Development and Evaluation of Push-Pull Strategies in Mosquito Control

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES
DER NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN
DER UNIVERSITÄT REGENSBURG

vorgelegt von

Ulla Obermayr (geb. Kröckel)

aus
Kronach

im Jahr
2016

Das Promotionsgesuch wurde eingereicht am:

21.03.2016

Die Arbeit wurde angeleitet von:

Prof. Dr. Joachim Ruther

Unterschrift:

Ulla Obermayr

List of Publications

Chapter 2 has been shortened and modified. It was originally published as **Obermayr, U. Excitorepellency. In: Insect Repellents Handbook. Editors: Debboun, M.; Frances, S.P. and Strickman, D. CRC Press, Taylor & Francis Group, Boca Raton, Florida, USA, 2015.** ISBN: 978-1-4665-5355-2.

Chapter 3 has been updated and shortened. It was originally published as **Obermayr, U., Ruther, J., Bernier, U., Rose, A. and Geier M. Laboratory Evaluation Techniques to Investigate the Spatial Potential of Repellents for Push-pull Mosquito Control Systems.** Journal of Medical Entomology, 2012. DOI: http//dx.doi.org/10.1603/ME12084

Chapter 6 has been updated and shortened. It was originally published as **Obermayr, U., Ruther, J., Bernier, U., Rose, A. And Geier, M. Evaluation of a Push-Pull Approach for *Aedes aegypti* (L.) Using a Novel Dispensing System for Spatial Repellents in the Laboratory and in a Semi-Field Environment.** PLoS ONE 10(6): e0129878. doi: 10.1371/journal.pone.0129878, June 26, 2015.

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Summary

Mosquito-borne diseases are a major threat to human health: malaria is responsible for a half million deaths, while dengue kills approximately 10,000 people per year. Dengue is transmitted by two important vector species, *Aedes aegypti* and *Ae. albopictus.* Especially *Ae. aegypti* is highly domestic and has a strong preference for human hosts. Control of the disease primarily relies on control of the vector but traditional intervention methods like outdoor adulticidal fogging frequently fail because *Ae. aegypti* tends to rest indoors or in secluded sites. In addition, emerging insecticide resistance in wild mosquito populations necessitates a shift in current control strategies. An interesting new concept is *push-pull*, a system that combines repelling and attracting stimuli to change the abundance of an insect pest in a given area (Pyke et al., 1987; Cook et al., 2007). Adult trapping systems like the BG-Sentinel trap (BGS) (Kröckel et al., 2006) have been suggested as a pull component for *Ae. aegypti* (Paz-Soldan et al., 2011; Salazar et al., 2012a) and several volatile pyrethroids have been tested for their spatial repellent (push) potential (Achee, 2012ab; Ogoma et al., 2012a, Wagman, 2014). However, the use of insecticides conflicts with the general idea of push-pull as a non-toxic means of pest management. In addition, constant human exposure to insecticides should be avoided and further insecticide resistance in the target population needs to be prevented which necessitates the screening for potent, non-toxic spatial repellents. Static air chambers are commonly used to assess spatial repellency (Grieco et al., 2005; Peterson & Coats, 2011), however, these set-ups may overestimate spatial repellent effects due to the confined air space. This thesis introduces a laboratory evaluation technique that allows for a more realistic evaluation of potential spatial repellents before they are tested in the field. In a first step, the efficacy of non-pyrethroid spatial repellents was evaluated in y-tube olfactometers and the most promising candidates were used for the development of a novel and larger scaled procedure. The new room test included a repellent dispensing system that created a repellent-loaded air curtain which had to be overcome by the mosquitoes in order to reach an attractive trap (BGS) located behind. Best results were obtained with a dispensing system based on conventional fans (*five fan system*, FFS) and a repellent sachet containing polymer granules with catnip (*Nepeta cataria*) essential oil. Mosquito attraction to a BGS-trap was reduced by 70 % in the presence of repellent volatiles (push-only trials). The indoor success of the FFS was attributed to a homogenous and constant dispersal of active ingredients (in contrast to a former set-up that used pressurized air), as confirmed by quantification experiments using thermal desorption gas chromatography coupled to mass spectrometry (TD-GC-MS). However, protection of a human volunteer was insufficient in push-only trials proving the strong attraction of *Ae. aegypti* to its natural host. Additional cues like exhaled carbon dioxide (CO_2) most likely rendered the spatial repellency of catnip ineffective. The integration of a potential CO_2 blocking blend in combination with catnip did not restore the attraction reduction to human odors. Successful

protection, however, was achieved in push-pull trials using catnip (push) in combination with a BGS trap (pull). In these trials, human landing collections were reduced by 50 %.

When tested under semi-field conditions, prominent spatial repellent effects of the FFS could not be detected. BGS trap catch rates in push-only trials were not reduced in the presence of catnip and human landing collections in push-pull trials were only slightly reduced. Future research needs to focus on (1) testing higher spatial repellent concentrations in an outdoor setting, (2) the use of additional attracting cues like CO_2 in the trapping system and (3) testing the impact of more than one trap as a pull component.

1. General Introduction

Mosquito-borne diseases are a major threat to human health all around the globe. Malaria kills approximately a half million people every year, the majority being African children under the age of five (World Health Organization (WHO) fact sheet on malaria, 2016). Dengue affects almost 400 million people per year and is responsible for the death of 10,000 (WHO fact sheet on dengue, 2015a). On April 25, 2014, Bill Gates declared the mosquito to be the deadliest animal in the world: "When it comes to killing humans, no other animal even comes close"[1]. Not even humans.

