ratio of molasses and dry yeast. More *An. gambiae* mosquitoes were attracted to a combination of the Mbita blend plus CO_2 produced from fermenting molasses than the Mbita blend plus CO_2 from yeast-fermented sucrose. In the field, significantly more female *An. gambiae* sensu lato mosquitoes were attracted to the Mbita blend augmented with CO_2 produced by fermenting 500 g of molasses compared to 250 g of sucrose or 250 g of molasses. Similarly, significantly more *An. funestus*, *Culex* and other anopheline mosquito species were attracted to the Mbita blend augmented with CO_2 produced from fermenting molasses than the Mbita blend with CO_2 produced from sucrose. Augmenting the Mbita blend with CO_2 produced from molasses was associated with high catches of blood-fed *An. gambiae* s.l. and *An. funestus* mosquitoes. Molasses is a suitable ingredient for the replacement of sucrose as a substrate for the production of CO_2 for sampling of African malaria vectors and other mosquito species. The finding of blood-fed malaria vectors in traps baited with the Mbita blend and CO_2 derived from molasses provides a unique opportunity for the study of host-vector interactions.

Introduction

Female mosquitoes rely mainly on odour cues to locate vertebrate hosts from which they obtain blood meals necessary for egg maturation (Takken, 1991). One of these cues, namely carbon dioxide (CO_2), activates and guides mosquitoes towards their hosts (Gillies, 1980, Costantini et al., 1996, Takken and Knols, 1999). It is for this reason that CO_2 is commonly added to mosquito traps, the ultimate aim being to increase mosquito catches during surveillance and/or sampling (Gibson and Torr, 1999, Mboera et al., 2000, Kline, 2007). Previous studies have shown that utilization of CO_2 to increase trap catches is dependent on mosquito species, release rate and structure of the host-odour plume (Costantini et al., 1996, Kline and Lemire, 1998, Spitzen et al., 2008, Cardé and Gibson, 2010). It is now evident that CO_2 enhances catches of the anthropophilic and anthropophagic malaria vector *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *An. gambiae*) when released together with human-related odorants (Cardé and Gibson, 2010, Smallegange et al., 2010, Jawara et al., 2011).

Laboratory findings indicated that higher catches of *An. gambiae* mosquitoes were recorded in traps baited with CO_2 combined with skin emanations or ammonia plus L-lactic acid than CO_2 alone (Spitzen et al., 2008). Under semi-field conditions, responses of *An. gambiae* to traps baited with foot odour were greatly increased by adding CO_2 (Njiru et al., 2006). In The Gambia, addition of CO_2 to synthetic odours substantially increased the catches of females of all mosquito species collected in MM-X traps (Qiu et al., 2007, Jawara et al., 2011). In addition, traps baited with CO_2 recorded significantly higher numbers of female *An. gambiae* sensu lato and *An. funestus* Giles than those without (Costantini et al., 1996, Mboera et al., 1997). Currently, CO_2 is incorporated in synthetic odour blends for sampling of malaria vectors (Okumu et al., 2010a, Mukabana et al., 2012a).

Artificial sources of CO_2 such as fermentation of refined sugar, dry ice, and CO_2 released from pressurized gas cylinders or from propane-powered traps are commonly used in mosquito traps (Mboera et al., 1997, Burkett et al., 2002, Oli et al., 2005, Kline, 2006, Njiru et al., 2006). However, utilization of CO_2 from the named sources is neither cost-effective nor sustainable for mass deployment of odour-baited devices in remote areas of the tropics. For instance, combustion of propane depends on costly gas tanks that are not widely available in rural sub-Saharan Africa where the greatest burden of malaria occurs. In the tropics, dry ice sublimes faster than in temperate areas and therefore, it has to be replaced more frequently. Where mass trapping is required, the use of industrially-produced CO_2 stored in pressurized cylinders will be prohibitively expensive. To overcome these limitations, CO_2 produced by fermentation of refined sugar is currently used to supplement synthetic odour-baited MM-X traps for sampling the African malaria vector *An. gambiae* and other mosquito vectors

(Smallegange et al., 2010). Nevertheless, increased cost of living and escalating prices of refined cane sugar in sub-Saharan Africa call for production of CO_2 from cheaper and locally available raw materials.

In this study the possibility of producing CO_2 by fermentation of sugar cane molasses (i.e., a by-product formed after crystallization of refined white sugar from the raw juice of crushed sugar cane) instead of refined sugar was explored. The objectives of this study were to: (a) determine average release rates of CO_2 produced by fermentation of different quantities of molasses and dry yeast; (b) evaluate the effect of release rates of CO_2 on behavioural responses of *An. gambiae;* (c) evaluate the effect of CO_2 produced from molasses on attractiveness of *An. gambiae* to a previously charatecterized synthetic odour blend; (d) assess the effect of CO_2 released from refined sugar and molasses on attractiveness of a synthetic odour blend to *An. gambiae*; and, (e) evaluate the effect of CO_2 released from refined sugar and molasses on attractiveness of a synthetic odour blend to outdoor-biting malaria and other mosquitoes.

Materials and Methods

Mosquitoes

Semi-field experiments were conducted (April-September 2011) at the Thomas Odhiambo Campus (TOC) of the International Centre of Insect Physiology and Ecology (*icipe*) located near Mbita Point Township in western Kenya. Laboratory-reared *An. gambiae* (Mbita strain) mosquitoes were used. Mosquito larvae were raised within a screen-walled greenhouse under ambient climatic conditions while adults were reared in a holding room under ambient conditions with a photo: scotophase of 12:12 h. For egg maturation, female mosquitoes were fed three times a week on blood by direct imbibition from a human arm for 10 min. Eggs were laid on moist filter paper and dispensed into plastic trays containing filtered water from Lake Victoria. Newly hatched larvae were transferred into plastic basins and fed on Tetramin® baby fish food (Melle, Germany) provided three times a day. Pupae were collected daily, placed in clean cups containing filtered water from Lake Victoria and enclosed in mosquito cages. Emerging adult mosquitoes were kept inside cages (30 × 30 × 30 cm) covered by mosquito netting and maintained on 6% glucose solution delivered on Whatman filter paper wicks. Water was provided on cotton towels placed on top of the mesh-covered cages. A total of 200 females aged 3-5 d old without prior access to a blood meal were randomly collected from holding cages, placed in a release cup covered with mosquito netting, starved for 8 h and released at the centre of a screen-walled greenhouse at the onset of each experiment (20:00 - 06:30 h) (Verhulst et al., 2011b). During starvation, mosquitoes were only provided with water on a moistened towel.

Field study site

Field studies were carried out in November 2011 at Kigoche village, situated near Ahero town, in the Kano plains of Kisumu County, western Kenya. Kigoche village is located 00°34ôS, 034°65ô E and 1,158 m above sea level along the northern boundary of the Ahero rice irrigation scheme (Bukhari et al., 2011, Mukabana et al., 2012a), approximately 110 km east of the *icipe,* TOC campus. Generally, Ahero experiences an approximate rainfall range of 1000 - 1800 mm, temperature of 17 ï 32°C and 44 - 80% relative humidity (RH) throughout the year. The long rainy season occurs between March and August while short rains are common in October-November. Irrigated rice farming is the main economic activity. Supplementary traditional farming of maize, millet, bananas, sweet potatoes, beans, cassava, sorghum and rearing of indigenous cattle, goats, sheep and poultry is also practiced. During the night, cattle, sheep and goats are tethered outdoors adjacent to houses occupied by dwellers. Most houses consist of mud walls with open eaves, corrugated iron-sheet roofs, without ceiling, and have either one or two rooms. Malaria transmission is mainly caused by *An. gambiae, An. arabiensis* Patton and *An. funestus* (Mathenge et al., 2005, Bukhari et al., 2011, Mukabana et al., 2012a). The vectors breed predominantly in rice paddies, shaded and unshaded irrigation water channels. Experiments were started at 18:30-06:30 h because light was not necessary under field conditions and wild the mosquitoes lured to trapping devices originate from random sources located in different directions

Ethical approval

Scientific and ethical clearance of the present study was granted by the Kenya Medical Research Institute (KEMRI/RES/7/3/1). Inclusion consent of houses into the study was obtained from household heads and local administration.

Preparation and dispensing of synthetic mosquito odour blend

Carbon dioxide was produced from a mixture of refined cane sugar (ñsugarò) or molasses from sugar cane (ñmolassesò) (Mumias Sugar Company Ltd, Kenya), instant dry yeast (ñyeastò) (Angel® Company, China) and 2 L of water. The sugar content, total dissolved solids and purity of molasses used were determined at the Kenya Sugar Research Foundation (KESREF) laboratory in Kibos, western Kenya. Both sugar and molasses were processed from sugar cane grown in a malaria-prone area of western Kenya. To achieve fermentation, 250 g of sugar was mixed with 17.5 g yeast and 2 L water (Smallegange et al., 2010a, Mukabana et al., 2012a). Molasses-produced CO_2 was obtained by mixing 2 L of water with (a) 125 g molasses plus either 8.75 g or 17.5 g yeast, (b) 250 g molasses plus 17.5 g or 35 g yeast and (c), 500 g molasses plus 17.5 g or 35 g yeast. Tap water was used during semi-field experiments while field bioassays were conducted using water from a nearby stream. The

ingredients were mixed by shaking for 30 sec. prior to fermentation, in 5 L plastic containers under ambient conditions.

A strip of laboratory Parafilm ñMò (Pechiney Plastic Packaging, Chicago, IL 60631, USA) was tied round the connection points along the CO_2 delivery system to prevent leakage. In addition, non-perfumed pure petroleum jelly (Vaseline ^{TM}, Unilever Kenya Ltd) was also applied to prevent leakage. There was no more shaking of ingredients in the container upon commencement of CO_2 emission. Released CO_2 from either source of carbohydrate was delivered through a 60 cm long silicon tubing (0.5 cm diameter) into individual MM-X traps (American Biophysics, North Kingstown, RI, USA) and dispensed singly or in combination with a mosquito attractant referred to as the Mbita blend. This blend contained ammonia (2.5%), L-lactic acid (85%), tetradecanoic acid (0.00025%) and 3-methyl-1-butanol (0.000001%) impregnated on nylon strips (Mukabana et al., 2012a). Nylon strips impregnated with components of the Mbita blend were hooked together on a wire ring and hung inside the plume tube of a trap supplied with CO_2 from either source of carbohydrate (Okumu et al., 2010b, Smallegange et al., 2010). Trap outlets were suspended 15 cm above ground level (Schmied et al., 2008, Jawara et al., 2011). The treated nylon strips for individual sets of experiments were re-used throughout without replacement or replenishment whereas CO_2 was prepared for each experimental night (Mukabana et al., 2012b).

General procedures

Semi-field and field experiments were performed from 20:00 - 06:30 h and 18:30 - 06:30 h, respectively. All MM-X traps were operated on 12 V. Vaseline pure petroleum jelly was also applied on suspension wire bars and electrical cables to prevent ants from preying on mosquitoes caught in the MM-X trap. Baited traps and an unbaited MM-X trap were randomly assigned and alternated daily between or among trap positions to eliminate confounding effects associated with site. A data logger (Tinytag® Ultra, model TGU-1500, INTAB Benelux, The Netherlands) was used to record ambient temperature and RH at an interval of 30 min. To terminate individual experiments, a plug was inserted into the outer tube of the MM-X trap, CO_2 supply was cut off, power was switched off (semi-field) or traps were disconnected from batteries (field study). Latex gloves were worn during preparation of sugar/molasses-yeast mixtures, nylon strips, application of attractants on nylon strips and baiting of traps to avoid contamination with human volatiles or other odorants compounds. Traps containing mosquitoes were placed in a refrigerator at 4^{o}C for 30 min. Immobilized mosquitoes were collected from each trap, counted and recorded for estimation of relative attractiveness due to the CO_2 released from sugar and different combinations of molasses mixed with yeast. Thereafter, traps were cleaned using 70% methanol to remove residual odours and dried for the next round of experiments. A manual aspirator was used to collect mosquitoes that had not been trapped in the screen-walled greenhouse on the previous night

and killed. The sand-filled floor of the greenhouse was moistened daily to increase humidity necessary for enhanced survival of *An. gambiae*.

Determination of average release rates of CO_2 from molasses

The amounts of molasses and yeast required to produce an optimal quantity of CO_2 necessary to elicit similar or higher catches of *An. gambiae* as the same quantity of gas produced from sugar were evaluated. The CO_2 produced by fermentation of 250 g of sugar mixed with 17.5 g yeast and 2 L water according to Smallegange et al. (2010) was used as a reference treatment. On this basis, the quantity of molasses and yeast reduced into a half, doubled or held constant. Two litres of water were used in each treatment. The average volume and duration of CO_2 produced by mixing 2 L of water with (a) 125 g molasses plus 8.75 g yeast, (b) 125 g molasses plus 17.5 g yeast, (c) 250 g molasses plus 17.5 g yeast, (d) 250 g molasses plus 35 g yeast, (e) 500 g molasses plus 17.5g yeast, and (f) 500 g molasses plus 35 g yeast was determined at ambient conditions within a screen-walled greenhouse. In addition, the time interval between mixing of ingredients and release of the first bubble of CO_2 was recorded.

A 60 cm long silicon tube (0.5 cm diameter) was used to lead CO_2 into a calibrated beaker held upside down in a plastic basin filled with 10 L of water. The quantity of CO_2 released was estimated by measuring and recording volumes of displaced water at an interval of 20 min until the end of each experiment (Smallegange et al., 2010). Individual experiments were replicated four times. A digital stopwatch was used to record time taken prior to and during CO_2 production. The presence of CO_2 as a constituent of volatile organic compounds (VOCs) produced by yeast-fermented molasses was confirmed by the formation of a white precipitate of calcium carbonate when passed through a calcium hydroxide solution. Selection of a suitable combination of molasses and yeast for substituting refined sugar as an alternative source of CO_2 was based on the length of the release period, volume of CO_2 produced, release rate, relative attractiveness to *An. gambiae*, bulk and cost-effectiveness.

Effect of release rates of CO_2 from molasses on behavioural responses of *An. gambiae*

The attraction of *An. gambiae* mosquitoes to traps releasing CO_2 at different rates due to variations in the quantities of molasses and yeast used was compared to those attracted to CO_2 derived from sugar under semi-field conditions. Individual experiments were achieved through a dual-choice assay repeated over four nights (Njiru et al., 2006, Smallegange et al., 2010). The traps assigned for each treatment were diagonally placed within a screen-walled greenhouse. The total number of mosquitoes caught in two unbaited MM-X traps was also recorded to determine the symmetry of the experimental design (Smallegange et al., 2010, Verhulst et al., 2011b). The MM-X traps were baited with CO_2 emitted by a mixture of 2 L of water, yeast, and sugar or molasses after an incubation period of 30 min (for 250 g sugar and

250 g or 500 g molasses) or 40 min for 125 g molasses prior to the onset of each experiment.

Effect of a synthetic odour blend on attractiveness of CO_2 released from molasses to *An. gambiae*

Semi-field experiments were conducted to ascertain whether attraction of *An. gambiae* mosquitoes to CO_2 released separately by two potential combinations of molasses and yeast was enhanced by the addition of the Mbita blend. These combinations were 250 g of molasses plus 17.5 g yeast, and 500 g molasses plus 17.5 g yeast. Therefore, two sets of dual-choice experiments were designed to investigate the responses of *An. gambiae* mosquitoes to CO_2 released from (a) 250 g of molasses, 17.5 g yeast and 2 L water alone, versus 250 g of molasses, 17.5 g yeast and 2 L water, in conjunction with the Mbita blend, and (b) 500 g of molasses,17.5 g yeast and 2 L water alone, versus 500 g of molasses, 17.5 g yeast and 2 L water, presented in conjunction with the Mbita blend.

Effect of CO_2 released from sugar and molasses on attractiveness of a synthetic odour blend to *An. gambiae*

Follow-up experiments were conducted to evaluate responses of *An. gambiae* to the Mbita blend augmented singly with CO_2 produced by fermentation of 250 g of sugar, 250 g molasses or 500 g molasses (each mixed with 17.5 g yeast and 2 L water). This was achieved through a 4 × 4 Latin square experimental design replicated for 16 nights. The treatments included (i) MM-X trap without odour (control), (ii) Mbita blend released in conjunction with CO_2 released from 250 g sugar, (iii) Mbita blend supplemented with CO_2 emitted from 250 g molasses, and (iv) Mbita blend supplemented with CO_2 released from 500 g molasses. These semi-field studies were subsequently validated under field conditions.

Responses of wild female malaria vectors

This study was designed to compare the effect of CO_2 produced by fermentation of 250 g of sugar, 250 g molasses or 500 g molasses (each mixed with 17.5 g yeast and 2 L water) on the attractiveness of the Mbita blend to outdoor-biting malaria and other mosquitoes. The treatments included (i) MM-X trap without odour, (ii) Mbita blend containing CO_2 emitted from sugar, (iii) Mbita blend containing CO_2 released from 250 g molasses, and (iv) Mbita blend containing CO_2 derived from 500 g molasses. A randomized 4 × 4 Latin square experimental design replicated over 20 nights was adopted.

Individual treatments were assigned to particular MM-X traps suspended outside the bedroom under the eaves of village houses (Figure 1). Each house was used and occupied routinely by two to five dwellers. The dwellers slept under untreated bed nets during experimental nights

(Jawara et al., 2011). The four village houses were spaced within a distance range of 40-481 m apart along a transect, approximately 100 m away from the edge of rice paddies to maximize homogeneity in mosquito density and exclude interactions between treatments.

Figure 1: A picture of a trapping system baited with the Mbita odour blend augmented with carbon dioxide produced by fermentation of 250 g of molasses using 17.5 g of dry yeast mixed with 2 L of water. Carbon dioxide was produced in a plastic container and delivered into the MM-X trap through a silicon tubing. The trapping system was deployed outside the bedroom under the overhanging roof of a village house to collect outdoor biting mosquitoes at night (18:30 ï 06:30 h). Photograph: C.K. Mweresa

At the end of each experimental night, all traps were transported to a field laboratory located at the Ahero Multipurpose Development Training Institute (AMDTI) and placed in a freezer for 30 min. The frozen adult mosquitoes were emptied into labeled Petridishes, identified morphologically (Gillies and Coetzee, 1987) counted, and recorded according to (i) gender as male or female *An. gambiae* s.l. *An. funestus*, *Culex*, *Mansonia* spp. and other anopheline mosquitoes (all collected *Anopheles* spp. except *An. gambiae* s.l. and *An. funestus*) and (ii) external abdominal appearance as unfed, blood-fed (partially and fully blood-fed), or gravid female of *An. funestus*, and *An. gambiae* s.l. (WHO, 1975). All female *An. gambiae* s.l and *An. funestus* were preserved in 2 mL Eppendorf tubes containing silica gel crystals.

Data analysis

Differences in the release rates of CO_2 produced from 250 g sugar, 125 g, 250 g and 500 g molasses were determined by using a General Linear Model (GLM), univariate analysis of variance. The Tukey test was used for pairwise comparison of release rates of CO_2 from sugar

versus 125 g, 250 g and 500 g molasses. Individual dual-choice bioassays were analysed using Chi-square tests. The Chi-square test determined whether the distribution of total number of mosquitoes caught in both MM-X traps differed from a 1:1 distribution [11]. Trap counts of mosquitoes collected from experiments conducted through a 4×4 Latin square design were analysed using a GLM assuming a Poisson distribution and logarithmic link function [22]. The effects of treatment, trap position or house on mosquito catches were tested and fitted as parameters in the model. Significant effect of trap or house on mosquito catches was fitted with treatment in the model to test for interaction. Effects were considered significant at $P < 0.05$. All analyses were performed using IBM SPSS statistical software, version 16.

Results

Determination of average release rates of CO_2 from molasses and sugar.

An average temperature of 23.2 ± 1.3^{o}C and 77.0 ± 2.6% RH were recorded during semi-field experiments (April-September 2011). The molasses used in all experiments was 44.7% pure, it contained 34.2% sugar and 76.4% of dissolved solids. The release rates of CO_2 were dependent on the quantity of molasses used ($P < 0.001$).

Fermentation of 125 g, 250 g and 500 g of molasses emitted CO_2 within a time range of 310 to 440 min, 490 to 645 min, and > 840 min, respectively (Figure 2). An increase in the quantity of molasses enhanced release rates and duration of CO_2 production. The post-mixing time range for emission of a first bubble of CO_2 from 125 g, and 250 g to 500 g of molasses was 29.00 - 37.50 min, 20.00 - 29.50 min. and 18.35 - 26.09 min, respectively.

Although release rates of CO_2 from 125 g of molasses mixed with 8.75 g (36.5 ± 3.3 ml/min) or 17.5 g (30.3 ± 1.6 ml/min) of yeast were not different ($P = 0.12$), the two release rates were significantly lower compared to 250 g of sugar + 17.5 g yeast (63.23 ± 2.82 ml/min) ($P < 0.001$) (Figure 2A). There was a greater increase in the release rate of CO_2 produced by a combination of 250 g of molasses + 17.5 g yeast (80.63 ± 2.82 ml/min) compared to 250 g of sugar or 250 g of molasses + 35 g yeast (63.9 ± 6.6 ml/min) ($P = 0.010$ for both) (Figure 2B). By contrast, release rates of CO_2 obtained from 250 g of sugar, and 250 g of molasses + 35 g yeast were not different ($P = 0.10$). The release rate of CO_2 was greatly increased by mixing 500 g of molasses with either 17.5 g (87.79 ± 2.14 ml/min) or 35 g (127.4 ± 8.9 ml/min) of yeast compared to 250 g of sugar ($P < 0.001$ for both) (Figure 2C).

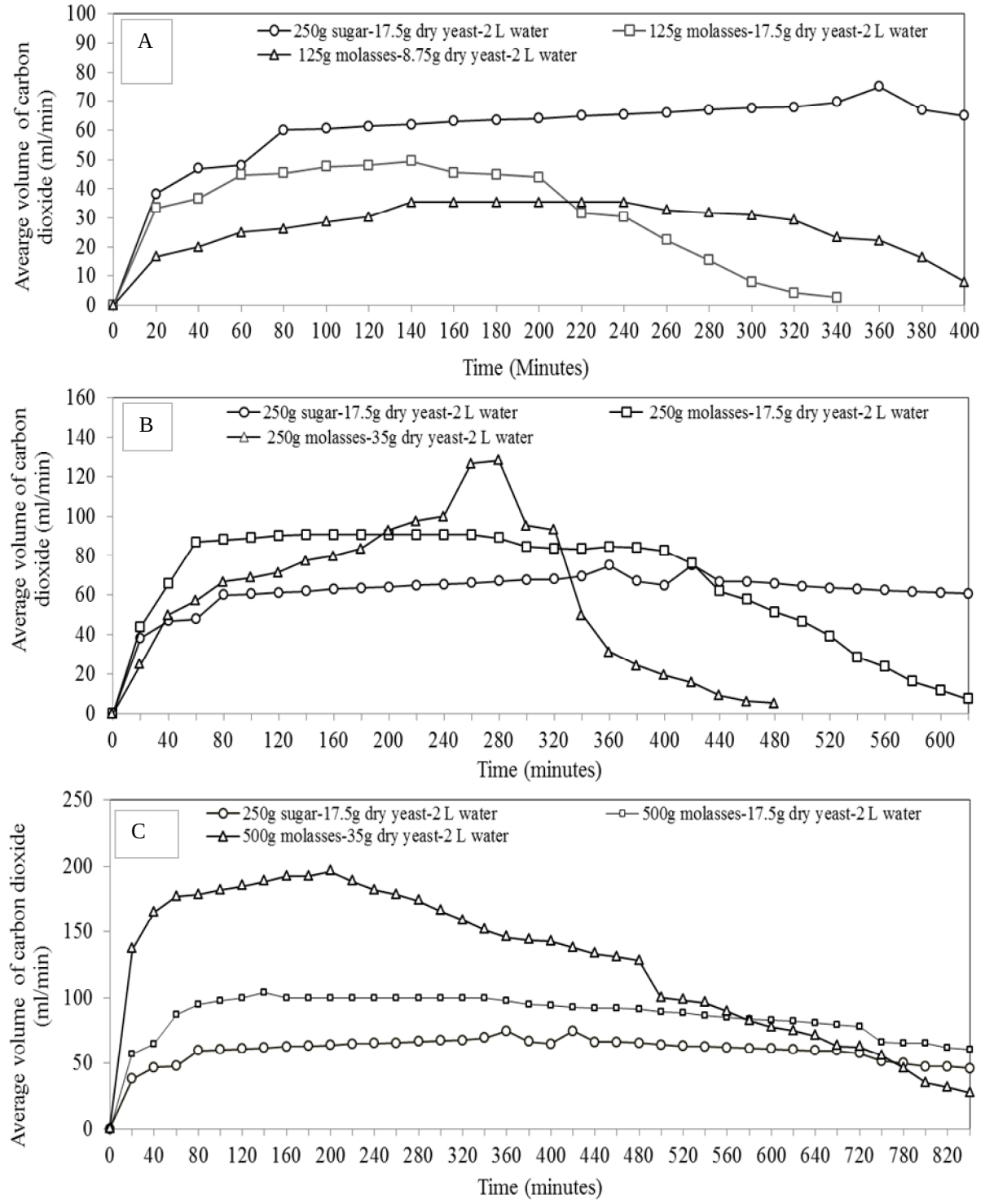


Figure 2: Effect of varying the quantity of dry yeast on average release rate (ml/min) of carbon dioxide produced by 2 L of water mixed with either 125 g (panel A), 250 g (panel B), or 500 g (panel C) of molasses compared to a combination of 250 g of refined sugar, 17.5 g dry yeast and 2 L of water (reference treatment). The volume of carbon dioxide released was measured and recorded at 20 min. intervals at ambient conditions within a screen-walled greenhouse.

Effect of release rates of CO_2 from molasses on catches of *An. gambiae*

Trap positions had no influence on mosquito catches within the screen-walled greenhouse (P = 0.11). Both traps without odour caught 15.5% (n = 124) of the released mosquitoes implying that all bioassays were symmetrical. There was a significant increase in the proportions of mosquitoes attracted to CO_2 released from 250 g of sugar + 17.5 g yeast than to a mixture of 125 g molasses + 8.75 g yeast (P < 0.001). However, the attractiveness of CO_2 produced by fermentation of 250 g of sugar + 17.5 g yeas, and 125 g of molasses + 17.5 g yeast to mosquitoes was not different (P = 0.51). A significantly higher proportion of mosquitoes responded to CO_2 released from 250 g molasses + 17.5 g yeast compared to 250 g sugar + 17.5 g yeast (P < 0.001). By contrast, mosquitoes responded equally to CO_2 derived from 250 g of sugar + 17.5 g yeast and 250 g of molasses + 35 g yeast (P = 0.07). Nonetheless, there was a higher response of mosquitoes to CO_2 emitted from a mixture of 500 g of molasses + 17.5 g yeast than to CO_2 released from sugar + 17.5 g yeast (P = 0.001). Although the release rate of CO_2 from 500 g of molasses + 35 g yeast was the highest, this combination attracted similar proportions of mosquitoes compared to sugar + 17.5 g yeast (P = 0.11). These results (Table 1) provided the baseline information used to select alternative combinations of molasses and yeast for replacement of the currently-used 250 g of sugar as a source of CO_2 bait for sampling malaria vectors.

Table 1: Total and mean (± SE) number of *An. gambiae* attracted by carbon dioxide produced in a dual-choice assay in a screen house between molasses treatments (test combinations of molasses and dry yeast dissolved in 2 L of water) and a reference treatment (250 g refined sugar, 17.5 g dry yeast and 2 L water).

Molasses treatments	N	n	Mosquitoes attracted to carbohydrate sources of CO_2		
			Molasses treatment (mean ± SE)	Reference treatment (mean ± SE)	P-value
125 g molasses - 17.5 g yeast - 2 L water	4	507	61.5 ± 4.0	65.3 ± 4.0	0.51
125 g molasses - 8.75 g yeast - 2 L water	4	417	35.8 ± 3.0	68.5 ± 4.1	0.001
250 g molasses - 17.5 g yeast - 2 L water	4	460	70 ± 4.2	45.0 ± 3.4	0.001
250 g molasses - 35 g yeast - 2 L water	4	371	50.8 ± 3.6	42.0 ± 3.2	0.07
500 g molasses - 17.5 g yeast - 2 L water	4	545	79.3 ± 4.5	57.0 ± 3.8	0.001
500 g molasses - 35 g yeast - 2 L water	4	440	50.8 ± 3.6	59.3 ± 3.9	0.11

N is the number of experimental nights, n is the total number of mosquitoes caught whereas SE is the standard error of the mean catch per night. A total of 200 female *An. gambiae* were released per night. Pairwise comparisons in the same row of mean catches differ significantly at P < 0.05 (Chi-square test).

Effect of a synthetic odour blend on the attractiveness of CO_2 released from molasses to *An. gambiae*

Preceding results indicated that CO_2 produced by fermentation of 250 g or 500 g of molasses (each mixed with 17.5 g yeast and 2 L water) were potential alternatives for the currently-used sugar source. The responses of mosquitoes to CO_2 produced from either 250 g or 500 g of molasses was significantly increased when released in conjunction with the Mbita odour blend compared to CO_2 alone from either quantity ($P < 0.001$ for both). Of the 272 trapped mosquitoes, 26.1% were attracted to CO_2 released from 250 g of molasses and 73.9% to the Mbita blend augmented with CO_2 derived from 250 g molasses (Figure 3A). In a similar bioassay, of the 407 mosquitoes collected, 37.1% were attracted to CO_2 emitted from 500 g of molasses, and 62.9% to the Mbita blend supplemented with CO_2 released from 500 g of molasses (Figure 3B).

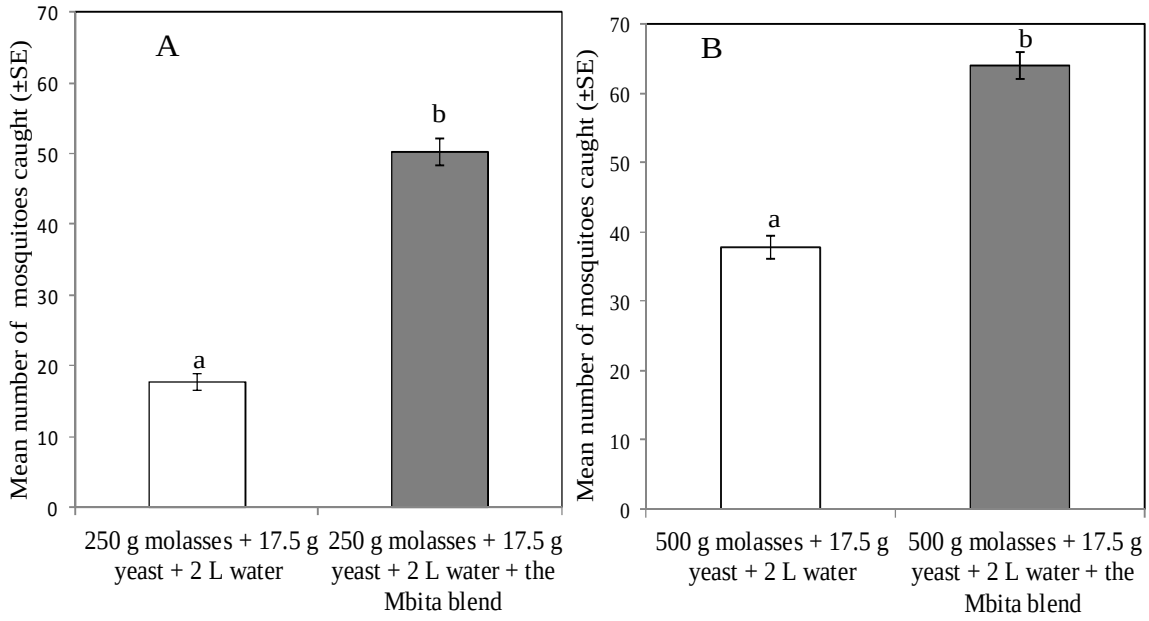


Figure 3: Effect of adding the Mbita odour blend to carbon dioxide produced by fermentation of 250 g (panel A) and 500 g (panel B) of molasses on mean number (± SE) of *An. gambiae* collected for four nights. A total of 200 female *An. gambiae* were released per night within a screen-walled greenhouse. Mean numbers with different letters on error bars in the same graph are significantly different ($P < 0.05$). Error bars represent the standard error of the mean number of mosquito collections.

Effect of CO_2 released from sugar and molasses on the attractiveness of a synthetic odour blend to *An. gambiae*

Preceding findings demonstrated that the attraction of *An. gambiae* to the Mbita blend was enhanced by addition of CO_2 produced by either 250 g of molasses and 17.5 g yeast or 500 g molasses and 17.5 g of yeast. Out of the 3,200 *An. gambiae* mosquitoes released, 1,801 (i.e.

56.3%) were trapped during 16 experimental nights. The mosquitoes were collected in a trap without odour (1.4%), the Mbita blend augmented with CO_2 released from 250 g of sugar (25%), 250 g molasses (33.9%) or 500 g molasses (38.9%) (Table 2). Addition of CO_2 from 250 g of molasses to the Mbita blend enhanced the mosquito catches compared to the sugar source ($P < 0.001$). Similarly, the attractiveness of the Mbita blend was significantly increased when combined with CO_2 produced by fermentation of 500 g of molasses than from 250 g sugar ($P < 0.001$). Moreover, the Mbita blend supplemented with CO_2 released from 500 g of molasses was more attractive to mosquitoes than with CO_2 released from 250 g of molasses ($P < 0.014$).

Table 2: Behavioral responses of *An. gambiae* to traps baited with the Mbita blend alone versus the Mbita blend supplemented with selected dilutions of test compounds released from nylon strips. Studies were conducted in a large outdoor screened cage.

Novel Blend (Mbita blend + optimal dilution of test compound)	N	Mean number ± SE of mosquitoes caught		
		Novel blend	Mbita blend alone	P-value
Mbita blend + butyl-2-methylbutanoate (1:10,000)	4	54. 0 ± 3.7	48.8 ± 3.5	0.30
Mbita blend + 2-pentadecanone (1:100)	4	70.8 ± 4.2	44.8 ± 3.3	0.001
Mbita blend + 1-dodecanol (1:10,000)	4	71.3 ± 4.2	47.3 ± 3.4	0.001
Mbita blend + 1-butylamine (1:100)	4	49.3 ± 3.5	42.5 ± 3.6	0.16
Mbita blend + 1-butylamine (1:10,000,000)	4	71.8 ± 4.2	58.5 ± 3.8	0.020

N is the number of experimental nights whereas SE is the standard error of the mean catch per night. A total of 200 female *An. gambiae* were released per night. Pairwise comparisons of mosquitoes caught differ significantly at $P < 0.05$ (Chi-square test).

Responses of wild female malaria vectors

Effect on female mosquitoes

The 20 nights over which field experiments were carried out (November 2011) were characterized by 71.9 ± 1.9% of RH, a mean wind speed of 3.2 ± 0.09 km/h, temperature of 23.5 ± 2.2^{o}C and a total rainfall of 263.4 mm. A total of 1,807 mosquitoes were caught outdoors. Of this number, 11.2% (n = 203) were males and 88.7% (n = 1,604) females. Both treatment and house effect played an important role in influencing trap collections of all female mosquitoes ($P < 0.001$ for both). The 1,604 female mosquitoes were collected in traps without odour (2.9%), with the Mbita blended supplemented with CO_2 released by 250 g of

fermenting sugar (22.6%), 250 g of fermenting molasses (35.0%) and 500 g of fermenting molasses (39.5%) (Figure 4). The female mosquitoes comprised *An. gambiae* s.l. (18.1%), *An. funestus* (20.2%), *Culex* spp. (37.8%), *Mansonia* spp. (10.2%) and other anophelines (13.6%). Trap collections of female *An. gambiae* s.l. and *An. funestus* were influenced by the carbohydrate source of CO_2 presented with the Mbita blend ($P < 0.001$, for both). Addition of CO_2 released from 500 g of molasses to the Mbita blend attracted the highest number of *An. gambiae* s.l. compared to either CO_2 derived from 250 g of molasses ($P < 0.001$) or from 250 g of sugar ($P < 0.001$). However, addition of CO_2 released from 250 g of molasses or 250 g of sugar on the Mbita blend caused no significant difference on trap collections of *An. gambiae* s.l. ($P = 0.32$).

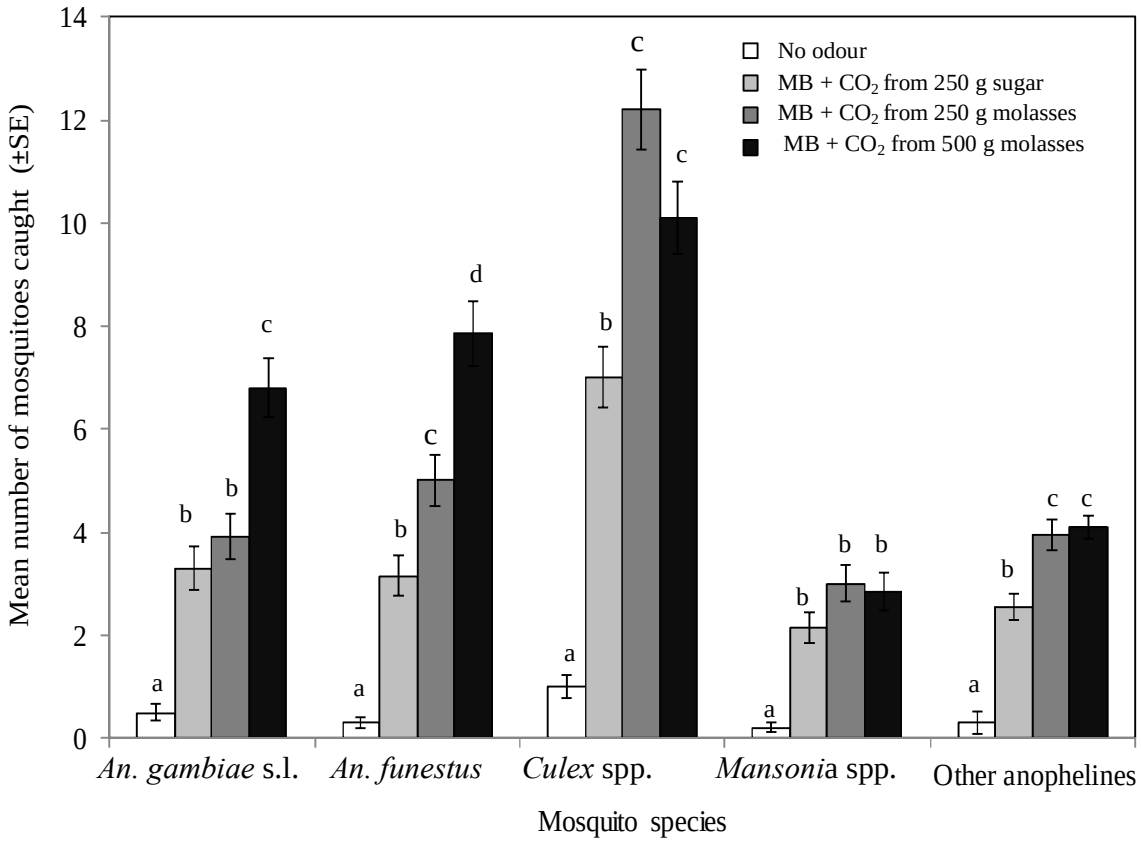


Figure 4: Mean number (± SE) of female mosquitoes caught overnight outdoors in a MM-X trap without odour, baited with the Mbita blend (MB) augmented with carbon dioxide produced by fermentation of either 250 g of refined sugar, 250 g or 500 g of molasses. Each quantity of refined sugar or molasses was mixed with 2 L of water and 17.5 g of dry yeast. The study was conducted in Kigoche village for 20 nights. Treatments are shown in the legend. Mean values within the same mosquito type having no letter in common were significantly ($P < 0.05$) different.

There was a significant increase in the responses of *An. funestus* to the Mbita blend supplemented with CO_2 released from 500 g or 250 g of molasses than to 250 g of sugar ($P < 0.001$ and $P < 0.007$, respectively). Nonetheless, the Mbita blend was more attractive to *An. funestus* when augmented with CO_2 derived from 500 g than 250 g of molasses ($P < 0.01$).

Combinations of the Mbita blend and CO_2 released from 250 g or 500 g of molasses were equally attractive to *Culex* spp. ($P = 0.12$) and other anopheline mosquitoes ($P = 0.18$). However, attractiveness of the Mbita blend supplemented with CO_2 from 250 g of sugar to *Culex* spp. was significantly lower compared to 250 g ($P < 0.001$) or 500 g ($P < 0.010$) of molasses. By contrast, trap collections of *Mansonia* spp. were not dependent on the carbohydrate source of CO_2 ($P = 0.42$).

Effect on malaria vectors in different physiological conditions

A combination between the Mbita blend and CO_2 produced by fermentation of molasses elicited a physiological stage-dependent behaviour of local malaria vectors. The 290 females of *An. gambiae* s.l. collected were unfed (57.6%), blood-fed (33.8%) or gravid (8.6%) (Figure 5A). The responses of unfed *An. gambiae* s.l. to the Mbita blend were significantly increased by addition of CO_2 derived from 500 g of molasses compared to 250 g of molasses ($P < 0.012$) or 250 g of sugar ($P < 0.001$). However, addition of CO_2 released from 250 g of molasses or 250 g of sugar had no effect on the attractiveness of the Mbita blend to unfed *An. gambiae* s.l. ($P = 0.73$).

There were more blood-fed *An. gambiae* s.l. attracted to the Mbita blend augmented with CO_2 emitted from 500 g of molasses than from 250 g sugar ($P < 0.001$) or from 250 g molasses ($P < 0.001$). Also, the attractiveness of the Mbita blend to blood-fed *An. gambiae* s.l. was significantly enhanced by addition of CO_2 released from 250 g of molasses compared to 250 g of sugar ($P < 0.034$). Treatment had no effect on trap catches of gravid *An. gambiae* s.l. ($P = 0.34$).

The 324 females of *An. funestus* collected were unfed (51.2%) blood-fed (36.1%) or gravid (12.6%) (Figure 5B). The attraction of unfed *An. funestus* to the Mbita blend was greatly enhanced by addition of CO_2 derived from 250 g or 500 g of molasses compared to 250 g sugar ($P < 0.013$ and $P < 0.001$, respectively). However, responses of gravid *An. funestus* to the Mbita blend supplemented with CO_2 released from 250 g of molasses or from 250 g sugar were not different ($P = 0.49$). By contrast, significantly more gravid *An. funestus* were attracted to the Mbita blend augmented with CO_2 produced by fermentation of 500 g of molasses compared to 250 g sugar ($P < 0.014$). Although baited traps collected high numbers of blood-fed *An. funestus*, the catches were not statistically different ($P = 0.14$).