Dengue, a viral disease transmitted by *Aedes aegypti* L. and *Ae. albopictus* Skuse, is no longer restricted to tropical or subtropical regions of the world. It has spread along with its vectors and now poses the threat of possible outbreaks in Europe, in areas wherever its vectors are found. For the first time in 2010, local transmission of dengue was reported from France and Croatia (La Ruche et al. 2010; Gjenero-Margan et al., 2011). A dengue outbreak on Madeira Island in 2012 resulted in over 1,300 cases (European Centre for Disease Prevention and Control, ECDC, 2012). Chikungunya, another viral disease transmitted by *Ae. aegypti* and *Ae. albopictus*, reached Europe in 2007, resulting in a local disease outbreak in northern Italy with almost 200 cases (Angelini et al., 2007). The WHO confirmed that "mosquito-borne disease outbreaks by *Ae. albopictus* are plausible in Europe" (WHO fact sheet on chikungunya, 2015b). Zika virus, another arthropod borne virus (arbovirus) transmitted primarily by *Ae. aegypti* has been recently introduced into the Americas and is rapidly spreading (Basarab et al., 2016). Zika virus infections have been associated with an increase in congenital microcephaly and Guillain-Barré syndrome (causing muscle weakness and paralysis) in Brazil and even though this association has not yet been confirmed, the WHO declared the recent cluster of neurological disorders a public health emergency of international concern (PHEIC)[2].

Aedes aegypti is a highly domestic species with a strong preference for human hosts. During her lifespan, the female takes up multiple blood meals that will provide nutrients for egg development, flight and survival (Nelson, 1986; Scott et al., 1993). Sometimes, a female may take up several blood meals within one gonotrophic cycle, a behavior that leads to increased host contacts and thereby raises the likelihood of acquiring and transmitting viral pathogens (Scott et al., 1997). In combination with its close connection to human habitats, this particular blood-feeding behavior turns *Ae. aegypti* into a very competent *vector* (Service, 1992); a term that refers to arthropods that carry and transmit pathogens to their

[1] Source: www.gatesnotes.com, accessed May 15, 2015.

[2] Source: http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/, accessed February 29, 2016.

hosts. Among the approximately 3.500 species of mosquitoes worldwide (Rueda, 2008; Becker et al., 2010), *Ae. aegypti* is the most important vector of human arboviral diseases and is the principal vector of yellow fever, dengue and zika (Gubler, 1989; Monath, 1989; WHO fact sheet on zika, 2016).

While yellow fever infections can be prevented through vaccination, there are currently no publicly available vaccines to protect against dengue[3], chikungunya or zika. Control of these diseases therefore relies primarily on control of the vectors, which is still widely performed through the use of insecticides (a brief summary on historical approaches in mosquito control can be found in the Supplemental Information, p. 109) (WHO, 1997, Horstick et al., 2010). Larval control relies on bacterial insecticides (*Bacillus thuringiensis* toxin, Bti), insect growth inhibitors (methoprene), organophosphate insecticides (temephos) (U.S. Environmental Protection Agency (EPA), 2000) and source reduction (physical elimination of breeding sites). Adults are controlled through insecticide spraying, especially during epidemics. Here, organophosphates (malathon, naled) and synthetic pyrethroids (permethrin) are the most commonly used active ingredients (EPA, 2015; WHO, 2012). All of these actions are labor intensive, strongly depend on the skills and motivation of the trained personnel and have often shown limited success in controlling *Ae. aegypti.* A major impediment to controlling *Ae. aegypti* is its tendency to rest indoors and in secluded sites that are difficult to reach by outdoor spraying (Matthews, 1996). Indiscriminate and inefficient insecticide application also has led to an increased development of insecticide resistance (Fonseca-González et al., 2010; Marcombe et al., 2011; Polson et al., 2011). In order to minimize insecticide use and augment *Ae. aegypti* control, innovative new techniques are essential.

One of these new approaches could be *push-pull*. Well-established and successfully applied in integrated crop pest management in sub-Saharan Africa (Khan & Pickett, 2004; Cook et al., 2007; Hassanali et al., 2008), push-pull has become a topic in mosquito control (Cook et al., 2007; Paz-Soldan et al., 2011; Salazar et al., 2012a; Wagman, 2014; Menger et al., 2015; Menger et al., 2016). However, data showing the potential impact of push-pull techniques on mosquitoes are scarce and ready-to-use systems are unavailable.

The push-pull concept takes advantage of the fact that insects use a variety of semiochemicals to locate and identify their necessary resources, like mating partners, oviposition sites or food sources. Through the combinatory application of behavior-modifying volatile stimuli that [1] deter the target species from its resource („push") and [2] lure it to an alternative source („pull"), the distribution or abundance of an insect pest can be changed (Cook et al., 2007). In mosquito control, push-pull could be based on

[3] Sanofi's "Dengvaxia" has been approved in Philippines, Brazil and Mexico for people aged 9-45. While it has been approved for use, it is not currently available as negotiations are underway to determine price and distribution plan (Sanofi Pasteur press release, December 9, 2015; Source: www.en.sanofi.com, assessed February 24, 2016)

components that keep the blood-seeking female away from her preferred host and attract her to an alternative target where she is trapped and removed. The successful implementation of such a strategy would require [1] a powerful spatial repellent that impedes the attraction to the natural host [2] a highly attractive and efficient trapping system that represents a substitute host.