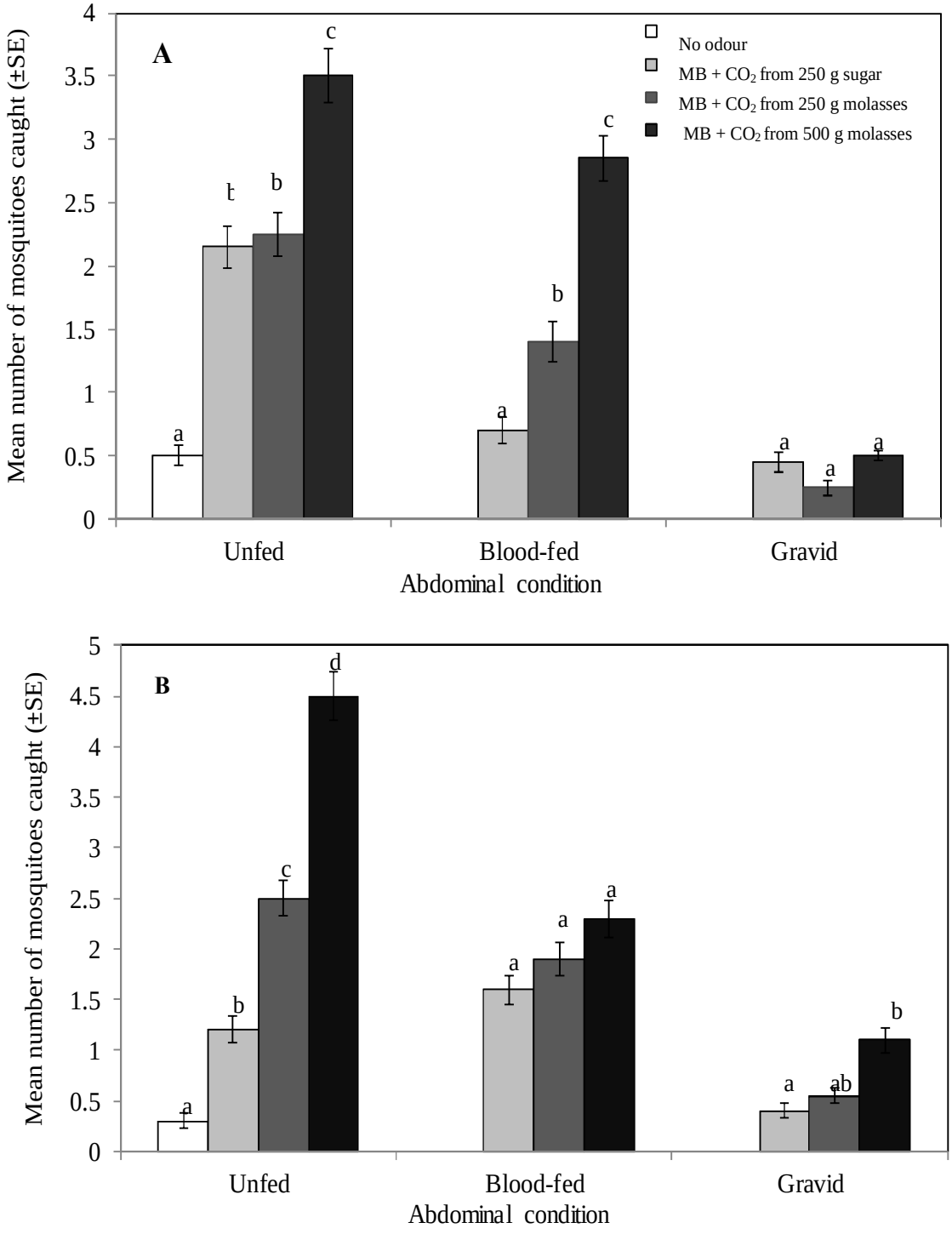


Figure 5: Mean number (± SE) of outdoor-biting *An. gambiae* s.l. (panel A) and *An. funestus* (panel B) in different abdominal conditions (unfed, blood-fed and gravid) collected overnight in a MM-X trap without odour, baited with the Mbita blend (MB) augmented with carbon dioxide released from 250 g of refined sugar, 250 g or 500 g of molasses for 20 nights. Each quantity of refined sugar or molasses was mixed with 2 L of water and 17.5 g of dry yeast. Treatments are shown in the legend of panel A. The study was conducted in Kigoche village for 20 nights. Treatments are shown in the legend. Mean values within the same mosquito type having no letter in common were significantly ($P < 0.05$) different.

Discussion

The findings of this study indicate that CO_2, and possibly other volatiles, produced by fermentation of molasses provide a suitable alternative to the CO_2 obtained from fermentation of the more expensive, refined cane sugar to lure malaria vectors towards traps. The release rate and proportion of *An. gambiae* mosquitoes caught increased consistently as the quantity of yeast-fermented molasses increased up to an optimal ratio of molasses and yeast. The attraction of mosquitoes to traps baited with CO_2 produced by fermenting molasses was enhanced when presented jointly with the Mbita blend of synthetic odours. More malaria vectors and female *Culex* spp. responded to the Mbita blend supplemented with CO_2 released from molasses compared to a combination of the Mbita blend and CO_2 derived from sugar. Total collections of wild mosquitoes comprised of 18.0% *An. gambiae* s.l. and 20.3% *An. funestus* in an unfed, blood-fed or gravid abdominal condition. Significantly more blood-fed *An. gambiae* s.l. were caught in traps baited with CO_2 derived from fermenting molasses compared to sugar.

Our results confirm that variation in the release rates of CO_2 influenced by ratios of molasses and dry yeast can be sufficiently large to induce differential activation and upwind orientation of mosquitoes towards baited traps (Gillies, 1980, Takken and Knols, 1999, Cardé and Gibson, 2010). For example, 125 g of molasses resulted in significantly lower release rates of CO_2 lasting for periods of 320 ï 440 min compared to a higher, stable flow rate and longer release period (over 840 min) demonstrated by the currently-used sugar source (Smallegange et al., 2010, Mukabana et al., 2012a). Utilization of 250 g of molasses mixed with 17.5 g of yeast released CO_2 for periods of 490 to 645 min, and this translated into significantly higher mosquito catches compared to those collected with CO_2 from 250 g of sugar with 17.5 g of yeast. By contrast, fewer mosquitoes responded to CO_2 derived from 250 g of molasses and 35 g of yeast, possibly because of a drastic drop in release rates. These findings suggest that doubling the quantity of yeast enhanced the fermentation rate of molasses, and reduced the release period thereby producing higher concentrations of CO_2 responsible for a possible inhibitory effect on host-seeking responses of *An. gambiae* (Costantini et al., 1996, Lefèvre et al., 2009, Smallegange et al., 2010). Such an inhibitory effect has been reported in other studies (Takken and Kline, 1989, Lefèvre et al., 2009) and it is likely to account for lower mosquito responses to high release rates of CO_2 from a mixture of 500 g of molasses + 35 g of yeast in 2 L. These results indicate that although CO_2 activates mosquitoes by inducing upwind flight, higher concentrations may reduce orientation to the source at close range (Gillies, 1980, Takken et al., 1997, Dekker et al., 2001).

The release rates of CO_2 produced by fermentation of 250 g (80.6 ± 2.82 ml/min) or 500 g (87.8 ± 2.14 ml/min) of molasses (each mixed with 17.5 g yeast and 2 L of water) were not

different. However, more mosquitoes responded to CO_2 produced by fermentation of 500 g than to 250 g of molasses, possibly because the higher quantity of molasses provided more substrate for an extended release period of optimal CO_2 concentration. In contrast, fermentation of 250 g molquantity of yeast and water. This suggests that, besides CO_2, yeast-fermented molasses produces additional VOCs that enhance mosquito catches. The VOCs emitted by fermented sugar were reported preliminarily by Smallegange et al. (2010). Unlike sugar, it has been reported that molasses contains a lower sugar content but relatively more water, solid matter, nitrogen and minerals (Patrascu et al., 2009).

During the current study, the numbers of *An. gambiae* mosquitoes attracted to CO_2 obtained from either 250 g or 500 g of molasses was greatly increased in the presence of the Mbita blend. This implies that *An. gambiae* mosquitoes locate and orient themselves towards odour-baited trapping systems by responding to CO_2 released together with host-specific cues (Gillies, 1980, Dekker et al., 2002, Spitzen et al., 2008, Verhulst et al., 2011a). Thus, our results demonstrate that, although CO_2 is one of the stimuli to which mosquitoes respond, trap catches are significantly increased in the presence of a plume containing human odour or synthetic odour than on its own (Gillies, 1980, Dekker et al., 2002, Spitzen et al., 2008). For example Dekker et al. (2001) demonstrated that trap catches of host-seeking *An. gambiae* and *Aedes aegypti* (L.) were influenced by the structure of host odour-plumes and that the effect of CO_2 on trap catches was concentration dependent. Moreover, Kline and Lemire (1998) also found that an additive effect of CO_2 on worn sock was responsible for the attraction of most species of mosquitoes in the genus *Aedes*, *Anopheles*, *Coquilletfidia*, *Culex*, *Culiseta*, and *Psorophora*.

Similarly, the abundance and diversity of mosquitoes caught outside village houses occupied by dwellers depended on CO_2 source and dose, hence corresponding to previous findings by Costantini *et al.* (1996). During the current study, a combination between CO_2 produced by 500 g fermenting molasses and the Mbita blend led to the highest catches of unfed and blood-fed females of *An. gambiae s.l.* However, the numbers of female *An. gambiae s.l.* collected in a trap baited with the Mbita blend augmented with CO_2 from 250 g molasses or 250 g sugar were similar, indicating that both CO_2 sources were equally attractive. Nonetheless, CO_2 produced by 250 g fermenting molasses attracted similar numbers of female *Culex* spp. and other anopheline mosquitoes as 500 g molasses when presented with the Mbita blend. These observations indicate that CO_2 produced by 250 g fermenting molasses is a suitable alternative to that produced from 250 g sugar. However, utilization of 500 g molasses would be a convenient alternative for mass-trapping of malaria vectors in situations where malaria-prone areas are endowed with sufficient resources and large scale production of sugar cane. It is then not necessary to increase the quantity of yeast, as it is, in congruence with enzyme kinetics (Patrascu et al., 2009, Smallegange et al., 2010), and has shown that 17.5 g yeast added to 2 L water and a sugar source is sufficient for optimal CO_2 production.

The attraction of significantly higher numbers of blood-fed *An. gambiae s.l.* mosquitoes to the Mbita blend augmented with CO_2 released from molasses is a very interesting finding. Blood-fed mosquitoes are rarely caught with indoor or outdoor human landing collections (Dia et al., 2005, Le Goff et al., 1997). The method is also not ethically accepted especially if the humans performing the catches are not properly protected from infection (Gimnig et al., 2013). Instead, more representative samples of unfed, blood-fed and gravid females of *An. gambiae s.l.* as well as males are obtained by collection of resting mosquitoes (Joshi et al., 1975, Bentley et al., 2009). This accounts for the use of resting boxes, pyrethrum spray catches, clay pots and manual aspiration of resting mosquitoes especially in studies associated with the estimation of the entomological inoculation rate (Dia et al., 2005, Mboera, 2005). Seemingly, cues derived from a combination asses showed a higher release rate of CO_2 and was more attractive to *An. gambiae* compared to 250 g of refined sugar though both carbohydrate sources were mixed with the same of the Mbita blend and CO_2 accompanied with other VOCs derived from fermenting molasses may have activated a temporal sensitivity of odour receptors thereby inducing host-seeking behaviour of unfed and blood-fed *An. gambiae* s.l. and *An. funestus* (Qiu and van Loon, 2010).

These findings are contrary to the expectations that after a blood meal, blood feeding responses of female mosquitoes are down-regulated for the next 48 to 72 h until eggs mature and increase sensitivity of odour receptors to oviposition cues (Qiu and van Loon, 2010). It has also been reported that small-bodied malaria vectors and those which take small-sized blood meals engage more frequently in multiple blood feeding during single gonotrophic cycles to meet their nutritional requirements (Foster and Takken, 2004, Scott and Takken, 2012). Such feeding behaviours are more likely to increase human-vector contact and risk of malaria transmission. Whereas there were cows sleeping adjacent to all houses occupied by dwellers in the surrounding of which outdoor-biting mosquitoes were collected, utilization of different synthetic odour baits supplemented with CO_2 derived from refined sugar attracted predominantly unfed *An. gambiae* s.l. and *An. funestus* in the same study site (Mukabana et al., 2012a).

From the foregoing it is clear that attraction of blood-fed malaria vectors by synthetic odour blends supplemented with CO_2 in conjunction with VOCs released from fermentation of molasses requires further investigation. This may provide an important bait for studies that require estimates of malaria transmission risk, as provided by the entomological inoculation rate (Smith et al., 2006). The collection of blood-fed mosquitoes can also be used for studies of the infectious reservoir of malaria; as such mosquitoes may have fed on *Plasmodium*-infected hosts. Clearly the additional volatiles produced by fermentation of molasses stimulate blood-fed females to respond to the odour-baited trap, which opens up new potential for malaria-epidemiological studies. In this study no attempts were made to identify other volatile

compounds produced by yeast fermentation of molasses. Likewise no efforts were made to remove/separate CO_2 from the other gaseous products (if any) in order to allow investigations on the potential effect of any other products on eliciting mosquito behavioral responses. Although this research was not aimed at resolving such issues, there is a need for further investigations. The investigations should also focus on (a) determining the relative composition of VOCs emitted by fermenting molasses and their effect on the capture rate of malaria and other mosquito vectors in different physiological conditions, (b)identification of blood meal source, and (c) how this technology may be used for estimation of entomological inoculation rate .

Whereas semi-field bioassays were conducted using *An. gambiae s.s.*, trapping of indoor mosquitoes in the same village confirmed that *An. gambiae* s.l. was represented by 96.7% *An. arabiensis* and 3.3% *An. gambiae s.s.* (Mukabana et al., 2012a). Our results agree with Smallegange et al. (2010) and Jawara et al. (2011) in that it is possible to intercept and reduce the number of malaria mosquitoes entering or leaving houses by deploying outdoor traps baited with a combination of CO_2 derived from molasses and synthetic blends that mimic human odour. Deployment of this innovative technology is extremely important against outdoor vectors responsible for maintaining residual transmission of malaria because intensive use of insecticide-treated nets and indoor residual spraying continues to reduce indoor transmission (Antonio-Nkondjio et al., 2006, Russell et al., 2011, Govella and Ferguson, 2012). Because baiting of traps with CO_2 affects host-seeking behaviour of different species of malaria vectors, further reduction of house entry of mosquitoes can be achieved by incorporation of complementary measures such as house screening (Lindsay et al., 2002) or application of novel repellents in a push-pull strategy (Takken, 2010).

Conclusion

Yeast-fermented molasses is an effective alternative source of CO_2 for odour-baited trapping systems of mosquitoes as it is equally or more attractive than CO_2 from fermenting sugar. This study presents an alternative use of molasses and a local solution for the development of cheaper and more sustainable lures for sampling and control of *An.* gambiae *s.l., An. funestus,* and other human-biting mosquitoes in rural areas. This research did not expect to find blood-fed mosquitoes inside traps baited with a synthetic odour blend supplemented with a combination of CO_2 and VOCs released from fermenting molasses as female mosquitoes are not attracted to their vertebrate hosts after blood feeding. Therefore, a need for further investigations is necessary.

Acknowledgements

We are grateful to Mr David Odhiambo Alila for providing the laboratory-reared mosquitoes used during the semi-field experiments, while Brian Andrew Abuya and Caroline Moseti are thanked for assisting us to determine release rates of CO_2. Mr Juma Wanyama and Mr Patrice Mulama are acknowledged for determining the quality of molasses used in our experiments. We are thankful to Dr Susan A Imbahale and Mr Fred Kisanya for laboratory facilities at the Ahero Multi-purpose Development Training Institute (AMDTI) and Mr Charles Oketch for conducting field studies alongside Philemon Omusula. Our gratitude goes to household owners at Kigoche village for allowing us to conduct this study on their houses. This investigation was supported by a grant from the Foundation for the National Institutes of Health (FNIH) through the Grand Challenges in Global Health initiative (GCGH #121) and a sandwich PhD scholarship provided to CKM by Wageningen University, the Netherlands. CKM was hosted by ICIPEï TOC as a dissertation research internship scholar.

Chapter 9

Textile substrates for dispensing synthetic mosquito attractants

Submitted for publication as: Mweresa, C.K., Mukabana, W. R., Gheysens, T., Omusula, P., Otieno, B., Takken, W. and van Loon, J.J.A. Textile substrates for dispensing synthetic mosquito attractants

Abstract

The full-scale impact of odour-baited technology on the surveillance, sampling and control of vectors of infectious diseases is partly limited by the lack of methods for the efficient and sustainable dispensing of kairomones. In this study we investigated whether locally-available and commonly used textiles can provide an efficient substrate for the release of synthetic attractant odorants of malaria vectors. The relative efficacy of polyester, cotton and cellulose + polyacrylate versus nylon textiles as odour-dispensing substrates using a synthetic attractant (i.e. Ifakara blend 1 (IB1) for malaria mosquitoes was evaluated in western Kenya. The study was conducted through competitive binary choice and Latin square experimental assays under semi-field and field conditions.

Traps charged with polyester, cotton and cellulose + polyacrylate materials that had been impregnated with the attractant IB1 caught significantly more *Anopheles gambiae sensu stricto* mosquitoes compared to IB1-treated nylon ($P < 0.001$) under semi-field conditions. Females and males accounted for 93.6% (n = 4,415) and 6.4% (n = 281) of outdoor mosquito collections, respectively. There was a significant increase in the responses of female *An. gambiae* sensu lato to IB1-treated polyester, cotton and cellulose + polyacrylate materials compared to nylon ($P < 0.001$). The IB1-impregnated cellulose + polyacrylate textile was the most attractive to female *An. funestus* mosquitoes compared to all other release substrates ($P < 0.001$). The responses of *An. funestus* mosquitoes to IB1-treated cotton and polyester were equal ($P = 0.45$). Significantly more female *Culex* mosquitoes were attracted to IB1-treated cotton than to the other treatments ($P < 0.001$). Whereas IB1-impregnated cotton and cellulose + polyacrylate material attracted equal numbers of *Mansonia* mosquitoes ($P = 0.44$), the catches due to these two substrates were significantly higher than those associated with the other substrates ($P < 0.001$). The results demonstrate that the number and species of mosquitoes attracted to synthetic odorant blends is influenced by the type of odour-dispensing material used. Thus, odour-based technologies directed towards surveillance, sampling and control of disease vectors should be wary of the release substrate employed.

Introduction

The application of semiochemicals as a novel means of monitoring and controlling mosquito vectors has been investigated under different environmental conditions with promising results (Kline, 2006, Kline, 2007, Eiras et al., 2010, Mukabana et al., 2010). This technology is pegged on the understanding that blood-questing mosquitoes are mainly guided to their hosts by olfactory cues (Takken, 1991, Takken and Knols, 1999). Indeed, host-specific attractant compounds have been identified and constituted into synthetic odour blends to provide a complementary tool for sampling and control of both outdoor- and indoor-biting malaria mosquitoes (Smallegange et al., 2009, Okumu et al., 2010a, Verhulst et al., 2011, Mukabana et al., 2012a). However, improvement of odour-baited trapping systems depends partly on efficacy and sustainability of selected odour-dispensing devices (Torr et al., 1997, Cork, 2004). Importantly, devices used to dispense odorants should ensure stable chemical properties of impregnated active ingredients, sustained release of optimal odour concentrations and should be easy to prepare for area-wide application (Torr et al., 1997, Okumu et al., 2010b).

Recent findings have shown that nylon strips treated with synthetic attractant odorants lured significantly higher numbers of host-seeking *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *An. gambiae*) mosquitoes into traps than glass vials and low density polyethylene (LDPE) sachets containing the same attractants (Okumu et al., 2010b, Mukabana et al., 2012b). Like other repellent- or insecticide-impregnated fabric materials (Yates et al., 2005, N'guessan et al., 2006, Pennetier et al., 2010), nylon strips treated with attractant odorants have also demonstrated a long-term residual activity to *An. gambiae* for over one year post-treatment under semi-field conditions (Chapter 4).

Besides nylon, cotton socks have been utilised to collect human foot odour in experiments evaluating the attraction of *An. gambiae* (Pates et al., 2001, Qiu et al., 2004, Njiru et al., 2006). In addition, suitability of both polyester and cotton materials to dispense a candidate contaminant insecticide inside an odour-baited station against wild malaria mosquitoes in southern Tanzania was demonstrated (Okumu et al., 2010c). On the other hand, the absorption layer of commonly used unscented, ultra-thin disposable sanitary pads consists of cellulose + polyacrylate for holding absorbed liquids. Although these materials are highly absorbent of fluids, it is not known whether the same substrates would also be effective in dispensing synthetic attractants optimised to lure malaria vectors into trapping tools. To answer this question, we investigated whether locally available and commonly used polyester netting, cotton clothing and cellulose + polyacrylate materials provided similar or better release matrices for synthetic attractants to host-seeking mosquitoes compared to nylon. The specific objectives of the current study were to (a) investigate behavioural responses of *An. gambiae*

to attractant-treated and untreated odour-dispensing substrates, (b) evaluate behavioural responses of *An. gambiae* to attractant-treated odour-dispensing substrates, and (c) evaluate efficacy of attractant-treated substrates to lure malaria and other mosquitoes into traps placed outdoors.

Materials and Methods

Mosquitoes

The Mbita strain of female *An. gambiae* mosquitoes was used for semi-field experiments conducted within a screen-walled greenhouse (November 2011 and April 2012). The mosquitoes were reared in the insectary at the Thomas Odhiambo Campus (TOC) of the International Centre of Insect Physiology and Ecology (*icipe*) located at Mbita Point, western Kenya. Adult mosquitoes were kept in 30 cm^3 gauze cages, fed on a human arm for a blood meal and provided with 6% glucose solution supplied through a Whatman filter paper wick. Female mosquitoes oviposited on a wet filter paper placed in a Petri dish. The eggs were thereafter dispensed in plastic trays half-filled with water obtained from Lake Victoria. Larvae were fed on Tetramin® baby fish food provided thrice a day. Pupae were collected daily and transferred into 30 cm^3 gauze cages for emergence. A total of 200 adult female mosquitoes aged 3 - 5 d old without prior access to a blood meal were randomly aspirated and kept in a plastic holding cup. The mosquitoes were starved for 8 h while being supplied with water through a wet cotton towel placed on top of the cage before they were released at the centre of a screen-walled greenhouse for each experiment (20:00 - 06:30 h). The roof of the greenhouse (11 m × 7 m × 2.5 m) was covered with a glass panel whereas a large mosquito netting cage (10 × 6 × 2.5 m; mesh width 3 mm) was suspended inside from the roof along the screened wall to a sand-covered floor (Njiru et al., 2006).

Field study site

Field studies were carried out at Kigoche village (00°34ôS, 034°65ôE and 1158 m above sea level) in May-June 2012. The village is situated near Ahero town, in the Kano flood plains of Kisumu County, western Kenya, approximately 110 km north east of the *icipe* -TOC campus. Annual rainfall ranges from 1000 ï 1800 mm, temperatures between 17 and 32°C and 65% average relative humidity (RH) are experienced. The long rainy season occurs between March and August while short rains are common in October-November. Ahero is a seasonally inundated flood plain adjacent to the river Nyando within the Lake Victoria basin in western Kenya. Irrigated rice farming is the dominant economic activity, but traditional farming of maize, millet, bananas, sweet potatoes, beans, cassava, sorghum and rearing of indigenous cattle, goats, sheep and poultry is also practiced. Malaria is transmitted primarily by *An.*

funestus Giles*, An. gambiae s.s.* and *An. arabiensis* Patton (Bukhari et al., 2011, Mukabana et al., 2012a).

Ethical approval

Scientific and ethical clearance of the present study was granted by the Kenya Medical Research Institute (KEMRI/RES/7/3/1). Inclusion consent of houses into the study was obtained from household heads and local administration.

Description of study houses

A total of five village houses (measuring 15.8 to 22.5 m^2) were randomly selected and labelled for trapping of outdoor mosquito populations. The houses consisted of mud walls and floors with open eaves, corrugated iron-sheet roofs, no ceiling, and they were either single or double roomed (Atieli et al., 2009). They were located on a transect oriented east-west along the northern edge of the Ahero rice irrigation scheme, approximately 28 - 150 m apart, 10 - 20 m away from cowsheds and within a range of 100 m from irrigation water channels and rice paddies (Okumu et al., 2010a-b, Mukabana et al., 2012a). The exact location of all houses was determined with a hand-held global positioning system receiver (Trex HC series, Garmin International, USA). The prevailing outdoor temperature, RH and rainfall were recorded from a weather station located at the Ahero Irrigation Research Station (AIRS), approximately 800 m away from the study houses. During experimental nights, the five houses were occupied routinely by 2-5 dwellers who slept under bed nets without insecticides or repellents (Jawara et al., 2011).

Preparation and dispensing of synthetic mosquito lures

A synthetic mosquito attractant blend called Ifakara blend 1 (IB1) was made from 10 chemical compounds (Okumu et al., 2010a-a, Mukabana et al., 2012) and supplemented with carbon dioxide. The carbon dioxide was produced nightly from a mixture of 2 L of tap or river water, 17.5 g of instant dry yeast (Smallegange et al., 2010, Mukabana et al., 2012a) and 250 cm^3 of molasses (44.7% pure, contained 34.2% sugar and 76.4% of total dissolved solids). Molasses is a by-product formed after crystallization of refined white sugar from raw sugarcane syrup (Mumias Sugar Company Ltd, Kenya).

Nylon strips (1 cm × 26.5 cm each) have commonly been used to dispense synthetic attractant odorants for studies on host-seeking mosquitoes (Okumu et al., 2010b, Mukabana et al., 2012b). Since the absorption layer embedded within a disposable sanitary pad was 24 cm long, this measurement was adopted for all four types of release substrates evaluated in the present study. A total of ten individual strips (1 × 24 cm) were cut from (a) nylon stockings

(15 denier microfibre, 90% polyamide and 10% spandex purchased from Bata Shoe Company Ltd, Kenya), (b) 100% polyester mosquito bed-net without insecticide (Country Mattresses Company Ltd, Kenya), (c) 100% woven cotton (Articot Golden quality duster, India) and (d) the absorbent layer (95% cellulose and 5% sodium polyacrylate fibres) of a disposable menstrual sanitary pad (unscented *Always* ultra thin, ultra-fine Gel-X, Fabricadona Egiptopor. EG Procter & Gamble Company, Egypt). Currently, sodium polyacrylate is the cheapest and most commonly used super absorbent polymer on the market. The composition of the absorbent layer embedded within the sanitary pad was determined at the Department of Textiles at Ghent University, Belgium.

Each of the ten strips from the four substrates was separately soaked in a glass bottle containing 1 ml of an optimal concentration of the individual chemical constituents of blend IB1 published by Okumu et al. (2010a). Thereafter, the strips were air-dried at room temperature for 5 h. All attractant-treated strips for each of the four substrates were hooked at one end and hung inside the odour plume tubes of separate Mosquito Magnet-X (MM-X) counter flow geometry traps (American Biophysics, North Kingstown, RI, USA). Traps containing IB1 dispensed from any of the four substrates were supplied with carbon dioxide (approximately 80.63 ml/min) through a 5 mm-wide silicon tubing during each experimental night (Smallegange et al., 2010). However, 10 untreated strips (no odour) as control were cut from each substrate soaked in 1 ml of water, air-dried for 5 h and tested during preliminary investigations against attractant-impregnated substrates.

Each trap was suspended on a separate tripod stand within a screen-walled greenhouse or under the eaves of a village house with trap opening positioned 15 cm above ground level, marked and used for one specific treatment throughout the experiment (Schmied et al., 2008, Jawara et al., 2009). The traps were operated on 12 V and alternated between or among different trapping positions to minimize site effects. Individual sets of attractant-impregnated substrates were separately stored at 4^{o}C between experimental runs. Latex gloves were worn when cutting and impregnating strips, and also when hanging them inside the plume tube of specified traps to avoid contamination from human volatiles. Prevailing temperature and RH humidity levels in the greenhouse were recorded at an interval of 30 min using a data logger (Tinytag® Ultra, model TGU-1500, INTAB Benelux, The Netherlands).

Response of *An. gambiae* to attractant-treated and untreated substrates

Although nylon has been confirmed to be a more effective matrix for dispensing synthetic mosquito attractants than LDPE sachets, we performed preliminary experiments to investigate whether alternative locally available materials performed similarly or better (Okumu et al., 2010c, Mukabana et al., 2012b). The first sets of competitive dual-choice assays included (a)

control and IB1-treated nylon, (b) control and IB1-treated polyester, (c) control and IB1-treated cotton, and (d) control and IB1-treated cellulose + polyacrylate material. Additional dual-choice assays were conducted to compare behavioural responses of *An. gambiae* to blend IB1 dispensed from nylon versus blend IB1 released from polyester, cotton and cellulose + polyacrylate material. Individual bioassays were run for four nights. Each untreated (control) and IB1-treated substrate was re-used throughout the four experimental nights (Mukabana et al., 2012b).

Responses of *An. gambiae* to attractant-treated substrates

The efficacy of different substrates to dispense chemical constituents of blend IB1 for attraction of *An. gambiae* was tested further in a semi-field enclosure through a 4 × 4 Latin square experimental design. The design included blend IB1 dispensed from (a) nylon as a positive control, (b) polyester, (c) cotton, and (d) cellulose + polyacrylate material. The IB1-treated substrates were tested for 16 successive nights and thereafter deployed for luring outdoor-biting malaria and other mosquitoes into traps for 25 nights at Kigoche village.

Efficacy of treated substrates to lure malaria and other mosquitoes into outdoor traps

The potential of traps containing IB1-treated substrates to intercept and attract mosquitoes under eaves of village houses occupied by the dwellers overnight was tested in a 5 × 5 Latin square experimental assay for 25 successive nights (18:30 - 06:30 h). The treatments included (a) an unbaited MM-X trap (no odour), (b) IB1-treated nylon, (c) IB1-treated polyester, (d) IB1-treated cotton, and (d) IB1-treated cellulose + polyacrylate material. The attractant-impregnated substrates were re-used for the entire study period of 25 nights and had previously been tested under semi-field conditions for 16 nights post-impregnation.

At the end of each experimental night, all traps were transported to a field laboratory located at the Ahero Multipurpose Development Training Institute (AMDTI) (approximately 5 km away) and placed in a freezer for 30 min. The frozen adult mosquitoes were emptied into labelled Petridishes, identified morphologically (Gillies and Coetzee, 1987), counted, and recorded according to (i) gender as male or female *An. gambiae* s.l. *An. funestus*, *Culex, Mansonia* spp. and other anopheline mosquitoes (all collected *Anopheles* spp. except *An. gambiae* s.l. and *An. funestus*) and (ii) external abdominal appearance as unfed, blood-fed or gravid female *An. gambiae* s.l. and *An. funestus* (WHO, 1975). All female *An. gambiae* s.l. and *An. funestus* were separately preserved in 2 mL Eppendorf tubes containing silica gel crystals and labelled. A randomly selected sub-sample of 125 females of *An. gambiae* s.l. from all treatments was analysed for species composition using a ribosomal Polymerase Chain Reaction (PCR) assay (Scott et al., 1993).

Data analysis

Differences between proportions of *An. gambiae* caught in both traps during dual-choice bioassays were analysed using a Chi-square test. This was aimed at determining whether the proportion of mosquitoes caught in each of the two MM-X traps differed from a 1:1 distribution. A generalized Linear Model fitted with a Poisson regression and a logarithmic link function, dispersion estimated, was used to investigate the effect of treatment on behavioural responses of mosquitoes to blend IB1 dispensed from different substrates tested in the 4 × 4 or 5 × 5 Latin square design experimental bioassays (Verhulst et al., 2011). Effects were considered to be significant at $P < 0.05$. The effects of treatment and trap, or house position on mosquito catches were tested as parameters in the model. All analyses were carried using IBM SPSS statistical software, version 16.

Results

Response of *An. gambiae* to untreated and IB1-treated substrates

Semi-field experiments were conducted between November 2011 and April 2012 at an average temperature and RH of 25.7 ± 2.5^{o}C and 62.8 ± 8.4%, respectively. In general, the attractiveness of nylon, polyester, cotton, and cellulose + polyacrylate material to *An. gambiae* was significantly enhanced after being treated with blend IB1 ($P < 0.001$). The total mosquito catches with control (untreated) and treated materials were as follows: (a) control (n = 18, 6%) and IB1-treated nylon (n = 284, 94%), (b) control (n = 20, 6%) and IBI-treated polyester (n = 325, 94%), (c) control (n = 31, 8%) and IB1-treated cotton (n = 362, 92%), and (d) control (n = 24, 6%) and IB1-treated cellulose + polyacrylate material (n = 354, 94%) (Figure 1).

A second series of dual-choice bioassays indicated that the responses of *An. gambiae* to IB1-treated nylon were significantly lower compared to IB1-treated polyester ($P < 0.001$), IB1-treated cotton ($P < 0.001$) and IB1-treated cellulose + polyacrylate material ($P < 0.010$) (Table 1).

Responses of *An. gambiae* to attractant-treated substrates

Of the 3,200 mosquitoes released, 65.2% (n = 2,087) were trapped (Table 2). The catches of *An. gambiae* were influenced by trap position ($P < 0.001$) and type of odour-dispensing substrate ($P < 0.001$). The responses of mosquitoes to blend IB1 dispensed from nylon were significantly lower compared to cotton ($P < 0.014$) and cellulose + polyacrylate material ($P <$

0.001). However, IB1-impregnated nylon was more attractive to mosquitoes than similarly treated polyester but the difference was not statistically significant ($P < 0.07$). The same treatments were tested in the field for 25 successive nights.

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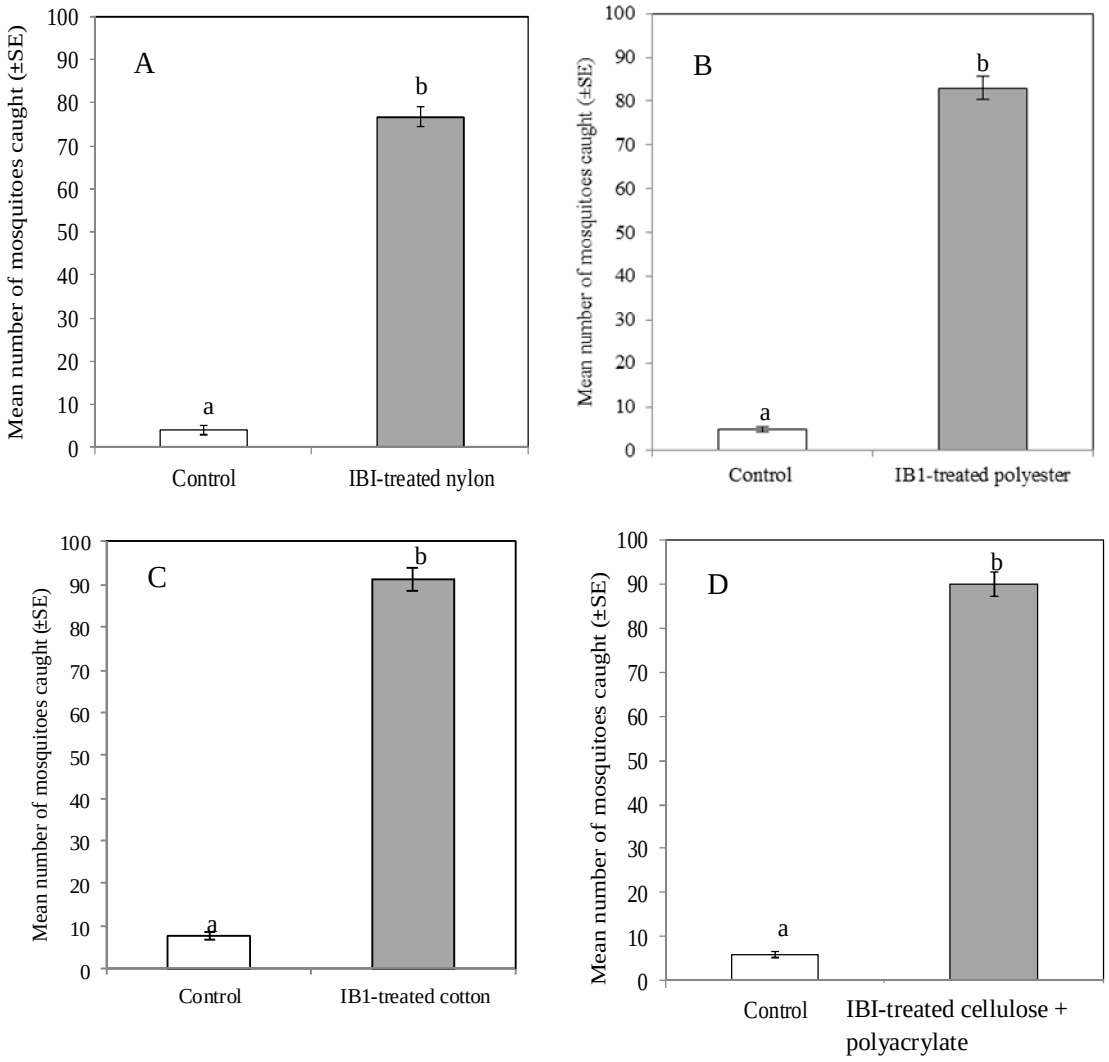

Figure 1: Mean number ± SE of female *An. gambie* caught in a dual-choice assay between a trap containing control (untreated) and IB1-treated (i) nylon (panel A), (ii) polyester (panel B), (iii) cotton (panel C) and (iv), cellulose + polyacrylate embedded within the sanitary pad (panel D) material for four nights. A total of 200 female *An. gambiae* were released per night within a screen-walled greenhouse. Means with different letters in the same panel are significantly different at $P < 0.05$ level.

Efficacy of treated substrates to lure malaria and other mosquitoes into outdoor traps

Female mosquitoes

An average outdoor temperature of 23.6 ± 3.0°C, 64.4 ± 13.7% RH and a total of 77.6 mm of rainfall (for 18 days) were recorded during the 25 nights of field experiments (May-June 2012). A total of 4,415 mosquitoes were collected in all traps combined, with 93.6% (n = 4,134) females and 6.4% (n = 281) males.

Table 1: Total and mean ± SE number of *An. gambiae* attracted in a dual-choice bioassay by blend IB1 dispensed from nylon (as a reference treatment) versus candidate odour-dispensing substrates (polyester, cotton and cellulose + polyacrylate material) within a screen-walled greenhouse.

Candidate odour-dispensing substrate	N	n	Mean ± SE mosquitoes caught		
			Nylon	Candidate substrate	P-value
Polyester	4	474	42.8 ± 3.3	75.8 ± 4.4	0.001
Cotton	4	434	43.0 ± 3.3	65.5 ± 4.5	0.001
Cellulose + polyacrylate	4	359	35.8 ± 3.0	54.0 ± 3.7	0.010

N is the number of experimental nights, n is the total number of mosquitoes caught whereas SE is the standard error of the mean catch per night. A total of 200 female *An. gambiae* were released per night. Pairwise comparisons in the same row of mean catches differ significantly at $P < 0.05$ (Chi-square test).

Table 2: Total and mean (± SE) number of *An. gambiae* collected in a trap baited with blend IB1 dispensed from nylon, polyester, cotton and cellulose + polyacrylate material within a screen-walled greenhouse.

Treatment	N	Mosquitoes caught	
		n	Mean (±SE)
IB1-treated nylon	16	428	26.8 ± 1.3^{a}
IB1-treated polyester	16	377	23.6 ± 1.2^{a}
IB1-treated cotton	16	503	31.4 ± 1.4^{b}
IB1-treated cellulose + polyacrylate	16	779	48.7 ± 1.7^{c}

N is the number of experimental nights, n is the total number of mosquitoes caught whereas SE is the standard error of the mean catch per night. A total of 200 female *An. gambiae* were released per night. Pairwise comparisons in the same column of mean catches differ significantly at $P < 0.05$ (Generalized Linear Models).

The female mosquitoes caught indoors comprised *An. gambiae* s.l. *(*25.4%), *An. funestus* (30.2%), *Culex* spp. (36.7%), *Mansonia* spp. (3.9%) and other anopheline spp. (3.9%) (Figure 2). Trap collections of female *An. gambiae* s.l. were influenced by house position ($P < 0.001$) and treatment ($P < 0.001$). The IB1-treated nylon was significantly less attractive to *An. gambiae* s.l. than similarly-treated polyester ($P < 0.001$), cotton ($P < 0.001$) and cellulose + polyacrylate material ($P < 0.001$). Although IB1-treated cotton and cellulose + polyacrylate material were the most attractive to *An. gambiae* s.l., catches between both substrates were not different ($P = 0.546$). Moreover, the cellulose +

polyacrylate material was the most effective substrate for dispensing IB1 to *An. funestus* compared to other materials ($P < 0.001$) whereas nylon was the least effective ($P < 0.001$) (Figure 2).

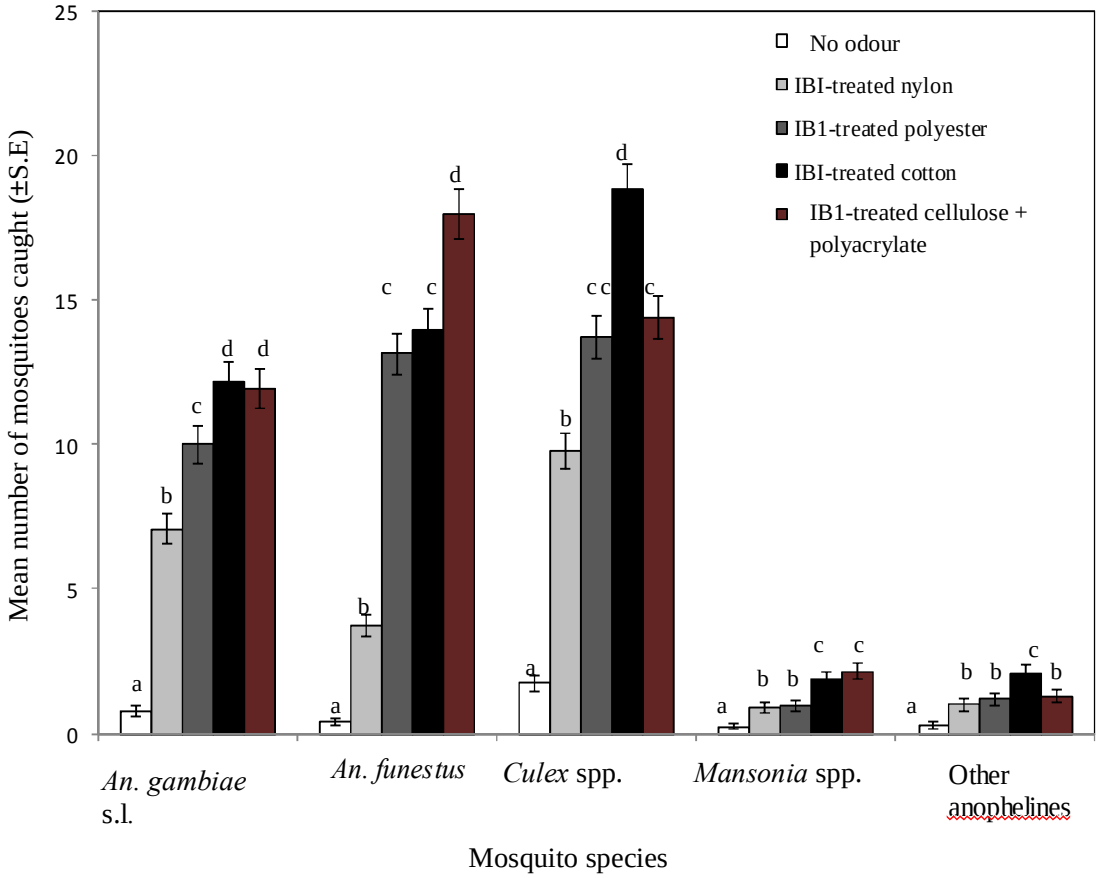

Figure 2 : Mean number ± SE of female mosquitoes caught in an outdoor trap without odour, baited with blend IB1 dispensed from nylon, polyester, cotton or cellulose + polyacrylate material for 25 nights in Kigoche village. Treatments are shown in the legend. Mean values within the same mosquito type having no letter in common differ significantly at $P < 0.05$.