Adult mosquito trapping systems have been in use since 1934, when the first sampling device, the *New Jersey light trap*, was introduced as an alternative to human landing collections (Mulhern, 1942; Service, 1993). Over decades, variations of this first trap model have been employed; they used light as an attractant and a fan to draw insects into a holding container beneath the trap. However, light is a poor and non-selective attractant, frequently resulting in the capture of non-target insects like moths and beetles outnumbering those of mosquitoes (Kline, 1999). The addition of carbon dioxide (CO_2), a strong attractant to the host-seeking female mosquito, has been demonstrated to greatly increase trap catch rates (Newhouse et al., 1966; Gillies, 1980; Service, 1993), however, the routine use of CO_2 in mosquito surveillance programs can be problematic due to cost and logistics requirements. To address these issues, researchers have continued to search for new and better attractants and improved trap designs to provide innovative options for mosquito surveillance and control. Learning more about the host-seeking behavior of *Ae. aegypti* played a prominent role in the optimization process.

The mosquito female locates her host through visual cues, heat, moisture and most importantly host-derived odor plumes that represent a strong olfactory cue (Day, 2005, Cardé, 2015). Such host-derived semiochemicals are defined as *kairomones*, trans-specific chemical messengers that benefit the recipient, not the transmitter (Brown et al., 1970; Ruther et al., 2002). The odor plume that is given off by a vertebrate host is very complex, more than 1,400 volatile organic compounds (VOCs) have been detected in human skin secretions and breath (De Lacey Costello et al., 2014). Among these, only a few have been identified to play key roles in the host-finding process of *Ae. aegypti:* carbon dioxide (Gillies, 1980; Eiras & Jepson, 1994; Geier et al., 1999a), lactic acid (Acree et al., 1968; Geier et al. 1996, Dekker et al., 2002), ammonia (Geier et al., 1999b) short chained fatty acids (Bosch et al., 2000) and acetone (Bernier et al., 2003). In general, a blend of different odors is more attractive than single compounds and most compounds only work as attractants in the presence of lactic-acid (Geier et al., 1999b; Bosch et al., 2000; Dekker et al., 2002).

In 2006, a novel trapping system for the surveillance of *Ae. aegypti* was introduced, the BG-Sentinel trap (BGS, Kröckel et al., 2006). The trap utilizes a dispenser that emits an attractive volatile mixture composed of lactic acid, caproic acid and ammonia. In combination with the artificial host odors emitted from the dispenser, the BGS uses visual cues and mimics convection currents produced by a human body to create a very attractive target to the host-seeking female mosquito. Initially designed as a highly sensitive population surveillance tool, the BGS trap has been proven to be superior in catching *Ae.*

aegypti (Maciel de Freitas et al., 2006, Williams et al., 2006a), *Ae. albopictus* (Meeraus et al., 2008; Farajollahi et al., 2009; Pagès et al., 2009) and *Ae. polynesiensis* Marks (Schmaedick et al., 2008; Hapairai et al., 2013) compared to other, currently available trapping devices. More important, the BGS trap is not only a surveillance tool, it can also be implemented to control the vector population. A recently published study from Brazil demonstrated that a mass trapping approach with BGS traps resulted in a significant reduction in the abundance of *Ae. aegypti* within the treatment area (Degener et al., 2014). Its effectiveness against *Ae. aegypti* turns the BGS trap into a very interesting candidate for a pull component (Paz-Soldan et al., 2011; Salazar et al., 2012a).

Finding potent repellents that work at a distance to render the natural host unattractive poses a greater challenge. Numerous substances have been identified that act as mosquito repellents but little is known about their specific mode of action on the sensory level (Davis, 1985; Bohbot & Dickens, 2010; Bohbot et al., 2011). In general, *spatial repellents* are defined as airborne chemicals that lead to a reduction in human-vector contacts through different behavioral changes, including movement away from the source, interference with host detection (attraction-inhibition) or feeding response (WHO, 2013). Their spatial potential is most likely related to (a) higher concentrations of active ingredients in the vapor phase (high volatility) or (b) their mode of action on the involved sensory receptors. The majority of the commonly known spatial repellents are pyrethroid-based active ingredients deployed in outdoor vaporizing devices (Achee et al., 2012a; Ogoma et al., 2012b). These devices facilitate volatilization to maintain high levels of active ingredients within a confined space. Pyrethroids are structural modifications of pyrethrins, insecticides that are produced from *Chrysanthemum* flowers. In contrast to their natural template, synthetic pyrethroids show a greater potency and stability (Casida, 1980). When used at sublethal doses, volatile pyrethroids like metofluthrin and transfluthrin exhibited spatial repellent effects that resulted in reduced mosquito-house entries and reduced human-biting rates (Charlwood et al., 2014; Achee et al., 2012b; Ogomo et al., 2012ab). The underlying physiological mechanisms that cause the avoidance reaction to sublethal doses of pyrethroids have not been fully explored, however there are indications that the exposure prevents blood-feeding (Ogoma et al., 2014), causes mosquitoes to rest and seek shelter and reduces their attraction to trapping systems (Kitau et al., 2010; Salazar et al., 2012b). In addition, little is known about the potential impact of low-dosed pyrethroids on emerging insecticide resistance (Wagman et al., 2015), a process that must be avoided in alternative control strategies.

Indeed, the use of insecticides as spatial repellents conflicts with the general idea of push-pull as a non-toxic strategy for pest management as defined by Pyke and colleagues (1987). Thus, more research needs to be directed towards finding spatial repellents with different, non-toxic modes of actions. To date, only few alternatives have been identified and among these, linalool and catnip oil (*Nepeta cataria* L.) showed very promising spatial effects in small-scaled laboratory trials (Kline et al., 2003; Bernier et al., 2005; Polsomboon et al., 2008), however, data from larger-scaled trials and application-oriented set-ups were lacking.

Thesis Outline

This work investigates the potential of a push-pull approach for *Ae. aegypti* control, based on a combination of non-pyrethroid spatial repellents and the BGS trap. The main focus of these studies is on the characterization and evaluation of spatial repellents through existing and novel test set-ups, embedding the most promising candidate materials in a push-pull approach and transferring findings from the laboratory to the field.

Chapter 2 is based on an extensive literature review and provides a thorough overview on the terminology, history, evaluation and use of spatial repellents. In chapter 3, laboratory set-ups are presented to investigate different active ingredients with respect to their ability to repel mosquitoes from a distance and to inhibit their response to attractive kairomones. In a first step, all compounds are screened in y-tube olfactometer assays and candidates providing the greatest attraction reduction to natural host odors are further evaluated under more rigorous conditions within a novel room test set-up using a repellent dispensing system. The novel system also allows investigating their potential as push components in a laboratory push-pull set-up using a BGS trap (pull) to protect a human volunteer. Chapter 4 focuses on compounds that have been described to act as CO_2 inhibitors. The impact of these compounds on mosquito host attraction is investigated in y-tube olfactometer- and room tests to verify if these compounds can serve as additional push components to further decrease *Ae. aegypti´s* attraction to a human host. In chapter 5, a search for additional spatial repellents using catnip essential oil and its properties as a paradigm is conducted and results of the first y-tube olfactometer screenings are presented. In chapter 6, a novel dispensing system is introduced that facilitates a homogenous dispersal of active ingredients and its potential as a more application-oriented push component is investigated. Finally, the laboratory push-pull set-up using the novel dispensing system and BGS trap is transferred to a semi-field environment to verify its efficacy under more realistic conditions.

2. Terminology, Evaluation and Application of Spatial Repellents

This chapter has been modified and shortened. It was originally published as **Obermayr, U. Excitorepellency. In: Insect Repellents Handbook. Editors: Debboun, M.; Frances, S.P. and Strickman, D. CRC Press, Taylor & Francis Group, Boca Raton, Florida, USA, 2015.** ISBN: 978-1-4665-5355-2.

2.1 Terminology and Concepts

The use of terminology in the field of repellents aims to create a unique and useful vocabulary to describe mosquito behavior in response to chemicals. As our knowledge of mosquito behavior has increased, the desire to introduce new terms to describe and categorize these behaviors has also increased. Consequently, the field of insect-chemical interactions and insect behavior is rife with terms that either attempt to describe behavioral reactions (effects) or delineate the mediating mechanisms involved (cause). The smaller, more general set of existing terms has been strained and expanded in an attempt to convey the complex interactions between mosquitoes and chemicals and terms have sometimes been misused (Dethier et al., 1960; Haynes, 1988; Miller et al., 2009).

The term *repellency* is derived from the Latin word *repellere* and has been traditionally used to describe an avoidance reaction, i.e. an insect´s movement away from a chemical source, that is repulsing or deterring (Kennedy, 1947; White, 2007) "The word repellent has (...) frequently been incorrectly used" (Dethier et al., 1960), "It is a loose term, looser than we can afford in view of the importance for applied entomology (...)" (Kennedy, 1947). Repellency was suggested to describe effects on the spatial distribution of insects, e.g. a surface is considered to be repellent if insects spend less time on it compared to other available surfaces. The term thereby describes an end result, including behavioral reactions but is not a reaction itself (Kennedy, 1947). Dethier et al. (1960) refined the definition by distinguishing between two types of repellency, one that causes an immediate and directed avoidance reaction (*taxis*) and the second one leading to a greater activity (*orthokinesis*) which also reduces the number of mosquitoes on a repelling surface. In 1977, Browne suggested defining a repellent as "a chemical that, acting in the vapor phase, prevents an insect from reaching a target to which it would otherwise be attracted". Such a definition, however, does not include chemicals that do not act through the vapor phase. Roberts (1993) used the term *excitorepellency* to encompass all chemically induced irritant and repellent behaviors. He further distinguished between movements of avoidance resulting from tarsal contact and non-contact actions by classifying chemicals as *irritants* when tarsal contact is required and repellents when avoidance is elicited through the vapor phase. The phenomenon of vapor phase based avoidance is more commonly described as *spatial* *repellency*. Spatial repellency refers to chemicals that deter mosquitoes at a distance (Gouck et al., 1967) and inhibit their ability to locate a host (Nolen, 2002).