There was no difference in the mean numbers of *An. funestus* collected in traps containing IB1 dispensed from cotton and polyester material ($P = 0.45$). The IB1-treated nylon was significantly less attractive to *Culex* spp. than similarly-treated polyester ($P < 0.001$), cotton ($P < 0.001$) and cellulose + polyacrylate material ($P < 0.001$). Although IB1-treated cotton was the most attractive to *Culex* spp. compared to other materials ($P < 0.001$), trap collections were not different between IB1-impregnated polyester and cellulose + polyacrylate material ($P = 0.53$). The attractiveness of blend IB1 dispensed from nylon to *Mansonia* spp. was not different from polyester ($P = 0.89$), but it was significantly lower compared to similarly-treated cotton ($P < 0.02$) and cellulose + polyacrylate material ($P < 0.010$). Furthermore, the responses of other anopheline mosquitoes to IB1 dispensed from nylon were not different from polyester ($P = 0.52$) and cellulose + polyacrylate material ($P = 0.72$), instead they were lower compared to IB1-treated cotton ($P < 0.023$).

Male mosquitoes

The 281 trapped male mosquitoes comprised *An. gambiae* s,l. (50.9%), *An. funestus* (30.6%), *Culex* spp. (14.2%), *Mansonia* spp. *(*1.4%) and other anopheline spp. (2.9%) (Table 3). Whereas traps baited with IB1-treated nylon collected similar catches of *An. gambiae* s.l. as the control (no odour) ($P < 0.87$), IB1-treated nylon was significantly less attractive than similarly-treated polyester ($P < 0.001$), cotton ($P < 0.015$) and cellulose + polyacrylate material ($P < 0.024$) to *An. gambiae* s.l. Dispensing blend IB1 from polyester, cotton and cellulose + polyacrylate material had no influence on the responses of *An. gambiae* s.l. ($P = 0.47$) and *An. funestus* ($P = 0.78$). Furthermore, there was a lower response of *An. funestus* to IB1-impregnated nylon than to polyester ($P < 0.022$), cotton ($P < 0.033$) and cellulose + polyacrylate material ($P < 0.012$). Treatment had no effect on trap collections of *Culex* ($P = 0.23$), *Mansonia* ($P = 0.79$) and other anophelines ($P = 0.45$).

Table 3: Mean number (± SE) of male mosquitoes caught in an outdoor trap without odour, baited with blend IB1 dispensed from nylon, polyester, cotton and cellulose + polyacrylate material in Kigoche village for 25 nights.

Treatment	N	Mean number ± SE of mosquitoes caught				
		An. gambiae s.l.	*An. funestus*	*Culex* spp.	*Mansonia* spp.	Other anophelines
No odour	25	0.32 ± 0.11^a	0.04 ± 0.04^a	0.12 ± 0.07^a	0.04 ± 0.04^a	0.04 ± 0.04^a
IBI-treated nylon	25	0.64 ± 0.16^a	0.35 ± 0.12^a	0.24 ± 0.09^a	___	0.08 ± 0.06^a
IB1-treated polyester	25	1.76 ± 0.27^b	0.94 ± 0.20^b	0.40 ± 0.13^a	0.04 ± 0.04^a	0.16 ± 0.08^a
IBI-treated cotton	25	1.60 ± 0.25^b	1.06 ± 0.21^b	0.36 ± 0.12^a	___	0.04 ± 0.04^a
IB1-treated cellulose + polyacrylate	25	1.40 ± 0.24^b	0.98 ± 0.20^b	0.48 ± 0.14^a	0.08 ± 0.06^a	__

N is the number of experimental nights, a dash (_____) implies no mosquito caught whereas SE is the standard error of the mean number of catches per night. Pairwise comparisons in the same column of mean catches differ significantly at $P < 0.05$. Mean ± SE mosquito catches within the same column assigned different letter superscripts are significantly

Major malaria vectors of different abdominal status

There were 1,049 female *An. gambiae* s.l. and 1,249 female *An. funestus* trapped. The majority of female *An. gambiae* s.l. collected were unfed (65.9%) whereas fewer were blood-fed (32.2%) and some were gravid (1.9%) (Figure 3A). Trap catches of unfed *An. gambiae* s.l. were greatly affected by treatment ($P < 0.001$). Dispensing of blend IB1 from nylon strips attracted a notably lower number of unfed *An. gambiae* s.l. compared to other substrates ($P <$

0.001). IB1-treated cotton and cellulose + polyacrylate material attracted the highest mean numbers of unfed *An. gambiae* s.l. that were similar for both materials (P = 0.74). Moreover, unfed *An. gambiae* s.l. responded equally to IB1-impregnated polyester and cellulose + polyacrylate material (P = 0.07).

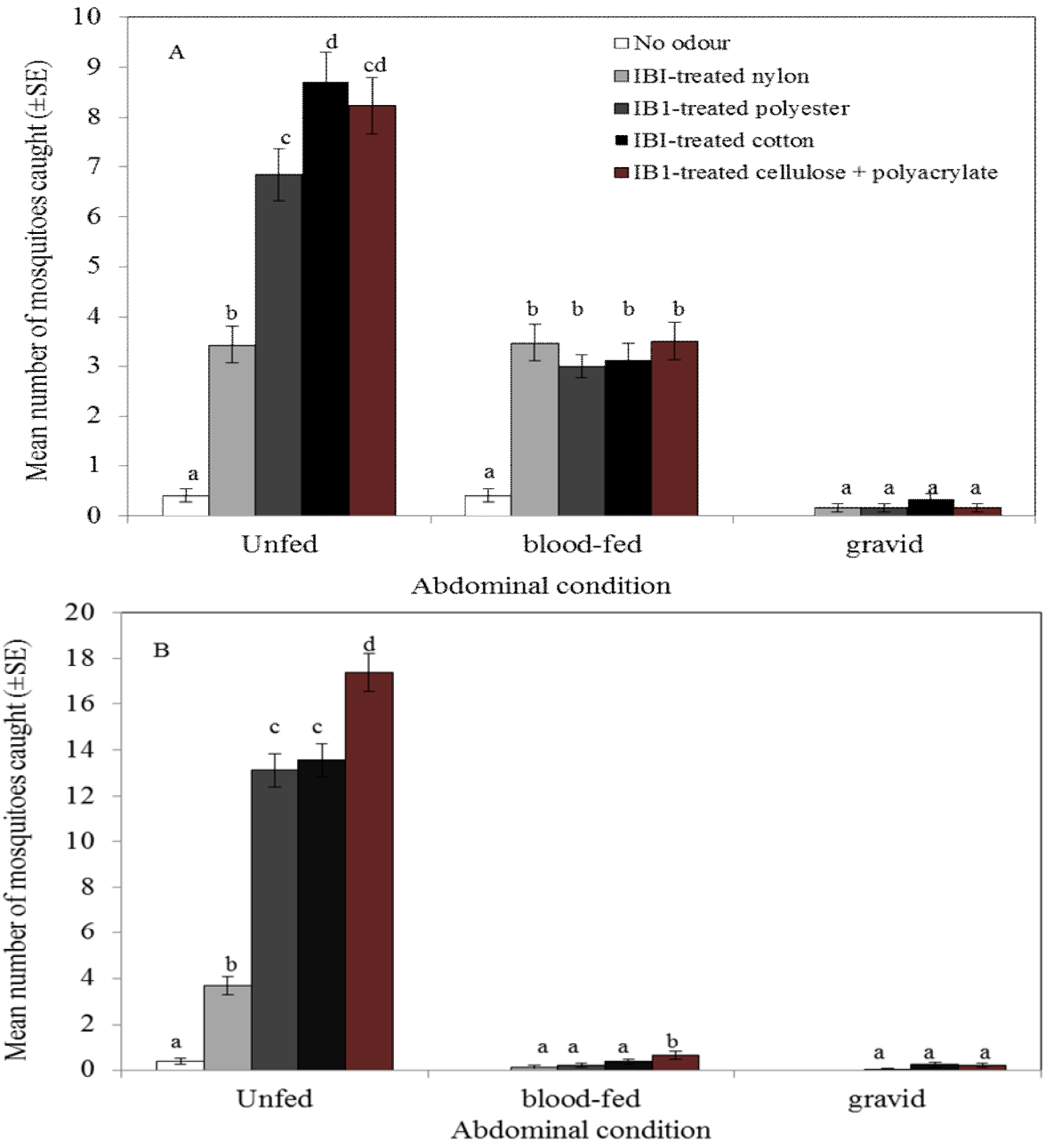

Figure 3: Mean number ± SE of *An. gambiae* s.l. (panel A) and *An. funestus* (panel B) in different abdominal conditions (unfed, blood-fed and gravid) collected in an outdoor trap without odour, baited with blend IB1 dispensed from nylon, polyester, cotton or cellulose + polyacrylate material for 25 nights in Kigoche village. Treatments are shown in the legend of panel A. Mean values within the same abdominal condition having no letter in common differ significantly at P < 0.05.

Trap catches of blood-fed and gravid *An. gambiae* s.l. among the four IB1-impregnated materials were similar (P = 0.36 and P = 0.50, respectively). The female *An. funestus* caught were largely unfed (96.4%), with few blood-fed (2.6%) or gravid (1%) mosquitoes (Figure 3B). The response of unfed *An. funestus* to IB1-baited traps was influenced by treatment (P < 0.001).

Dispensing of blend IB1 from nylon caught significantly fewer unfed *An. funestus* compared to polyester (P < 0.001), cotton (P < 0.001) and cellulose + polyacrylate material (P < 0.001). Although there was no difference between the numbers of unfed *An. funestus* attracted to IB1-treated polyester and cotton materials (P = 0.67), each of these catches was significantly lower compared to IB1-impregnated cellulose + polyacrylate material (P < 0.001). Blood-fed *An. funestus* responded equally to blend IB1 dispensed from nylon, polyester and cotton (P = 0.43), however, cellulose + polyacrylate material was the most efficient substrate for dispensing of attractants (P < 0.041). Moreover, selection of dispensing material for blend IB1 had no impact on trap collections of gravid *An. funestus* (P = 0.25).

Analysis of *An. gambiae* s.l. by PCR

Results from PCR analysis indicated that 117 out of 125 samples of *An. gambiae* s.l. were successfully identified. All the 117 sub-samples were confirmed to be *An. arabiensis* and no *An. gambiae s.s* was identified.

Discussion

The release of Ifakara blend 1 from strips of cotton, polyester and cellulose + polyacrylate materials consistently lured more *An. gambiae* into traps compared to nylon under semi-field conditions. Similarly, IB1-impregnated cotton, polyester and cellulose + polyacrylate materials attracted significantly more *An. gambiae* s.l., *An. funestus, Culex* and *Mansonia* species than IB1 dispensed from nylon strips under field conditions. Field collected mosquitoes were in different physiological conditions (unfed, blood-fed and gravid), and the majority were unfed females of *An. gambiae* s.l. and *An. funestus*. *Anopheles arabiensis* was the only sibling species of the *An. gambiae* complex identified.

In all experiments, carbon dioxide was added to the synthetic blend to synergistically improve the attractiveness of synthetic odorants released from all four textile materials to target mosquitoes (Gillies, 1980, Gibson and Torr, 1999, Dekker et al., 2002) (Chapter 8). Although it was recently established that nylon strips were more effective than LDPE sachets in

dispensing synthetic mosquito attractants (Okumu et al., 2010b, Mukabana et al., 2012b), the present results suggest that alternative textile materials may perform equally well or even better than nylon for monitoring malaria mosquitoes. The better effect of polyester, cotton and cellulose + polyacrylate materials is possibly caused by a larger adsorbent surface area which allows for an even and constant dispensing of odorants to the environment. This seems to apply especially to the sanitary pads, consisting of cellulose + sodium polyacrylate. Cellulose provides fine fibres covered with sodium polyacrylate as a super adsorbent material. It is highly likely that a combination of the cellulose and the polyacrylate creates microfibers that are ideally suited for adsorption and slow-release of odorant compounds, thereby resulting in increased mosquito catches compared to nylon material.

The repeated utilization of the same IB1-impregnated substrates over 16 nights post-treatment under semi-field conditions followed by 25 consecutive nights of field testing confirmed the potential of residual activity reported by Mukabana et al. (2012b). This suggests that all substrates caused minimal change of the chemical properties of the impregnated active ingradients, leading to a sustained release of an attractive odour blend, thereby inducing a behavioural response over extended periods of time (Torr et al., 1997, Cork, 2004). These results demonstrate that the search for alternative and easy-to-prepare odour-dispensing systems can improve the effectiveness and sustainability of odour-baited technology.

Both cotton and polyester materials are preferable for disruption of the host-seeking process of endophilic malaria vectors as they can be impregnated with mosquito repellents and used as ceiling materials, window or door curtains (Lines et al., 1987, Kline and Lemire, 1998). Repellent-impregnated cotton clothing could also be worn as an alternative solution against outdoor-malaria transmission or outbreaks of dengue transmitted by day-active *Aedes aegypti* (L.) (Dia et al., 2005, Pennetier et al., 2010). Polyester bed net material has also contributed substantially towards malaria reduction as such nets provide a long-term protection against mosquito bites and subsequent mosquito-borne diseases when impregnated with insecticides (Yates et al., 2005, N'guessan et al., 2006, Mendis et al., 2009). On these grounds, we conclude therefore that attractant-impregnated cotton and polyester materials can effectively be utilised to sample and possibly control mosquitoes through a lure-and-kill technology (Kline and Lemire, 1998, Kline, 2007). The textile materials were easy in use, locally available in different sizes and relatively cheap to be considered for area-wide application. Nonetheless, these candidate attractant-treated matrices should be tested further for their wash -resistance and long-lasting residual activity on target mosquitoes as in the case of long-lasting insecticide-treated or repellent nets (N'guessan et al., 2006).

This study also demonstrated the possibility of developing novel odour-release technologies for dispensing of human-derived kairomones to monitor host-seeking malaria and other mosquito vectors (Mboera, 2005, Okumu et al., 2010b, Okumu et al., 2010c). Additionally,

such technologies may also be tested for dispensing of synthetic semiochemicals directed towards disruption of mating, sugar-feeding and oviposition behaviour of mosquitoes (Takken and Knols, 1999, Zwiebel and Takken, 2004). This was confirmed by a significant variation in abdominal conditions of *An. gambiae* s.l. and *An. funestus* that responded to attractant-treated substrates. Although we did not analyse the source of blood meals, the results of this study present a possibility of using a single synthetic blend to lure malaria vectors with heterogeneous feeding behaviour and malaria transmission potentials.

Females constituted 93.6% of all mosquitoes lured into outdoor traps baited with attractant-treated substrates compared to 6.4% males, thereby demonstrating the feasibility for mating disruption (Kline, 2007, Mukabana et al., 2012a). Considering that the odour blend released from test substrates was optimised for female malaria vectors and that males feed on plant nectar, captured males are assumed to have been in pursuit of virgin females. It is also likely that a combination of synthetic odorants and volatiles produced by fermenting molasses mimic those of plants thereby attracting males to the traps.

The collection of significantly higher mean numbers of unfed female mosquitoes compared to blood-fed and gravid females irrespective of the type of odour-dispensing substrate proved that IB1 is a potent lure for mosquitoes assumed to be host seeking (Takken, 1991, Smallegange et al., 2009, Okumu et al., 2010a). Whereas *An. arabiensis* was the only sibling species of the *An. gambiae* complex identified, previous studies have reported the existence of *An. gambiae s.s.* in the study area (Bukhari et al., 2011, Mukabana et al., 2012a). Possibly, *An. gambiae s.s*. were absent in our outdoor collections because of temporal and seasonal variation (Minakawa et al., 2002, Koenraadt et al., 2004). Whereas *An. funestus* were highly abundant and highly anthropophilic as *An. gambiae s.s.* (Githeko et al., 1996), it is likely that there is a gradual but consistent decline of *An. gambiae s.s*. associated with reduced levels of malaria infection in East Africa (Meyrowitsch et al., 2011).

The high catches of *An. gambiae* s.l. in a village where *An. arabiensis* is a primary vector of malaria coupled with the fact that cows, goats and sheep were present adjacent to human dwellings indicates that dispensing of blend IB1 from the tested materials competed favourably as a human proxy (Pates et al., 2001, Tirados et al., 2011). These results suggest that additional protection of people who are highly exposed to *An. arabiensis* and *An. funestus* bites could be enhanced by deploying outdoor and indoor traps containing human-derived attractant-treated substrates and possibly by keeping insecticide-treated cattle to maximize the effects of zooprophylaxis (Duchemin et al., 2001, Mahande et al., 2007, Torr and Vale, 2011).

Recent encouraging results have shown that a novel synthetic odour blend dispensed on nylon strips attracted as many *An. gambiae* s.l. but significantly more *An. funestus* compared to humans (Mukabana et al., 2012a). Similarly in Tanzania, a synthetic odour blend released

from nylon attracted significantly higher numbers of *An. gambiae* s.l., *An. funestus, Culex* spp. and other anophelines than human volunteers when both were placed in separate huts (Okumu et al., 2010a). Such findings demonstrate the prospects of deploying odour-baited technology to disrupt indoor malaria transmission. With an intensified search for more potent synthetic attractants than humans and the addition of spatial repellents, a push-pull system could also be integrated into the prevention of both indoor and outdoor malaria transmission (Takken, 2010). Targeting of outdoor-biting mosquitoes is currently important as recent studies have reported a shift from indoor- to outdoor-biting behaviour and transmission of malaria (Bayoh et al., 2010, Russell et al., 2011, Govella and Ferguson, 2012).

Conclusion

As a proof of principle, selection of appropriate odour-dispensing substrates for mosquito synthetic lures can significantly enhance the effectiveness of odour-baited technology for surveillance, sampling and disruption of malaria transmission. In such systems, locally available cotton, polyester and cellulose + polyacrylate materials can effectively replace nylon.

Acknowledgments

We acknowledge Mr. David Alila for rearing the mosquitoes used during semi-field bioassays and Mr. Charles Oketch for assisting Philemon during field experiments. We thank Dr. T. Gheysens, Department of Textiles at Ghent University in Belgium, for analysing the composition of an absorption layer of sanitary pads used during our experiments. This study was funded by a grant provided from the Foundations for National Institutes of Health (NIH) through the Grand Challenges in Global Health Initiative (GCGH#121) and a PhD fellowship provided to CKM by Wageningen University, The Netherlands.

Chapter 10

Summarizing discussion

Introduction

Over the last decade, increased funding for malaria control programs has contributed to a reduction in malaria morbidity and mortality through increased use of insecticide-treated bed nets (ITNs), indoor residual spraying (IRS) and highly effective anti-malarial drugs as a first line therapy (WHO, 2012). In spite of this major achievement, observed reductions fall below internationally agreed targets for elimination, especially in malaria endemic parts of the world (Alonso et al., 2011, WHO, 2012). In sub-Saharan Africa, the burden is mainly attributed to potential threats from the continued emergence and spread of parasite resistance to antimalarial medicines, mosquito resistance to insecticides (WHO, 2012), diverse mosquito breeding, resting and feeding habits and vectorial capacities of malaria vectors, as well as lack of a standardised reliable tools for monitoring malaria vectors in different abdominal conditions (Alonso et al., 2011).

Recent progress in research on olfactory behaviours of disease vectors, including mosquitoes, has led to the discovery of potent chemicals used by such insects to identify their sugar sources, mates, hosts (blood) and oviposition sites (Chapter 2). Thus, odour-based tools have been successfully exploited for control of tsetse flies and crop pests (Foster and Harris, 1997, Cook et al., 2007). Such tools are currently considered for monitoring and intervention of mosquitoes and other haematophagous insects (Logan and Birkett, 2007, Pickett et al., 2010). However, deployment of odour-baited trapping systems in malaria-endemic areas has been undermined by lack of (a) a synthetic lure to malaria vectors that is more potent than natural human odour, (b) effective and sustainable slow-release systems for odorants, (c) efficient trapping devices that rely on cheap or renewable sources of energy (Kline, 2006), and (d) alternative sources and more effective methods of delivering carbon dioxide (CO_2) and other volatile kairomones (Mukabana et al., 2010, Smallegange and Takken, 2010).

In this thesis, the efficacies of various odorants, odour-dispensing matrices and molasses as a source of CO_2 and other volatile organic compounds (VOCs) for sampling malaria vectors and other mosquito species were investigated under semi-field and field conditions in western Kenya. The effect of different odour-release matrices was evaluated using proven synthetic odour blends, termed Ifakara blend 1 (IB1) and the Mbita blend, for the malaria vectors *Anopheles gambiae* Giles *sensu stricto* (henceforth termed as *An. gambiae*), *An. arabiensis* Patton and *An. funestus* Giles. The residual activity of the IB1-impregnated nylon strips on malaria vectors over time was assessed for 40 consecutive nights and at weekly intervals for 48 and 52 nights post-impregnation. The efficacy of fermenting molasses as a CO_2 producer was tested on the above described mosquito species, with the aim of providing a more affordable and alternative source of the kairomone than sugar. In an additional study, the effects of attractive stimuli ñlightò and ñodourò on mosquito trap catches were compared with

the natural stimuli provided by a human host.

The findings of this thesis demonstrate that odour-baited technology may provide a reliable complementary tool for sampling multiple adult malaria vectors and other mosquito species over prolonged periods of time. Application of the odour-baited technology may also increase chances of targeting selected mosquito species, thereby reducing the need for reliance on broad-spectrum insecticides, the application of which is associated with insect resistance and environmental damage (Ranson et al., 2011). Odour baits were more selective than light as a stimulus for sampling malaria vectors as the latter also attracted non-target species and higher proportions of male mosquitoes. Unlike the commonly used methods for monitoring of malaria vectors, the odour-baited systems attracted and caught both indoor and outdoor biting malaria vectors in different physiological conditions. The main results of this thesis are discussed in a broader scientific and social context, and suggestions for future research are presented.

Potential of odour-based technology as a complementary tool for malaria control

The IB1, the Mbita blend and prototypes of the Mbita blend used as trap lures were prepared for host-seeking *An. gambiae* (Okumu et al., 2010a, Mukabana et al., 2012a). However, the baited traps also attracted and caught large numbers of indoor- and outdoor-biting *An. arabiensis*, *An. funestus* and *Culex* mosquitoes. This demonstrates a high prospect of using odour-baited devices for reliable vector surveillance. Moreover, odour-baited traps are now being evaluated as tools for malaria control by removal trapping, leading to interruption of parasite transmission (Hiscox et al 2012). Because the odour-baited traps also caught non-anopheline mosquitoes including *Culex* spp., some of which are vectors of arboviruses or filariasis, this novel technology could be used for sampling and reducing transmission of other mosquito-borne diseases. Currently such diseases do not pose a serious medical challenge in western Kenya.

Mosquito populations were more responsive to synthetic odour baits used than any other group of insects, with female mosquitoes responding at a higher rate (86.7%) than males (13.3%). This may have been possible because the baits mimic human odorants (Smallegange et al., 2009, Okumu et al., 2010a) to which male mosquitoes do not respond (Takken and Knols, 1999). Moreover, mosquito diversity observed in the odour-baited traps were consistent with catches obtained with a lit CDC miniature trap (Chapter 5 and 6) and previous studies (Chandler et al., 1975, Githeko et al., 1996). The *An. gambiae* sensu lato collected under field conditions were predominantly *An. arabiensis* (Chapter 5 and 9). Survival of this opportunistic malaria vector was favoured by lowland conditions compared to *An. gambiae s.s* as it thrives in higher altitude areas (Minakawa et al., 2002a, Imbahale et al., 2011) and possibly due to prolonged use of ITNs and IRS (Russel et al., 2011, Gatton et al., 2013). The

foregoing results suggest that synthetic odour blends may be applied for sampling and possibly control of multiple mosquito vectors found either indoors and outdoors (Chapter 2) as advocated in the research agenda for malaria eradication through vector control (Alonso et al., 2011). Odour-baited traps may be used to reduce the survival rates of malaria vectors through mass trapping (Kline, 2007). Further impact is likely to be achieved by treating the long-lasting attractant-treated substrate materials (Chapter 9) with insecticides (Okumu et al., 2010b), entomopathogenic fungi (Mnyone et al., 2012) or bacteria in a lure and kill system.

Anopheles funestus is likely to play a more important role in malaria transmission because the majority had fed on human blood while *An. arabiensis* fed mainly on bovine than human blood (Chapter 5 and 6). These results suggest that outdoor traps containing synthetic odour blends could be used to attract and intercept mosquitoes as they exit from human-occupied houses or before entry thereby disrupting malaria transmission. This approach would reduce the use of insecticides and offers an alternative solution to the increasing resistance of malaria mosquitoes to the chemicals impregnated on to mosquito bed nets and sprayed indoors (Russell et al., 2011). At present, prolonged use of ITNs and IRS does not confer complete protection against malaria due to physiological resistance to previously potent insecticides, changes in mosquito feeding behaviour, time of biting and outdoor transmission capacity of some malaria vector species (Russell et al., 2011, Gatton et al., 2013). Changes in species composition have been reported following large scale introduction of ITNs and differences in vectorial capacity of the newly dominant species may alter the impact of intervention strategies (Rielhe et al., 2011).

In this study, the odour-baited traps were deployed in a human-occupied or an empty house while outdoor traps were placed under the eaves of human-occupied houses (Hiscox et al., 2012) (Chapters 4 - 9). It has also be demonstrated that more anopheline mosquitoes are caught outdoors when traps baited with host-seeking odorants were placed under the eave of a human-occupied house than 25 m away (Mburu et al., unpublished data). Nonetheless, it has been suggest that baited intervention traps should be positioned far away from houses so as to minimize the risk of excessive mosquito bites and increased exposure to mosquito-borne pathogens (Okumu et al., 2010c, Sumaye et al., 2012). This is based on the capacity of the odour-baits including CO_2 to activate and attract mosquitoes from long distances (Gillies, 1980, Costantini et al., 1996). By so doing, mosquitoes are lured away from the homes, thereby reducing mosquito bites and malaria transmission. For example, baited traps or bait stations may be placed in a section of the village area where the majority of infective mosquitoes are likely to be caught (Okumu et al., 2010c, Bousema et al., 2012, Sumaye et al., 2012). Such traps could be located at least 10 m from the nearest house, near to the larval breeding sites and around areas where natural human aggregations occur (Qiu et al., 2007) (Chapter 2). As a result, there is a need for more research on optimal odour-baited trap positions.

The demand for high impact odour-based tools for sampling host-seeking malaria vectors was investigated by addition of novel attractants to the Mbita blend previously shown to be more attractive than the IB1 blend (Mukabana et al., 2012a). Both odour baits contained lactic acid, ammonia, tetradecanoic acid and CO_2 as a basic blend. The Mbita blend was simpler of composition as it comprised 3-methyl-1-butanol as a fifth component while the IB1 blend contained 11 components in total (Okumu et al., 2010a). Although the attractiveness of the Mbita blend to mosquito populations was improved by addition of other volatile chemicals (Chapter 7), direct comparisons with human volunteers and blend IB1 (Chapter 5 and 6) were not performed. Therefore, the possibility of enhancing the potency of the odour bait to surpass that of human odour should be explored as the attractiveness of IB1 did not exceed that of humans (Chapter 5 and 6). The robustness of potent synthetic odour baits for sampling and disruption of malaria transmission could also be improved by applying a combination of baits and spatial repellents in a push-pull system (Takken, 2010). Whereas a push-pull strategy has been tested for protection of cattle against tsetse fly vectors of trypanosomiasis (Chapter 2), much effort is required for its application in malaria control (Takken, 2010) as currently there are no suitable delivery systems, effective spatial and long-lasting repellents. In order to enhance malaria elimination and eradication in endemic areas, odour-based technology should be used to complement rather than replace existing malaria control strategies (Mendis et al., 2009). This is why the impact of deploying odour-based tools for the improved performance of ITNs, IRS or both in malaria elimination is called for. In Kenya, a novel approach to eliminate malaria at a local level by using odour-baited traps for mass trapping of adult mosquitoes is currently being implemented in partnership with the local community on an island in Lake Victoria (Hiscox et al., 2012).

Potential of odour baits for sampling malaria vectors in various physiological conditions

The reliability of using the IB1 and Mbita blends as trap lures for sampling indoor- and outdoor-biting *An. gambiae* s.l., *An. funestus* and *Culex* spp., demonstrates the potential of using odour-baited tools to replace human landing catches (HLC) for monitoring of mosquito populations. A previous model had shown that the highest proportions of infectious mosquitoes occur in localities with older mosquitoes and those found far away from breeding sites (Smith et al., 2004). In this study, parity status of female mosquitoes caught was not determined even though they were largely unfed. The highest proportion of unfed malaria vectors and *Culex* spp. were mainly observed in traps baited with a combination of the IB1 blend and CO_2 produced by yeast-fermented sugar (Chapter 4 and 5), and this effect was enhanced by the presence of light (Chapter 6). Although determination of the parity status or age structure of the collected mosquitoes would have been useful, it was assumed that majority of female mosquito caught were virgins as the sampling sites were adjacent to the breeding sites (Minakawa et al., 2002b, Smith et al., 2004, Nyaguara et al., 2012). Male mosquitoes responded more strongly to light than to odour cues (Chapter 6). This implies that

male mosquitoes can be sampled by using light-baited tools, while those attracted to the synthetic odour blends were assumed to be in pursuit of virgin females for mating (Klowden 1994). It has also been reported that male *Aedes aegypti* mate around the host and respond well to host-derived cues (Takken and Knols, 1999). More recently *An. gambiae* s.l. were observed mating indoors in a West African village (Dao et al., 2008). This observation suggest that *An. gambiae* s.l. employs several mating strategies, thereby explaining why some males were attracted to the synthetic odour baits, but this should be validated under diverse malaria epidemiological settings.

The use of light traps as a substitute for human-landing catches for estimation of human biting rates, and hence risk of malaria transmission, has been evaluated under different settings with varying degrees of success (Service, 1993, Mboera, 2005, Kelly-Hope and McKenzie, 2009). Evidence has shown that the light-baited CDC miniature trap is suitable for (a) sampling endophagic malaria and bancroftian filariasis vectors (Odetoyimbo, 1969, Costantini et al., 1998), (b) determination of specific composition of the *Anopheles* fauna during a period of high mosquito density (Joshi et al., 1975), and (c) attraction and collection of resting mosquitoes with a higher sporozoite rate than host-seeking ones (Petrarca et al., 1991, Smith et al., 2004). In view of the foregoing experimental results, baiting of MM-X traps with IB1-treated nylon strips demonstrated a high potential of serving as an alternative sampling tool. The IB1-treated nylon strips attracted similar catches of indoor-biting *An. gambiae* s.l. as humans and equal numbers of female *An. funestus* compared with a lit CDC miniature trap (Chapter 6). Higher numbers of outdoor-biting *An. gambiae* s.l. and more outdoor-biting *An. funestus* were also caught in the odour-baited MM-X trap than a light-baited CDC miniature trap (Chapter 5). Furthermore, *Plasmodium falciparum* sporozoites were identified in one sub -sample of female *An. funestus*.

Alit CDC light trap hung beside a human-occupied bed net offered the best lure for malaria vectors and this has also been show-cased by the synergistic effect between CO_2 and light on enhanced collections of phlebotomine sand flies (Andrade et al., 2008). Thus, sampling of malaria vectors could be improved by a combination of light and a synthetic odour stimuli and hence, the need for further investigations. Deployment of both stimuli may increase selectivity and collection of live mosquitoes than use of light alone which attracted many non-target insects, some of which may prey on the mosquitoes or damage them through violent movements.

In Chapter 7 of this thesis, selective and improved attraction of the Mbita blend for sampling different populations of *An. gambiae* s.l., *An. funestus* and *Culex* spp. was influenced by addition of 1-butylamine, 1-dodecanol or 2-pentadecanone in various concentrations. Besides increasing mosquito catches, notably with 1-butylamine and 1-dodecanol, the most interesting potential of this study lies in the attraction of equal proportions of unfed and gravid *An. gambiae* s.l. mosquitoes, as opposed to predominantly high catches of unfed *An. funestus*.

Therefore, it is evident that manipulation of existing synthetic blends, attractive to mosquitoes may provide a trap lure for collection of both unfed and gravid *An. gambiae* s.l. This finding has an important implications as the chemical ecology of oviposition behaviour, including oviposition pheromone and site-derived cues, has only been studied extensively for *Culex* spp. (Mboera et al., 2000, Seenivasagan and Vijayaraghavan, 2010) but not for anopheline mosquitoes (Pickett et al., 2010). Follow-up investigations are required as they may lead to the discovery of appropriate synthetic kairomones for sampling gravid and infected malaria vectors. By coincidence, the need to enhance robustness, sustainability and cost-effectiveness of odour-baited traps by the use of molasses as a locally available, alternative carbohydrate source of CO_2 demonstrated a potential bait for sampling both unfed and blood-fed malaria vectors (Chapter 8).

Whereas attraction of blood-fed *An. gambiae* s.l. to the Mbita blend was enhanced by the addition of CO_2 released from fermenting molasses compared to the addition of CO_2 released from fermenting sugar, the responses were directly dependent on the quantity of molasses used. In contrast to *An. gambiae* s.l., the high catches of blood-fed *An. funestus* observed were not influenced by the source of CO_2 used. It is likely that the responses of blood-fed malaria vectors was modulated by the carbohydrate source of CO_2 and not by the composition of the synthetic attractant blend used. However, a higher number of unfed and blood-fed *An. gambiae* s.l. and *An. funestus* responded to a combination of blend IB1 and CO_2 produced by fermenting molasses (Chapter 9) when compared to refined sugar (Mukabana et al., 2012a), which attracted especially unfed malaria vectors (Chapters 4 - 7). These results should be tested further and exploited for estimation of entomological inoculation rates as blood-fed malaria vectors are rarely caught with indoor or outdoor human landing collections (Le Goff et al., 1997, Mboera, 2005).

Although a few blood-fed malaria vectors were caught in experimental houses with a lit CDC trap + human volunteer sleeping under mosquito bed nets and no other potential hosts, it is possible that such mosquitoes were searching for resting sites after they had fed elsewhere (Chapter 6). Thus, the potential synergy between the Mbita blend and volatiles released by fermenting molasses may offer an alternative to the use of resting boxes, pyrethrum spray catches, clay pots and manual aspiration used for resting collections of unfed, blood-fed and gravid mosquitoes (Dia et al., 2005, Mboera, 2005, Bentley et al., 2009). The collection of blood-fed mosquitoes can also be used for studies of the infectious reservoir of malaria, as such mosquitoes may have fed on *Plasmodium*-infected hosts. It is likely that, additional volatiles produced by fermentation of molasses stimulate blood-fed females to respond to the odour-baited trap, which opens up new potential for malaria-epidemiological studies.

Efficacy and residual activity of attractant-treated nylon strips

This thesis demonstrates that the effectiveness and sustainability of applying odour-baited technology for sampling of insect populations is influenced by the choice of synthetic odour bait used (Chapters 6 ï 8) and slow-odour release devices (Chapters 3, 4, 5 and 9) (Torr et al., 1997, Okumu et al., 2010d, Heuskin et al., 2011). The higher catches of host-seeking *An. gambiae* mosquitoes achieved with IB1-treated nylon strips than LDPE sachets were unrelated to differences in surface area or sheet thickness of LDPE sachets filled with IB1 (Chapter 3). There was a need to reduce costs of odorants and nylon used, time and labour required to prepare fresh baits for area-wide sampling and control of malaria vectors. The IB1 -treated nylon remained consistently more attractive to female *An. gambiae* mosquitoes over 40 consecutive nights (Mukabana et al., 2012b) and at weekly intervals for 52 nights (Chapter 4) post-treatment than the attraction exerted by LDPE sachets containing the same blend. These were the first studies to demonstrate the residual activity of attractant-treated nylon strips to host-seeking mosquitoes in a semi-field setting. The concept of residual activity is commercially applied in the manufacture of long-lasting fabric materials impregnated with repellents or insecticides (Yates et al., 2005b, N'guessan et al., 2006a) used against biting flies and insect vectors of disease. Similar results were also observed for indoor-biting *An. gambiae* s.l. as well as outdoor- and indoor-biting *An. funestus* when traps baited with IB1-treated nylon strips were repeatedly deployed at weekly intervals for 52 nights post-treatment over one year (Chapter 5). Contrary to semi-field results (Chapters 3 and 4), both LDPE sachets and nylon strips were equally suitable for dispensing blend IB1 and collection of outdoor-biting *An. gambiae* s.l. (Chapter 5).

Potential underlying mechanisms of residual activity

The attractiveness of IB1-treated nylon to host-seeking mosquitoes was expected to diminish over time due to the volatile nature of the applied components. However, this was neither the case for *An. gambiae* under semi-field conditions (Chapters 3 and 4) nor for wild *An. gambiae* s.l. and *An. funestus* populations over one year post-treatment (Chapter 5). The findings of this thesis suggest that upon deployment, microbes colonised attractant-treated nylon strips and produced volatile compounds that may have interacted with odorants previously applied on the strips (Chapter 4) (Verhulst et al., 2009). This interaction seems to influence the suitability and long-term attractiveness of attractant-treated nylon strips to host-seeking mosquitoes. This is the first study to suggest the role of microbial interaction on long-term differential attraction of treated nylon strips to laboratory-reared and wild populations of host-seeking mosquitoes.

The crucial role of microbial activity in the attractiveness of human skin to host-seeking mosquitoes has recently been demonstrated (Verhulst et al., 2009, Verhulst et al., 2011a) and

explored for monitoring and disruption of malaria transmission by use of blends of chemicals present in microbial emanations (Verhulst et al., 2011b). Nevertheless, the role of microbial volatiles in differential attractiveness of treated nylon strips to host-seeking mosquito populations over time needs further investigation as this may lead to the discovery of novel attractants or repellents. Whereas 30-minute autoclaving of treated nylon strips prior to deployment improved trap collections of host-seeking *An. funestus*, this treatment had no effect on the responses of *An. gambiae* s.l., *Culex* and *Mansonia* species (Chapter 4). A follow -up of these results could account for the effect of storage on the residual activity of attractant -treated nylon and other odour-dispensing devices for mosquitoes between experimental nights and in the course of area-wide application. Consequently, a preliminary study (i.e. not part of this thesis) has also shown that storage of IB1-treated nylon in a freezer or in a trap under ambient conditions between experimental nights does not affect the attractiveness to *An. gambiae* mosquitoes when deployed at weekly intervals over a 12-week period (Mweresa et al., unpublished data). Thus, attractant-treated nylon strips can be left in a MM-X trap between experimental nights without necessarily storing them in a refrigerator or freezer as such facilities are often lacking in remote malaria-endemic regions.

Impact of textile substrates for dispensing synthetic mosquito attractants

Locally available and regularly used textile fabrics including polyester, cotton and cellulose + polyacrylate fibres embedded within the absorbent layer of sanitary pad materials, can provide a more efficient matrix for the release of synthetic attractant odorants of malaria vectors, *Culex* and *Mansonia* species compared to nylon (Chapter 9). The abundance and diversity of mosquitoes attracted to the IB1-baited traps was influenced by the type of odour-release material used over 25 consecutive nights without odour re-impregnation. It is of interest to evaluate the same odour-dispensing materials for the release of synthetic semiochemicals directed towards monitoring and disruption of mating, sugar-feeding and oviposition mosquitoes (Takken and Knols, 1999, Zwiebel and Takken, 2004) and other insect pests.

The cellulose + polyacrylate fibres within the sanitary pad material have the capacity to absorb and hold liquid substances, therefore, may be effectively used as a slow-release material for mosquito attractants (Chapter 9) as well as repelents. There is a need to explore whether a material, consisting of a super-absorbent polymer (Gheysens et al., unpublished data) can be made available with a larger surface area, to further exploit it for the release of kairomones and/or allomones. Both cotton and polyester materials are already available in different sizes and they are preferable for behavioural disruption of the host-seeking process of malaria vectors as they can be impregnated with mosquito repellents and used as ceiling materials, window or door curtains (Lines et al., 1987, Lengeler, 2004). Repellent-impregnated cotton clothing and nylon stockings could also be worn for personal protection

against outdoor transmission of vector-borne diseases including outbreaks of dengue transmitted by day-active *Ae. aegypti* (Pennetier et al., 2010). Polyester bed net material has also contributed substantially towards malaria reduction by providing a long-term protection against mosquito bites and subsequent mosquito-borne diseases when impregnated with insecticides (Yates et al., 2005, N'Guessan et al., 2006, Mendis et al., 2009). The textile materials were easy in use, locally available in different sizes and relatively cheap to be considered for area-wide application. Presently, the maximum duration of residual activity and release rates of odorant chemical compounds released from nylon and other odour-dispensing substrates (Chapter 9) remains unknown but would be worthwhile studying. Such measurements would allow for a direct comparison of the active aerial odorant concentration that host-seeking mosquitoes encounter.

Suggestions for future research

To achieve a better understanding of the possible impact of odour-based tools, further research is recommended on:

1. Attraction of malaria vectors in various physiological conditions: Although unfed *An. gambiae* were used in all semi-field experiments, addition of sugar-produced CO_2 to the IB1 blend increased trap collections of field populations of unfed *An. gambiae* s.l. and *An. funestus*. However, attraction of wild blood-fed *An. gambiae* s.l. (primarily *An. arabiensis*) to blend IB1 was increased by the use of CO_2 produced from molasses. Interestingly, addition of sugar-produced CO_2 to the Mbita blend augmented with 1-butylamine or 1-dodecanol enhanced trap collections of wild gravid *An. gambiae* s.l. The attractiveness of the Mbita blend to wild, blood-fed *An. gambiae* s.l. was also increased in the presence of CO_2 produced by fermentation of molasses compared to fermenting sugar. However, it remains to be confirmed whether similar results can be reproduced with *An. gambiae* under both semi-field and field conditions, and to establish whether attracted mosquitoes had fed on human or bovine (or other mammalian) blood meals. The chemical composition and influence of VOCs released from yeast-fermented molasses on mosquito responses should also be determined. The findings will underscore the significance of deploying odour-baited tools to reduce survival rates of newly emerged malaria vectors, sample blood-fed malaria vectors and provide novel synthetic odorants for the collection of gravid malaria vectors.