Some highly volatile pyrethroid insecticides like allethrin, transfluthrin and metofluthrin are also frequently defined as spatial repellents in applications like mosquito coils, mats or electric vaporizers that affect mosquitoes by causing knock-down, mortality, repellency or inhibition of feeding (Achee et al., 2012a; Ogoma et al., 2012ab; Xue et al., 2012; Kawada et al., 2008). The variety of terms found in the literature describing mosquito-insecticide interactions is bewildering (Fig. 2.1). Muirhead-Thomson (1960) regretted that when it came to describing behavioral responses of mosquitoes to residual insecticides "a rather confused terminology has grown up around this basic fact of irritability". If a mosquito settles down on an insecticide-treated surface and manages to take-off unharmed before absorbing a lethal dose, it was advised to use the term "protective avoidance". In case such a behavior was not observed at the first exposure but evolved after a certain number of years of being exposed, the term "behavioristic resistance" was suggested. As it is difficult to distinguish natural from developed behavior, Muirhead-Thomson proposed to use the term "behavioristic avoidance" to cover both.

In 1960, Dethier et al. published their classic paper characterizing chemicals through their modes of action using five basic terms. Chemicals act in different and sometimes multiple ways on an insect. They might cause the insect to stop or rest (arrestant), start or speed up (locomotor stimulant), make an oriented movement towards (attractant) or away (repellent) from the source or inhibit (deterrent) a certain behavior, e.g. feeding, mating or oviposition. It was advised to use the terms *attractant* and *repellent* only if an oriented movement to or from the source could clearly be detected. Dethier´s definitions provided great progress in the field of terminology and have remained in entomological literature since then.

The terms repellent, irritant, excitant or stimulant were commonly used to describe an insect´s behavioral response to insecticides, but new terms were frequently introduced while existing definitions were broadened to cover as many aspects as possible. Some of the existing terms, like repellent and irritant, were considered to be too vague to distinguish between neurotoxic effects and regular sensory inputs (Haynes, 1988) and a new discussion arose around the terminology used for insect-insecticide interactions (Miller et al., 2009).

Miller et al. (2009) updated Dethier´s definitions and introduced a new terminology to complement the original terms. Miller used the terms *engagent* and *disengagent* to describe a chemical´s effect on insect locomotion, which can either yield an increase (engagent) or decrease (disengagent) in encounters between insect and source. Both effects can be the result of tactic (oriented) or kinetic (non-oriented) movement patterns. Miller disagreed with the definition of contact irritants and spatial repellents, which include an oriented movement away from the source (Roberts, 1993; Roberts et al., 2000; Grieco et al., 2007). Accelerated flight behavior and non-oriented diffusion may also lead to a decrease in encounters and it was advised to use the latter-named terms only when a steered displacement was clearly detectable (Miller et al., 2009). Miller´s terminology has not yet gained wide acceptance and contact irritancy, excitorepellency and spatial repellency are still the more commonly used terms (Achee et al., 2012ab; Ogoma et al., 2012ab; Chauhan et al., 2012; Obermayr et al., 2012).

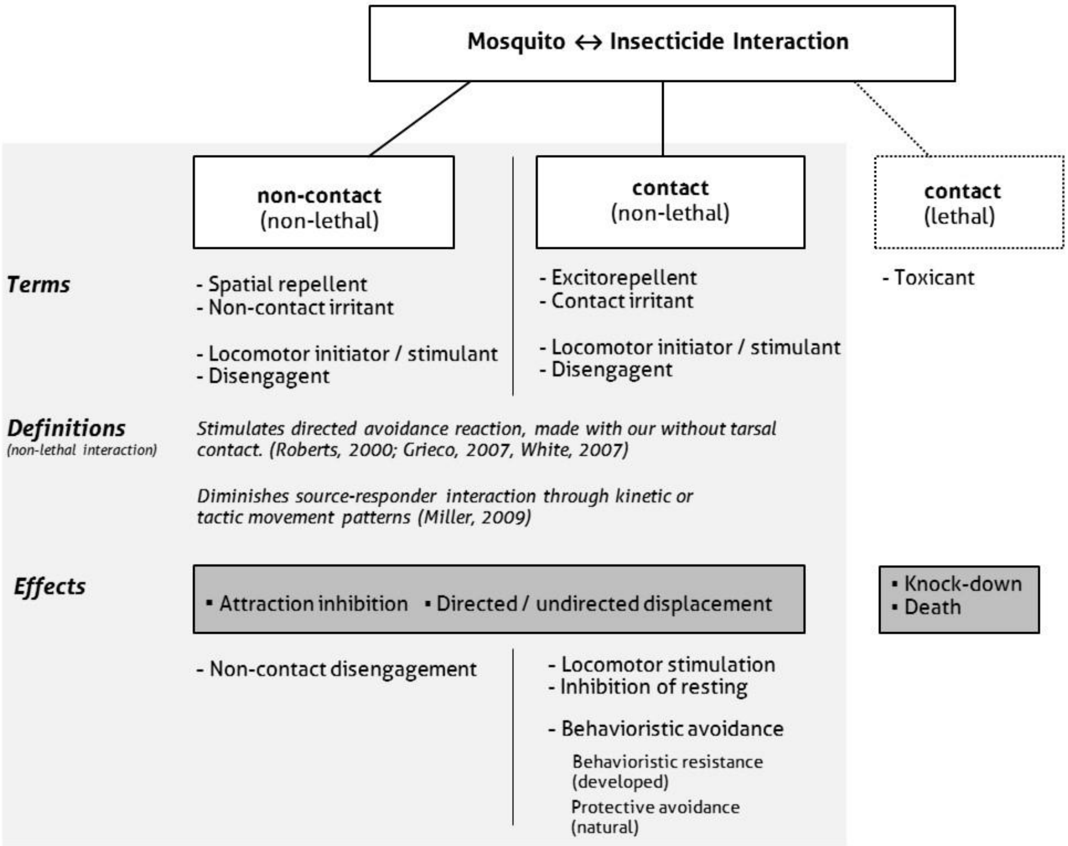

Fig. 2.1: Terms and definitions used to describe mosquito-insecticide interactions.