2. The role of microbes in residual activity of attractant-treated nylon strips to host-seeking malaria vectors: Preliminary findings reported in this thesis suggest that long-lasting attraction by attractant-treated nylon strips of *An. gambiae* and wild malaria vectors was potentially modulated by microbial interactions with attractants already applied on the strips. It will be interesting to investigate how microbes that colonise individual treated nylon strips interact with applied attractants and influence mosquito attraction over time. This may lead to

identification of novel repellent and attractant compounds for monitoring and disruption of malaria transmission in endemic regions.

3. The impact of complementing existing methods of sampling and control of malaria vectors with odour-based technology: This thesis demonstrates the prospect of deploying odour-baited traps to catch outdoor and indoor malaria vectors in numbers equal to mosquitoes attracted to a human host. Therefore, there is a need to determine the effect of applying LLINs, IRS and house screening in combination with odour-baited tools on the potential to disrupt malaria transmission. This may enhance monitoring and control of outdoor malaria transmission and prevention of house-entry or -exit of malaria vectors.

4. The effect of augmenting synthetic odour baits with light as a stimulus: It was clearly demonstrated that a combination of light + a human under a bed net offered the most attractive bait for sampling *An. gambiae* s.l. and *An. funestus*. Further knowledge on how interaction between light and synthetic odour stimuli influences the diversity and abundance of malaria vectors is required. This is important for developing a replacement for light + human-occupied bed combination and for outdoor sampling of such vectors.

5. Novel trapping devices: In order to be more effective in providing communal benefits, the traps used during an intervention should be cheap to deploy. The baits should also exceed the attractiveness of humans to capture the greatest possible proportion of mosquito populations, thus increasing the impact on anopheline biting rates and malaria transmission. Consequently, novel alternative trapping devices should be developed as the MM-X trap model used in this thesis is more convenient for monitoring than for intervention. Although the Mbita blend + 1-butylamine displayed a higher potential for attraction of malaria vectors than the Mbita blend alone, the relative level of attraction should be compared directly against human volunteers and the IB1 blend under natural conditions. It is also important to incorporate and evaluate the potential of such novel trapping devices and synthetic odour lures in a push-pull system and in different epidemiological settings.

Conclusion

The results presented in this thesis demonstrate the possibility of developing effective odour-release technologies with long-lasting residual activity for dispensing human-derived kairomones to monitor host-seeking vectors of malaria and other diseases. Addition of novel compounds to the Mbita blend and selection of fermenting molasses as an alternative carbohydrate source of CO_2 indicated the prospect of manipulating the concentration and composition of synthetic odour baits to monitor malaria vectors in different physiological conditions. The abundance and diversity of mosquitoes attracted to and collected in traps is

also influenced by the choice of the odour-release material used. Improved effectiveness of odour-based technology can accelerate the deployment of proxy hosts to replace human subjects in human landing catches or provide barriers that disrupt normal host-seeking behaviour and contact between vector-susceptible human hosts. The results provide great promise that odour-based technologies, based on the behavioural manipulation of malaria vectors, can be employed to improve monitoring in studies associated with the epidemiology of malaria and other vector-borne diseases, and can contribute to the reduction of malaria incidence.

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Summary

Malaria exerts a huge toll on human health and imposes a heavy socio-economic burden in developing countries, particularly in sub-Saharan Africa. Although major gains have been made in malaria control and elimination in the past decade, existing strategies are undermined by the emergence and spread of drug and insecticide resistance to malaria parasites and vectors, respectively. Also malaria vectors vary in terms of species diversity, breeding ecology, resting behaviour, feeding habits and vectorial capacities. The current change from indoor to outdoor biting behaviour and outdoor malaria transmission has resulted in different disease risks. These challenges suggest that novel strategies are required to complement existing methods for sampling, surveillance and control of indoor and outdoor biting malaria vectors.

Human body odours are the most important cues modulating host-seeking behaviour of malaria vectors. Identification and isolation of such odours can be exploited for the development of odour-baited mosquito traps or spatial repellents (**Chapter 2**). However, the robustness and success of applying odour-baited technologies in malaria-endemic areas can be enhanced by searching for a more potent synthetic odour bait to malaria vectors and which can also attract multiple malaria vectors in various abdominal conditions. In addition, more effective and sustainable odour-release systems, efficient trapping devices that rely on cheap or renewable sources of energy and alternative sources of carbon dioxide are needed. The present PhD research project was aimed at providing thorough knowledge on the practical applications of semiochemicals for the manipulation of haematophagous insect vectors. The residual activity of attractant-dispensing systems and alternative strategies for enhancing the effectiveness of odour-baited technologies for surveillance and disruption of African malaria transmission in western Kenya were also evaluated. Laboratory-based experiments were carried out at Wageningen University and Research Centre in The Netherlands while semi-field and field studies were conducted in western Kenya.

In **Chapter 3,** the suitability of low density polyethylene (LDPE) sachets and nylon strips for dispensing synthetic attractants of host-seeking *Anopheles gambiae sensu stricto* (henceforth termed *An. gambiae*) mosquitoes was evaluated under semi-field conditions. Nylon strips performed continuously better than LDPE satchets in dispensing synthetic mosquito attractants namely Ifakara blend 1(IB1) for 40 consecutive nights after treatment **(Chapter 3)**. Consequently, the long-lasting residual activity of IB1-treated nylon strips to host-seeking *An. gambiae* was investigated at weekly intervals over one year post-treatment period in a semi-field enclosure (**Chapter 4**). Each attractant chemical was released from nylon strips and LDPE sachets. The role of microbes colonizing the nylon strips in modulating the emission of the attractive chemicals initially impregnated and used over one year as mosquito trap lures was also tested. Significantly and consistently higher proportions of mosquitoes were

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attracted to IB1-treated nylon strips than other treatments. After one year of intermittent exposure, analysis of headspace volatiles of the attractant-treated nylon strips by gas chromatography and mass spectrometry detection (GC/MS) resulted in identification of additional volatile organic compounds (VOCs). Plating of IB1-treated nylon strips on trypticase soy agar resulted in the growth of various bacterial populations. Unlike natural populations of *An. funestus* and other anopheline mosquitoes, the responses of host-seeking *An. gambiae* s.l. and *Mansonia* spp. to baited traps were not influenced by autoclaving IB1-treated strips prior to the start of experiments (**Chapter 4**).

The long-lasting attraction of malaria vectors to synthetic odour baits demonstrated in **Chapter 4** was confirmed under ambient climatic conditions in a rice agro-ecosystem village **(Chapter 4 - 9).** The study investigated whether IB1-treated nylon strips and LDPE sachets used at weekly intervals can remain attractive to indoor and outdoor host-seeking malaria vectors up to one year after treatment (**Chapter 5**). IB1-treated nylon strips and LDPE sachets were consistently attractive to indoor and outdoor biting *An. gambiae* s.l. and *An. funestus* malaria vectors up to one year post-treatment. Whereas IB1 dispensed from both nylon strips and LDPE sachets had no effect on outdoor trap catches of *An. gambiae* s.l., the latter was significantly more attractive to *An. funestus*. When collecting indoors, both humans and IB1-treated nylon strips attracted similar catches of *An. gambiae* s.l. and each of them was more attractive compared to IB1 dispensed from LDPE sachets. Humans lured significantly more *An. funestus* into houses compared to IB1 released from nylon strips or LDPE sachets. There was a higher response of indoor-biting *An. funestus* to IB1 dispensed from nylon than LDPE sachets. Only one sub-sample of female *An. funestus* collected indoors was confirmed to have *Plasmodium falciparum* sporozoites. *Anopheles gambiae* s.l. comprised *An. gambiae s.s.* (2.1%) and *An. arabiensis* (97.9%) (**Chapter 5**).

Based on ample evidence of the long-lasting residual activity of IB1-treated nylon strips and their potential to attract high numbers of mosquitoes (**Chapter 5**), field experiments were carried out to evaluate whether a synthetic odour bait can be considered as a standard tool for routine sampling of malaria vectors indoors (**Chapter 6**). The attractiveness of a house without any bait, with a synthetic odour blend (i.e. IB1 blend), light, a human, or a human + light for malaria and other mosquitoes was assessed in five village houses. The responses of indoor-biting *An. gambiae* s.l. to a human and IB1-treated nylon strips were similar, but light alone and a human + light were more attractive than IB1 or a human without light. Attraction of indoor- biting *An. funestus* mosquitoes to IB1-treated nylon strips or light were not different. A human + light attracted the highest mean numbers of female *An. gambiae* s.l., and *An. funestus* compared to all other treatments (**Chapter 6**). Although trapped mosquitoes were predominantly unfed females, no blood-fed *An. gambiae* s.l. and *An. funestus* were trapped in houses occupied by a human alone (**Chapters 5 and 6**). *Anopheles funestus* fed more frequently on humans than bovines while *An. gambiae* s.l. displayed a higher feeding

preference for bovines than humans (**Chapters 5 and 6**). As a result, *An. funestus* seems to be the most important local malaria vector.

The findings presented in **Chapters 4 - 6** indicated that, although blend IB1 was an attractive stimulus for a wide range of natural populations of unfed mosquitoes, there is an opportunity to search for novel synthetic odour baits that are highly potent. Such odour baits should be dispensed in a more effective system, thereby providing a robust and reliable tool for sampling and control of mosquitoes in different physiological conditions (**Chapters 7 - 9**). Recently, a synthetic mixture of mosquito attractants referred to as ñthe Mbita blend,ò was shown to attract as many host-seeking malaria mosquitoes as were attracted to human subjects and it was more attractive than the IB1 blend. Thus, the capacity of the Mbita blend to attract more host-seeking malaria mosquitoes was tested under semi-field and field conditions by adding selected dilutions of butyl-2-methylbutanoate, 2-pentadecanone, 1-dodecanol and 1-butylamine. There was a significant increase in the proportion of *An. gambiae* caught in traps containing the Mbita blend augmented with certain dilutions of 1-butylamine, pentadecanone and 1-dodecanol in the semi-field facility. When tested in the village, addition of 1-butylamine to the Mbita blend enhanced indoor collections of female *An. gambiae* s.l, *An. funestus* and *Culex* mosquitoes. One-dodecanol increased attraction of *An. gambiae* s.l. to the Mbita blend while addition of 2-pentadecanone improved trap catches of *An. funestus* and *Culex* mosquitoes. The majority of female *An. gambiae* s.l. caught was either unfed or gravid as *An. funestus* were predominantly unfed (**Chapter 7**).

In **Chapter 8**, the possibility of replacing refined cane sugar (sucrose) with locally available molasses as a substrate for producing CO_2 to lure malaria vectors to traps was explored under semi-field and field conditions**.** The release rate of CO_2 and proportion of *An. gambiae* mosquitoes attracted increased in tandem with an increase in the quantity of yeast-fermented molasses up to an optimal ratio of molasses and dry yeast. The attraction of *An. gambiae* mosquitoes to CO_2 produced by fermenting 250 g and 500 g of molasses was significantly enhanced by addition of a synthetic mosquito attractant called Mbita blend (comprised ammonia, L-lactic acid, tetradecanoic acid and 3-methyl-1-butanol). Significantly more unfed and blood-fed *An. gambiae* s.l. mosquitoes responded to the Mbita blend augmented with CO_2 produced by fermenting 500 g of molasses compared to 250 g of sucrose or 250 g of molasses. Similarly, significantly more *An. funestus, Culex* and other anopheline mosquito species were attracted to the Mbita blend augmented with CO_2 produced from fermenting molasses than the Mbita blend with CO_2 produced from sucrose. Augmenting the Mbita blend with CO_2 produced from molasses was associated with high catches of blood-fed *An. gambiae* s.l. and *An. funestus* mosquitoes (**Chapter 8**).

The studies described in **Chapters 6 - 8** suggested a need to investigate whether locally available and regularly used textile fabrics can provide similar or more efficient release

matrices for synthetic odorant cues of outdoor malaria vectors compared to nylon (**Chapter 9**). Traps charged with IB1-impregnated polyester, cotton and cellulose + polyacrylate materials caught significantly more *Anopheles gambiae sensu stricto* mosquitoes than IB1-treated nylon under semi-field conditions. There was a significant increase in the responses of female *An. gambiae* s.l. to IB1-treated polyester, cotton and cellulose + polyacrylate materials compared to nylon ($P < 0.001$). Whereas IB1-impregnated cellulose + polyacrylate textile was the most attractive to female *An. funestus* mosquitoes compared to all other dispensing substrates, IB1-treated cotton was the most attractive to *Culex* mosquitoes than other substrates (**Chapter 9**).

The main points from this thesis can be concluded as follows**:** attractant-treated nylon strips sustain the attractiveness to host-seeking malaria vectors over a one year post-treatment period when deployed at weekly intervals under semi-field and field conditions. It appears that bacterial action is responsible for the production of VOCs involved in the prolonged attractiveness of treated nylon strips to mosquitoes over time, and therefore, frequent re-treatment of strips is not necessary. The residual activity of IB1-treated nylon strips to *An. gambiae* s.l. was similar to that of human emanations *in vivo* and can be used to replace a human in mosquito sampling devices such as the CDC light trap + bed net combination. Visual and olfactory cues are both useful in modulating mosquito host-seeking behaviour, and interaction between the two cues can provide a more robust and reliable tool for sampling malaria and other mosquito vectors. Improved synthetic mosquito attractant blends may provide efficient trap lures for sampling, surveillance and control of multiple indoor and outdoor host-seeking malaria vectors in unfed, blood-fed and gravid conditions. Besides nylon, locally available and regularly used textile fabrics can also serve as efficient and sustainable substrates for dispensing synthetic mosquito attractant kairomones. Thus, odour-based technologies directed towards surveillance, sampling and control of disease vectors can be improved by taking into account the type of release substrate employed. Although the possibilities of enhancing the capacity of odour-based technology to collect malaria and other mosquito vectors were explored in this thesis, appropriate trap designs should also be sought. Once such novel tools are available, the possibility of area-wide application could be considered in diverse epidemiological situations. This would reduce the number of malaria mosquitoes, the human biting frequency, and the intensity of *Plasmodium* transmission. As a result, the morbidity, mortality and socio-economic losses imposed by malaria and other mosquito-borne diseases on human would be minimized.

Samenvatting

Samenvatting

Malaria vergt een enorme tol van de volksgezondheid en legt een zware socio-economische last op ontwikkelingslanden, vooral in tropisch Afrika. Hoewel er grote vooruitgang is geboekt in de bestrijding en eliminatie van malaria in de afgelopen tien jaar, worden bestaande strategieën ondermijnd door de opkomst en verspreiding van resistentie van malariaparasieten tegen medicijnen en van vectoren tegen insecticiden. Daarnaast verschillen malariavectoren op het gebied van soortendiversiteit, voortplantingsecologie, rustgedrag, voedingsgewoonten en vectoriële capaciteit en reageren daarom niet allemaal hetzelfde op vergelijkbare bestrijdingsmethoden. De huidige transitie van endofiel naar exofiel voedingsgedrag en malariatransmissie buitenshuis heeft geresulteerd in gewijzigde ziektepatronen. Deze uitdagingen laten zien dat nieuwe strategieën nodig zijn om bestaande methoden voor bemonstering, monitoring en controle van binnenshuis- en buitenshuis bijtende malariavectoren aan te vullen.

Humane lichaamsgeuren zijn de belangrijkste signaalstoffen die het gastheerzoekgedrag van malaria vectoren reguleren. De identificatie en isolatie van dergelijke geuren kan worden benut voor de ontwikkeling van geurvallen of insectenwerende middelen met een ruimtelijk effect (Hoofdstuk 2). Echter, de robuustheid en het succes van de toepassing van technologieën die gebaseerd zijn op een lokstof in gebieden waar malaria endemisch is, kan worden verbeterd door te zoeken naar meer potente synthetische lokstoffen voor malaria vectoren die meerdere malariavectoren aantrekken in een gevarieerde fysiologische conditie. Daarnaast zijn nodig: effectievere en duurzame geur-afgiftesystemen, efficiënte vangstmechanismen die afhankelijk zijn van goedkope of hernieuwbare energiebronnen en alternatieve bronnen van koolzuur (CO_2). Het huidige promotieonderzoek was gericht op het verschaffen van grondige kennis over de praktische toepassingen van gedragsbeïnvloedende geurstoffen voor het manipuleren van haematofage insecten. Ook geëvalueerd werden: de residuele activiteit van afgiftesystemen van lokstoffen en alternatieve strategieën om de effectiviteit van lokstof technologieën voor monitoring en verstoring van de Afrikaanse malaria transmissie in het westen van Kenia te verbeteren. Laboratoriumexperimenten werden uitgevoerd aan het Wageningen University en Research Centrum in Nederland terwijl semi-veld en veldstudies werden uitgevoerd bij het International Centre for Insect Physiology and Ecology in het westen van Kenia.

In hoofdstuk 3 wordt, onder semi-veld omstandigheden, de geschiktheid van zogenaamde ólow density polyethyleenô (LDPE) materiaal en nylon strips geëvalueerd, voor het afgeven van synthetische lokstoffen voor gastheerzoekende *Anopheles gambiae sensu stricto* (hierna *An. gambiae*) muggen. Nylon strips presteerden continu beter dan LDPE in de verstrekking van de synthetische muggenlokstof Ifakara mix 1 (IB1) gedurende 40 opeenvolgende nachten na de behandeling (Hoofdstuk 3).

Vervolgens werd de langdurige residuele activiteit van met IB1 behandelde nylon strips op gastheerzoekende *An. gambiae* onderzocht in een semi-veld opzet, met tussenpozen van een week voor een periode van meer dan een jaar na de behandeling van de strips (Hoofdstuk 4). Elke chemische lokstof werd verspreid vanaf nylon strips en LDPE materiaal. De rol van micro-oranismen, die de nylon strips koloniseren, in het reguleren van de emissie van de aantrekkelijke chemicaliën die aanvankelijk geïmpregneerd en meer dan een jaar gebruikt waren als muggenval-lokstoffen, werd ook onderzocht. Een significant en consequent hoger aandeel van de muggen werd aangetrokken door met IB1 behandelde nylon strips in vergelijking met andere behandelingen. Na een jaar van alternerende blootstelling resulteerde een analyse van opgevangen geurstoffen van de nylon strips, door middel van gaschromatografie en massaspectrometrie-detectie (GC/MS), in de identificatie van bijkomende vluchtige organische stoffen (VOS). Het uitplaten van met IB1 behandelde nylon strips op trypticase soja-agar resulteerde in de groei van verschillende bacterie populaties. In tegenstelling tot de natuurlijke populaties van *An. funestus* en andere *Anopheles* soorten, werden de reacties van de gastheerzoekende *An. gambiae* s.l., en *Mansonia* spp. op met lokstof uitgeruste vallen, niet beïnvloed door het autoclaveren van met IB1 behandelde stroken voor aanvang van de experimenten (Hoofdstuk 4).

De langdurige aantrekkingskracht van synthetische geur-lokstoffen op malaria vectoren welke was aangetoond in hoofdstuk 4 werd bevestigd onder klimatologische omstandigheden die gelijk waren aan de omgeving in een rijst agro-ecosysteem dorp (Hoofdstukken 4-9). Tijdens deze studie werd onderzocht of nylon strips behandeld met IB1 en LDPE welke wekelijks gebruikt werden, aantrekkelijk bleven voor binnenshuis en buitenshuis gastheerzoekende malaria vectoren tot een jaar na de behandeling (Hoofdstuk 5). Met IB1 behandelde nylon strips en LDPE materiaal bleken langdurig aantrekkelijk voor binnen- en buitenbijtende *An. gambiae* s.l. en *An. funestus* tot een jaar na de behandeling. Terwijl de afgifte van IB1 vanaf zowel nylon strips als LDPE materiaal geen effect had op de buitenvangsten van *An. gambiae* s.l., was LDPE beduidend aantrekkelijker voor *An. funestus*. Wanneer er binnenshuis gevangen werd, werd er zowel door mensen als door met IB1 behandelde nylon strips vergelijkbare aantallen *An. gambiae* s.l. aangetrokken en elk van hen was aantrekkelijker dan IB1 afgegeven vanuit LDPE materiaal. Mensen lokten aanzienlijk meer *An. funestus* de huizen binnen dan IB1 verspreid vanaf nylon strips of LDPE materiaal. Er was een hogere respons van binnenshuis bijtende *An. funestus* op IB1 afgegeven door nylon strips dan van LDPE materiaal. Slechts één proefmonster van vrouwelijke *An. funestus* die binnenshuis verzameld waren bleek *Plasmodium falciparum* sporozoïeten te bevatten. De populatie van *Anopheles gambiae* s.l. bestond uit *An. gambiae s.s.* (2.1 %) en *An. arabiensis* (97,9%) (Hoofdstuk 5).

Gebaseerd op het bewijs van de langdurige restactiviteit van met IB1 behandelde nylon strips en hun vermogen om grote aantallen muggen (Hoofdstuk 5) aan te trekken werden

veldproeven uitgevoerd om te evalueren of een synthetisch geur-lokaas kan worden beschouwd als een standaard instrument voor routinematige bemonstering van malaria vectoren binnenshuis (Hoofdstuk 6). De aantrekkelijkheid voor malaria- en andere soorten steekmuggen van huizen zonder geur-lokaas, met een synthetische geur-lokstof (IB1), verlichting, een mens, of een mens + verlichting werd beoordeeld in vijf huizen. De reacties van binnenshuis bijtende *An. gambiae* s.l. op een mens en met IB1 behandelde nylon strips waren vergelijkbaar, maar verlichting alleen en een mens + verlichting waren meer aantrekkelijk dan IB1 of een mens alleen zonder verlichting. De aantrekking van binnenshuis bijtende *An. funestus* muggen tot met IB1 behandelde nylon strips of verlichting was niet verschillend. Een mens + verlichting trok het hoogste gemiddelde aantal vrouwelijke *An. gambiae* s.l. en *An. funestus* aan in vergelijking met alle andere behandelingen (Hoofdstuk 6). Hoewel de gevangen muggen overwegend niet-bloed-gevoede vrouwtjes waren, werden er geen bloed-gevoede *An. gambiae* s.l. en *An. funestus* gevangen in huizen met een mens alleen (Hoofdstukken 5 en 6). *Anopheles funestus* voedde zich vaker met mensen dan met runderen terwijl *An. gambiae* s.l. een hogere voedingsvoorkeur toonde voor runderen dan voor mensen (Hoofdstukken 5 en 6). Hierdoor lijkt *An. funestus* de belangrijkste lokale malariavector te zijn.

De bevindingen die in de hoofdstukken 4-6 gepresenteerd werden geven aan dat, hoewel IB1 een aantrekkelijke stimulus is voor een breed scala van natuurlijke populaties van niet-bloed-gevoede muggen, er een gelegenheid is om te zoeken naar nieuwe synthetische geur-lokazen die zeer krachtig zijn. Dergelijke geur-lokazen moeten worden verspreid met een doeltreffender systeem, waardoor een robuust en betrouwbaar hulpmiddel voor de bemonstering en controle van muggen in verschillende fysiologische omstandigheden geleverd kan worden (Hoofdstukken 7-9). Onlangs werd aangetoond dat een synthetisch mengsel aangeduid als de "Mbita blend" evenveel gastheerzoekende malariamuggen aantrekt als menselijke proefpersonen en aantrekkelijker was dan het IB1 mengsel. Daarom werd de capaciteit van de Mbita blend om meer gastheerzoekende malariamuggen aan te trekken getest onder semi-veld en veldomstandigheden door het toevoegen van verdunningen van butyl-2-methylbutanoaat, 2-pentadecanone, 1-dodecanol en 1-butylamine. Er was een significante toename in de proportie *An. gambiae* die gevangen werd in vallen met de Mbita blend aangevuld met bepaalde verdunningen van 1-butylamine, pentadecanone en 1-dodecanol in de semi-veld faciliteit. Bij testen in het dorp verbeterde de toevoeging van 1-butylamine aan de Mbita blend binnen-vangsten van vrouwelijke *An. gambiae* s.l., *An. funestus* en *Culex* muggen. 1-dodecanol verhoogde de aantrekkelijkheid van de Mbita blend voor *An. gambiae* s.l. terwijl de toevoeging van 2-pentadecanone de vangsten van *An. funestus* en *Culex* muggen verbeterde. De meeste vrouwelijke *An. gambiae* s.l. die gevangen werden waren niet-bloedgevoed of met eireren terwijl *An. funestus* overwegend niet-bloedgevoed waren (Hoofdstuk 7).

In hoofdstuk 8 werd de mogelijkheid om geraffineerde rietsuiker (sacharose) te vervangen met lokaal beschikbare melasse als substraat voor de productie van CO_2 om malariavectoren te lokken onderzocht onder semi-veld en veldomstandigheden. De afgiftesnelheid van CO_2 en de proportie van *An. gambiae* muggen die werden aangetrokken nam gelijkmatig toe met de verhoging van de hoeveelheid gefermenteerde melasse tot een optimale verhouding van melasse en droge gist. De aantrekking van *An. gambiae* muggen door CO_2 geproduceerd door fermentatie van 250 g en 500 g melasse werd aanzienlijk verbeterd door toevoeging van een synthetische mug lokstof genoemd de Mbita blend (bestaande uit ammoniak, L-lactic acid, tetradecanoic acid en 3-methyl-1-butanol). Significant meer niet-gevoede en bloed-gevoede *An. gambiae s.l.* muggen reageerden op de Mbita blend aangevuld met CO_2 geproduceerd door het vergisten van 500g melasse in vergelijking met 250g sucrose of 250g melasse. Op dezelfde manier werden significant meer *An. funestus*, *Culex* en andere *Anopheles* muggensoorten aangetrokken tot de Mbita blend aangevuld met CO_2 geproduceerd door het vergisten van melasse dan de Mbita blend in combinatie met CO_2 geproduceerd uit sucrose. Het verbeteren van de Mbita blend met CO2 geproduceerd uit melasse werd geassocieerd met hoge vangsten van bloed-gevoede *An. gambiae* s.l. en *An. funestus* muggen (Hoofdstuk 8).

De studies die beschreven worden in de hoofdstukken 6-8 wijzen op de noodzaak om te onderzoeken of lokaal beschikbare en regelmatig gebruikte weefsels soortgelijke of efficiëntere afgiftematrices kunnen bieden voor synthetische geur-lokstoffen voor malariavectoren buiten, in vergelijking met nylon (Hoofdstuk 9). Muggenvallen die uitgerust waren met met IB1 geïmpregneerd polyester, katoen en cellulose + poly-acrylaat materialen vingen aanzienlijk meer *Anopheles gambiae sensu stricto* muggen dan met IB1 behandeld nylon onder semi-veld omstandigheden. Er was een significante toename in de respons van vrouwelijke *An. gambiae* s.l. op met IB1 behandeld polyester, katoen en cellulose + poly-acrylaat materialen ten opzichte van nylon ($P < 0.001$). Terwijl met IB1 geïmpregneerd cellulose + poly-acrylaat textiel het meest aantrekkelijk was voor vrouwelijke *An. funestus* muggen vergeleken met alle andere afgifte-substraten, was met IB1 behandeld katoen het meest aantrekkelijk voor *Culex* muggen in vergelijking met andere substraten (Hoofdstuk 9).

De belangrijkste conclusies die uit dit proefschrift kunnen worden getrokken: met lokstof behandelde nylon strips behouden hun aantrekkelijkheid voor gastheerzoekende malariavectoren voor meer dan een jaar na de behandeling wanneer deze worden ingezet met wekelijkse intervallen onder semi-veld en veldomstandigheden. Het blijkt dat bacteriële activiteit verantwoordelijk is voor de productie van VOS die betrokken zijn bij de langdurige aantrekkelijkheid van behandelde nylon strips voor muggen en daarom een frequente herbehandeling van de strips niet noodzakelijk is. De residuele activiteit van met IB1 behandelde nylon strips op *An. gambiae* s.l. was vergelijkbaar met die van humane geurstoffen *in vivo* en kan gebruikt worden om een mens te vervangen in muggenvallen zoals de CDC lichtval + klamboe combinatie.

Zowel visuele en olfactorische signalen zijn nuttig bij het manipuleren van gastheerzoekgedrag van muggen en de interactie tussen de twee signalen kan een meer robuust en betrouwbaar gereedschap bieden voor de bemonstering van malariamuggen en andere vectoren bieden. Verbeterde synthetische mengsels van lokstoffen kunnen efficiënte lokstoffen bieden voor de bemonstering, surveillance en controle van meerdere binnen- en buiten-gastheerzoekende malariavectoren in niet-bloedgevoede, bloedgevoede en ei-dragende condities. Naast nylon kunnen lokaal beschikbare en regelmatig gebruikte weefsels ook dienen als efficiënte en duurzame substraten voor het afgeven van synthetische muggen-lokstoffen. Aldus kunnen op geur gebaseerde technologieën die gericht zijn op de bemonstering, surveillance en controle van de ziektevectoren verbeterd worden door rekening te houden met het type substraat dat gebruikt wordt. Hoewel in deze thesis de mogelijkheden om de capaciteit te verbeteren van op geur gebaseerde technologieën om malariamuggen en andere vectoren te verzamelen werden onderzocht, dienen geschikte ontwerpen van muggenvallen ook onderzocht te worden. Wanneer dergelijke nieuwe instrumenten beschikbaar zijn, kan de mogelijkheid van grootschalige toepassing in diverse epidemiologische situaties overwogen worden. Dit zou het aantal malariamuggen, de frequentie waarmee mensen gebeten worden en de intensiteit van *Plasmodium* verminderen. Als gevolg daarvan zouden de morbiditeit, mortaliteit en sociaal-economische verliezen veroorzaakt door malaria en andere door muggen overgebrachte ziekten op de mens geminimaliseerd worden.

Acknowledgements

The successful completion of this PhD study was characterized by challenging and happy moments. I am glad to note that many players contributed differently to the results presented in this thesis. I will not mention names of all people and institutions that were involved but I will acknowledge their significant support that led to the successful completion of the study. Special thanks are accredited to the Foundation for the National Institutes of Health (FNIH) through the Grand Challenges in Global Health Initiative (GCGH) and a sandwich PhD scholarship of Wageningen University and Research Centre in The Netherlands for funding this study. The funding enabled me to conduct my research activities at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (*icipe*-TOC) in Mbita Point, western Kenya. I am greatly indebted to my doctoral supervisors Prof. Willem Takken, Prof. Marcel Dicke, Prof. Wolfgang R. Mukabana and Prof. Joop J.A. van Loon. This PhD thesis is a brainchild of your excellent professional guidance and supervision through a result-based approach, strict adherence to time plan, brainstorming sessions, firm but friendly and prompt responses to all aspects of my studies. You played a parental role and ensured that I was socially-adjusted while at Wageningen University and the *icipe*-TOC. You have made a mark in my life and you remain my role-models forever. I look forward to continued scientific collaborations with you in the future. I thank Margriet Huisman, Angelique Bosch and Marion Rodenburg for their administrative support in terms of travel, registration, and accommodation while I was in The Netherlands.

I commend Gabriella Bukovinszkiné-Kiss, Hans Smid, Tobias Hosman, Susan Imbahale, Samuel Guto, Marren Radeny, Leah Ombis, Sammy Latema, David A. Mulama, Teun Bousema, Elie A. Dannon, Berhane T. Weldegergis, Fred. W. de Boer, Ruijven Jasper van, Ties Huigens, Tullu Bukhari, Renate C. Smallegange, Jeroen Spitzen, Niels O. Verhulst, Erik Poelman and Sander C.J. Koenraadt for their kindness, useful advices, discussions and encouragements. Although I have not mentioned everyone by name, I thank all members at the Laboratory of Entomology in The Netherlands. The good moments and discussions shared during coffee breaks, colloquia, parties, vector group and PhD student meetings were extremely important as they cultivated a sense of belonging, team spirit and cultural integration. I acknowledge Dr. Claudius van de Vijver of the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE & RC) for promoting team work among PhD students from different chair groups through organization of PE & RC weekends, meetings, competence strengthening and skills courses. Alex Hiscox and David Menger are acknowledged greatly for their role as paranymphs during my PhD thesis defense while Sopher Ondiaka was very helpful in designing the final layout of this thesis. I appreciate the home welcoming and dinner parties provided by Françoise Kaminker and family as well as other staff of the Laboratory of Entomology. Thanks a lot to the Kenyan and East African

community at Wageningen University for the monthly parties. The shared sessions with many friends including Ben Okita, Fred Maati, Raphael Mkhize, George Agogo, Geoffrey Ogutu, Hellen Kimanthi, Yussuf Wato**,** Jackline, Lagat, Buddhi Achamii, Prisca Oria, Joel Opondo and church services conducted by Pastor Farai Maphosa provided wonderful moments of my life in The Netherlands. I always felt at home whenever we met.

My internship on the Disruption of Malaria Transmission (DMT) project at the *icipe*-TOC, opened an additional chapter of colleagues and friends. I enjoyed working with Bruno Otieno, Philemon Omusula, Monica M. Mirai, Brian A. Abuya and Charles Oketch. The team was open-minded, flexible, and very objective. I thank David Alila and Elisha Obudho for rearing the mosquitoes used in our semi-field experiments while Marjolein de Rijk, Frans Jacobs, Juma Wanyama and Dr. Tom Gheysens are acknowledged for analysis of samples. The success of our field studies at Kigoche Village is attributed to the commitment of Philemon and Charles, the hospitality of Kigoche residents as well as Fred Kisanya (Principal at the Ahero Multipurpose Development Training Institute (AMDTI)). Faith Kyengo is acknowledged for providing logistic support and Benedict Orindi for data analysis. I am very grateful to Francis Onyango, Patrick Sawa, Fred Orwa, Peter Nyongesa, Susan Akinyi, Mary Etuku, Mustafa Juma, Jakton Arija and all other staffs at the *icipe*-TOC, for providing a suitable environment for my stay and semi-field experiments. The interactive moments shared during Integrated Vector Disease Meetings (IVDM) were quite valuable as they offered a platform for fine-tuning my work. Oscar, George, Magara, Pauline, Kidiavai, Mutiambai, Moseti, Kiche, Mendi, Roselyn, Omweri, the entire SolarMal and AFIRM team members are acknowledged for their moral support.

My sincere gratitude is directed to my daughters Lyn, Ella, Rachael and Finney including my wife Pamella and her niece Babra for enduring long moments of my absence while I was in The Netherlands. Your regular calls, encouragements and consistent prayers provided enough proof of your concern for me. Additional communication from my brothers (Benson, Nickson and Benjamin) and sisters (Carol, Joyce, Russel and Flo) re-kindled my sense of belonging to a family unit. I am greatly indebted to my parents Mr. and Mrs. Nelson Kegode Mweresa and grandparents (Mr. and Mrs. Jeffer Mweresa) for the endless love, care, encouragement and guidance extended to me and my family. My academic successes are attributed to your inspiration and strictness. You maintained that nothing comes easily and that one must train hard to win easily. May this PhD thesis provide a source of inspiration to all of you and future generations. Finally, I thank God for the gift of good health. Dank Jullie wel!

Curriculum Vitae

Collins Kalwale Mweresa was born on 28 January 1970 in Kakamega County, western Kenya. He completed his secondary school education in 1988 at Chavakali boysô high School in Vihiga County. In 1989 he attended a pre-university military training course at Gilgil prior to joining Egerton University in Nakuru County. His studies at Egerton University resulted in the award of a Bachelor of Science degree (zoology, botany and geography) in 1993 and a Post Graduate Diploma in Education in1998. He was employed by the Teachers Service Commission (TSC) of Kenya as a high school teacher in 1994. In 2006, He acquired a study leave from the TSC to pursue a Masters of Science degree (Applied Parasitology) at the University of Nairobi until 2009. The subject of his MSc thesis was entitled: ñAbundance and control of malaria mosquito larvae in the traditional water management agro-ecosystem of Kasagam, western Kenyaò. The study was conducted at the Kenya Medical Research Institute (KEMRI) in Kisumu County and was financially supported by the Diorapthe Foundation through Wageningen University in The Netherlands. He quit the TSC in September 2008 and worked as an entomology research assistant for the REDHOT (Reducing malaria burden by targeting HOTspots of malaria transmission) and MALACTRES (Multi-drug resistance in malaria under combination therapy) projects until March 2010. Both projects were coordinated from the Moshi-based Kilimanjaro Christian Medical Centre (KCMC) in Tanzania. He obtained a sandwich PhD scholarship provided by the Wageningen University in The Netherlands in 2010. He was hosted at the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology (TOC-*icipe*) at Mbita Point, Homa Bay County in western Kenya as a dissertation research internship scholar on a Disruption of Malaria Transmission (DMT) project. The internship was supported by a grant from the Foundation for the National Institutes of Health (FNIH) through the Grand Challenges in Global Health initiative (GCGH #121). His PhD research was centred on manipulation of odour-based strategies for surveillance and disruption of host-seeking malaria and other mosquitoes of which the results are presented in this thesis. After defending his PhD dissertation, Collins will continue to work for the SolarMal Project at the TOC-*icipe* in Mbita Point. He is also involved in research activities associated with the determination of the human and mosquito infectious reservoir for malaria in western Kenya.

List of Publications

Imbahale, S., **C.K. Mweresa,** W. Takken and W.R. Mukabana. 2011. Development of environmental tools for anopheline larval control. Parasites and Vectors, 4:130.

Hiscox, A., N. Maire, I. Kiche, M. Silkey, T. Homan, P. Oria, **C.K. Mweresa,** B. Otieno, M. Ayugi, T. Bousema, P. Sawa, J. Alaii, T. Smith, C. Leeuwis, W.R. Mukabana and Takken. W. 2012. The SolarMal project: innovative mosquito trapping technology for malaria control. Malaria Journal, 11:45.

Mukabana, W.R., **C.K. Mweresa,** B. Otieno, P. Omusula, R.C.Smallegange, J.J.A. van Loon and W. Takken. 2012. A novel synthetic odorant blend for trapping of malaria and other African mosquito species. Journal of Chemical Ecology, 38:235-244.

Mukabana, W.R., **C.K. Mweresa**, B. Otieno, P. Omusula, B. Orindi, R.C. Smallegange, J.J.A. van Loon and W. Takken. 2012. Evaluation of low density polyethylene and nylon for delivery of synthetic mosquito attractants. Parasites and Vectors, 50:123-133.

Sawa P, S.A. Shekalagh, C.J. Drakeley .2013.C.J. Sutherland, **C.K. Mweresa**, A.Y. Baidjoe, A. Manjurano, R.A. Kavishe, K.B. Beshir, R.U. Yussuf, S.A. Omar , C.C. Hermsen, L. Okell, H.D. Schallig, R.W. Sauerwein, R.L. Hallett and T. Bousema. Malaria transmission after artemether-lumefantrine and dihydroartemisinin-piperaquine: a randomized trial. Journal of Infectious Diseases**,** 207(11):1637-45.

Beshir, K.B., C.J. Sutherland, P. Sawa, C.J. Drakeley, **C.K. Mweresa,** S.A. Omar, S.A. Shekalaghe, H.A. Kaur, A. Ndaro, J. Chilongola, H.D.F.H. Schallig, R. W. Sauerwein, R.L. Hallett and T. Bousema. 2013. Residual *Plasmodium falciparum* parasitemia in Kenyan children after Artemisinin-Combination Therapy is associated with increased transmission to mosquitoes and parasite recurrence. Journal of Infectious diseases, 208 (12):2017-2024.

Mweresa, C.K., P. Omusula, B. Otieno, J.J.A. van Loon, W. Takken and W.R. Mukabana. 2014. Molasses as a source of carbon dioxide for the malaria mosquito *Anopheles gambiae* and *An. funestus.* Malaria Journal, 13:160.

Mweresa, C.K., W.R. Mukabana, P. Omusula, B. Otieno, W. Takken and J.J.A. van Loon, J.J.A. Textile substrates for dispensing synthetic mosquito attractants. **Accepted by the Journal of Parasites and Vectors in April 2014.**

In preparation

Mweresa, C.K., van Loon, J.J.A., Mukabana, W.R., Dicke, M. and Takken, W. Potential application of semiochemicals for monitoring and manipulation of haematophagous insects: a review.

Mweresa, C.K., B. Otieno, P. Omusula, B.T. Weldegergis, N.O. Verhulst, M. Dicke, J.J.A. van Loon, W. Takken and W.R. Mukabana. Long-lasting behavioural responses of malaria mosquitoes to attractant-impregnated nylon: potential role of microbes.

Mweresa, C.K., P. Omusula, B. Otieno, J.J.A. van Loon, W. Takken and W.R. Mukabana. Long-lasting attraction of malaria vectors to synthetic odour baits placed indoors and outdoors in western Kenya.

Mweresa, C.K., Omusula, P., Otieno, B., van Loon, J.J.A., Takken, W. and Mukabana, R. W. Comparison of light and odours as stimuli for sampling malaria and other mosquitoes.

Mweresa, C.K., W.R. Mukabana, P. Omusula, B. Otieno, J.J.A. van Loon and W. Takken. Enhanced attraction of African malaria vectors to a synthetic odour blend.

PE&RC PhD Education Certificate

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

Potential application of semiochemicals for monitoring and manipulation of haematophagous insects: a review (2010-2011)

Writing of project proposal (4.5 ECTS)

Odour-based strategies for surveillance and behavioural disruption of host-seeking malaria and other mosquitoes (2010-2011)

Post-graduate courses (4.5 ECTS)

Innovation for sustainability; PE&RC, WUR (2010)
Generalized Linear Models with R; *icipe*, Kenya (2011)

Laboratory training and working visits (4.5 ECTS)

Field-based ecological research at *icipe*, Kenya; *icipe*, Kenya and SLU Sweden (2011)
10th Annual East African regional workshop of immunology and parasitic diseases at Morogoro, Tanzania; Seattle Biomedical and East Africa Research Institutes (2011)

Deficiency, refresh, brush-up courses (3 ECTS)

Ecological aspects of bio-interactions (2010)
Ecological methods (2010)
Basic statistics (2010)
Analysis and prevention of health risks in the tropics (2011)

Competence strengthening / skills courses (3.7 ECTS)

PhD Competence assessment; WGS (2010)
Effective behaviour in your professional surroundings; WGS (2012)
Techniques for writing and presenting a scientific paper; WGS (2012)
Project and time management; WGS (2012)
Stress identification and management workshop; WGS (2012)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

PE&RC Weekend (2010)
The last stretch of the PhD programme (2012)

Discussion groups / local seminars / other scientific meetings (6.6 ECTS)

Annual meeting of the Netherlands entomological society; Ede / Wageningen, the Netherlands (2012)
Local seminars and meetings on mosquito vector control, Kenya and the Netherlands (2010-2014)
PhD Students discussion group at the Laboratory of Entomology, the Netherlands (2010-2013)

International symposia, workshops and conferences (5 ECTS)

Gordon Research conferences on malaria ; Lucca (Barga), Italy (2011)
A platform for African and non-African scientists on insect chemical ecology and integrated pest management held at Duduville Campus; *icipe*, Nairobi, Kenya (2011)

Supervision of a MSc student

Evaluation of 2-butanone as a substitute for carbon dioxide in a malaria mosquito attractant blend (2012-2013)

Funding for the project: This project was funded by a grant from the Foundation for the National Institutes of Health (NIH) through the Grand Challenges in Global Health Initiative (GCGH #121)

Cover design: Collins K. Mweresa

Thesis layout: Collins K. Mweresa assisted by Sopher N. Ondiaka

Printed by: Wöhrman Printing Service, Zutphen, The Netherlands