This thesis will utilize the following definition:

Spatial repellency: Interaction with a chemical in the vapor phase, resulting in an avoidance reaction and reducing an arthropod´s ability to locate a host.

2.2 Test Methods to Assess Spatial Repellency

2.2.1 High Throughput Screening System (HTSS)

The high throughput screening system (HTSS) is used to test the effects of new chemicals on the behavior of adult mosquitoes, including contact irritancy and spatial repellency. The modular device (Fig. 2.2) uses different arrays of aluminum (test) and plexiglas (control) cylinders, depending on the objective of the assay (Grieco et al., 2005).

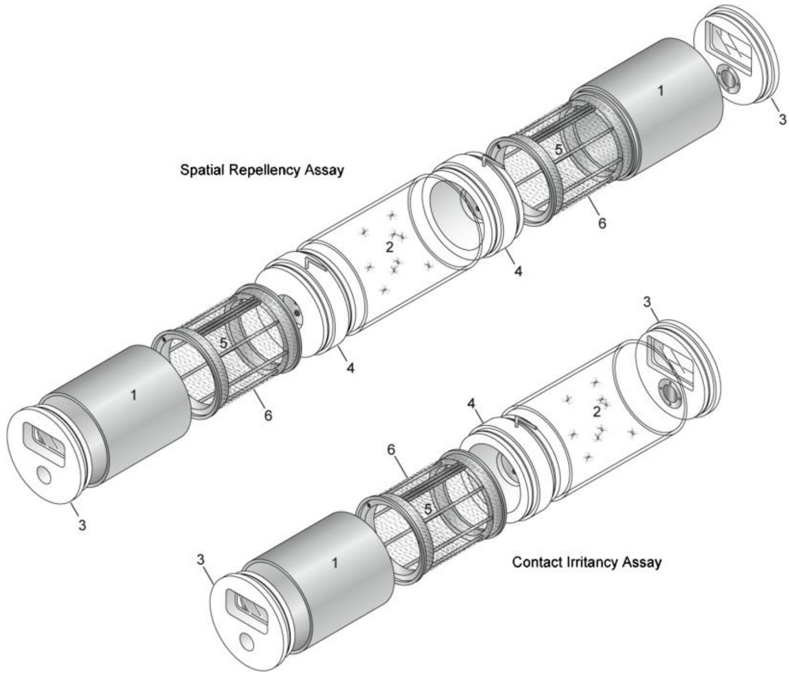

Fig. 2.2: Schematic drawing of the HTSS showing the spatial repellency assay (top) and contact irritancy assay (bottom) assemblies. Major components include: 1, treatment (metal) cylinder; 2, clear (Plexiglas) cylinder; 3, end cap; 4, linking section; 5, treatment drum; and 6, treatment net. (from Grieco et al., J. Am. Mosquito Contr., 21 (4), 404, 2005).

In contact-irritant assays, a test cylinder lined with a treated net is fixed to a darkened control cylinder. A valve between test and control unit is closed and 10 test mosquitoes are released into the treated cylinder; after an adaptation period of 30 seconds, the valve is opened and the distribution of the mosquitoes between the two compartments is recorded after 10 minutes. Individuals found in the control cylinder at the end of the test represent the proportion of escaping mosquitoes. Their numbers are compared to control trials (with ethanol treated nets) in order to examine the level of contact irritancy provided by a test chemical.

In spatial repellency assays, a metal test cylinder containing a treated net and another containing a solvent-treated net are connected by a clear cylinder that is placed in the middle. The valves of the intersections are closed and 20 mosquitoes are introduced into the clear central cylinder which is darkened by opaque felt. The end caps of the test cylinders are not covered in order to allow light to enter the system and help mosquitoes to orient. After an adaptation period of 30 seconds, valves are opened and the distribution of test mosquitoes among the test chamber (with treated net), the central chamber and control chamber (with solvent-treated net) is recorded after 10 minutes. With these numbers, a spatial activity index (SAI) can be calculated (WHO, 2013):

$$SAI = [(N_C - N_T) / (N_C + N_T)] \times (N_m / N)$$

with N_C being the number of mosquitoes inside the control chamber, N_T the number inside the test chamber, N_m the number of mosquitoes in both metal chambers and N the total number of mosquitoes inside the system. The SAI varies between -1 and 1, with 1 indicating a high level of spatial repellency and -1 no spatial repellency.

The HTSS system was used to learn more about the effects of standard vector control compounds on the behavior of *Ae. aegypti*. Pyrethroids like alpha-cypermethrin, deltamethrin or permethrin elicited great contact irritancy but still caused high knock-down and mortality while the action of dieldrin was toxic with no indications of contact-irritant or spatial repellent properties (Achee et al., 2009).

These studies demonstrate that commonly used insecticides have different impacts on mosquito behavior, which can exceed their role as a killing agent. Sublethal effects like contact irritancy and spatial repellency can contribute to a reduction in human-vector contact. (Grieco et al., 2007; Achee et al., 2009, Dusfour et al., 2009).

2.2.2 Y-Tube Olfactometer

Recently, a WHO guideline (WHO, 2013) on test methods for spatial repellents was published, which complements protocols on testing insecticidal activities (WHO, 2009). The new guideline addresses testing methods for airborne chemicals that may elicit an oriented movement away from the source, interfere with host-finding or change feeding responses and thereby reduce host-vector contact.

The exposure to airborne chemicals does not always result in a steered motion into the opposite direction. Some chemicals impede the host-finding process and are therefore called *attraction-inhibitors* (Bernier et al., 2007). Such a feature is of particular interest as spatial repellents that interfere with the mosquitoes´ ability to locate a host are promising candidates to be used in push-pull vector control strategies (Kline et al., 2003; Bernier et al., 2007; Obermayr et al., 2012).

Y-tube olfactometers (Fig. 2.3) are generally used to measure the level of attraction or repulsion of host seeking mosquitoes to volatile stimuli in choice experiments (Feinsod &

Spielman, 1979; Posey et al., 1988; Geier & Boeckh, 1999; Bernier et al., 2007). Clean and conditioned air constantly runs through the tube system to the end of the base leg, where mosquitoes are connected. During stimulus application, mosquitoes are allowed to fly upwind into a decision chamber to choose between a test cage that holds the test stimulus and a control cage with clean air.

In attraction-inhibition assays, repelling stimuli are presented in combination with attractive odors (either coming from a synthetic blend or human hand) in order to measure the attraction reduction elicited by the repellent (Kline et al., 2003; Bernier et al., 2005; Obermayr et al., 2012). The use of synthetic blends containing combinations of L-lactid acid, ammonia, hexanoic acid and acetone (Geier et al., 1999b; Bernier et al., 2003; Williams et al., 2006b) help to create more standardized conditions by reducing the variability that is known for human odors.

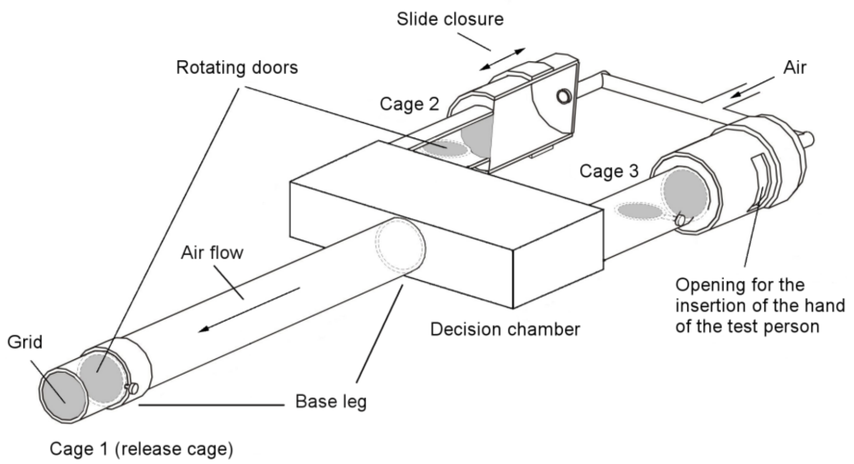

Fig. 2.3: Y-tube olfactometer according to Geier et al. (1999). Cage 2 and 3: control or treatment cage. (From Geier, M., Bosch, O. and Boeckh, J., Chem. Senses 24, 1999.)

2.2.3 Semi Field Tests in Screened Outdoor Cages

Screened outdoor cages with a volume of 300 m^3 to 815 m^3 have been used to simulate true environmental conditions but offer the benefit of reduced variability in comparison to field tests (Bernier et al., 2007; Ogoma et al., 2012ab). Outdoor cage studies allow the use of a defined number of test mosquitoes at a certain age and physiological stage and can be performed with standardized arrangements of huts, traps, and release devices. The WHO (2013) recommends the use of semi-field environments for the evaluation of formulated products. Semi-field tests have been used to investigate the spatial repellency potential of new materials and commercially available repellent products (Bernier et al., 2007; Kitau et al., 2012; Ogoma et al., 2012ab).

The typical study design involves at least two identical cages, one for treatment and one for control trials, built in close proximity to each other. The efficacy of a formulated product is evaluated by measuring human landing and feeding rates in comparison to control trials. The test treatment is installed in the center of the cage, with the volunteer sitting on one end and the mosquitoes released at the opposite end. Typically, 100 mosquitoes are released within one trial and human landing collections are performed by one volunteer. Mean mosquito collection rates during treatment trials are compared to controls. To ensure adequate host-seeking activity, the landing and feeding response in control trials should be greater than 50%.

Kline suggested the use of outdoor cages to evaluate the attraction-inhibiting potential of spatial repellents (D.L. Kline, unpublished data). Attractive traps using synthetic blend dispensers are installed in the center of the cage, surrounded by 4 spatial repellent releasing devices attached to 4 poles around the trap. The trap and release devices are switched on at least 30 minutes before mosquitoes are released into the cage and trap catches are documented after a certain sampling period, e.g. 12 hours. Afterwards, human landing collections are performed at different locations in order to evaluate the extent of spatial repellency produced by the dispensed chemical.

2.3 Spatial Repellency of Pyrethroids

Pyrethroids target voltage-gated sodium channels in the nerve axons and behavioral effects can be attributed to a disruption in the organization of the peripheral sensory system (Matsunaga, 1999). The spatial repellency of pyrethroids is believed to be caused by the high knock-down activity and intrinsic sublethal effects, which disrupt the orientation to the natural host or inhibit feeding or both (MacIver, 1964; Haynes, 1988; Winney, 1975; Birley et al., 1987).

There is growing evidence that repellents interact with odorants and odorant receptors (ORs) thereby interfering with the odorant-driven host-seeking process (Xia et al., 2008; Jones et al., 2011; Bohbot & Dickens, 2012; Ogoma et al., 2012b). Bohbot et al. (2011) tested the molecular effects of different insect repellents and one novel synthetic pyrethroid on *Ae. aegypti* ORs. The pyrethroid inhibited the OR response to an attractant in a similar way to 3,8-para-menthan-diol (PMD), a common insect repellent. Results indicated that repellent effects of pyrethroids may be due to a combination of sublethal neurotoxic excitement and interactions with the olfactory system (Ogoma et al., 2012b; Ujihara et al., 2004).

Pyrethroids with a high vapor pressure, like metofluthrin, transfluthrin, and allethrin, evaporate faster at ambient temperatures resulting in high vapor phase concentrations of active ingredient that can produce a barrier effect (Bernier et al., 2007; Kawada et al., 2008). Evaporation rates are further enhanced in product applications like plug-in vaporizers, mosquito coils, and mats. However, heating is not necessarily required to vaporize the active ingredients in impregnated plastic resins and passive paper emanators, offering new and cost-saving ways of dispensing the active ingredient.

Metofluthrin (SumiOne®, Eminence®) was synthesized by Sumitomo Chemical Co Ltd., Japan (Ujihara et al., 2004), and has been extensively studied over the past decade. Argueta et al. (2004) evaluated the spatial efficacy of metofluthrin impregnated paper strips in an outdoor setting in Japan and found a 95 % to 100 % reduction in *Ae. albopictus* trap catches in the presence of metofluthrin (which lasted for more than 6 weeks after treatment). Field tests of metofluthrin impregnated plastic strips also yielded a significant decrease in *Culex quinquefasciatus* Say, *Ae. aegypti,* and *Anopheles gambiae* Giles house density indices in intervention areas, reaching a reduction of 70 to 100% for up to 11 weeks after treatment (Kawada et al., 2004; 2005; 2006 & 2008). Laboratory wind tunnel tests of metofluthrin impregnated paper strips indicated that the presence of airborne active ingredient not only reduced the proportion of landing *Ae. aegypti* but also inhibited those that succeeded in landing from feeding (Lucas et al., 2007). Recently, metofluthrin became commercially available as a spatial repellent clip-on (OFF! Clip On Mosquito Repellent). The device contains 31.1 % active ingredient enclosed in a cartridge with a fan to dispense the chemical into the air. The clip-on was evaluated in a field study in Florida with 6 volunteers

(Xue et al., 2012). During a testing period of 3 hours, biting rates by *Ae. albopictus* and *Ae. taeniorhynchus* Wiedemann were reduced by 70 % to 79 %.

2.4 Spatial Repellency of Natural Compounds

Plant derived materials have been used for centuries to repel biting arthropods, e.g. by hanging bruised plant parts in houses, burning plant materials or applying essential oils to the skin (Maia & Moore, 2011). Recently, outdoor plantings of repellent plants like wild sage, neem, lemongrass, and West Indian *Lantana* were studied for their effect on mosquito house entry in rural tropical areas (Mngongo et al., 2011). When *Lantana camara* L. was planted outdoors, up to 83 % fewer *An. funestus* Giles were collected indoors compared to control houses.

There is a growing interest in using plant-derived compounds as alternatives to synthetic chemicals. Plant sesquiterpenes are especially active against mosquitoes and other pests (Paluch et al., 2009a). Twelve sesquiterpenes that share structural similarities and represent a range of mosquito-repellent activities were evaluated for spatial and contact repellency against *Ae. aegypti.* Based on the results, quantitative structure-activity relationship (QSAR) models were developed to identify key properties of the sesquiterpenes that could be used to predict spatial and contact repellent actions (Paluch et al., 2009b).

Over the past decade, increased efforts have been directed towards the discovery and analysis of non-insecticidal spatial repellents and a few promising substances and mixtures with such properties have been discovered, like linalool and catnip. Kline et al. (2003) observed spatial effects of linalool, a volatile compound contained in a variety of essential oils. When used in traps baited with CO_2 and 1-octenol-3-ol, linalool provided up to 50 % reduction in mosquito collection rates compared to control traps without linalool. In triple-cage olfactometer trials, linalool and dehydrolinalool exhibited spatial repellency against *Ae. aegypti*, causing a decrease in the overall flight activity and reducing the ability to locate a human derived attracting blend (Kline et al., 2003).

Linalool´s spatial repellent properties have also been studied against wild mosquito populations in Israel (Mueller et al., 2008). The bite reducing effects of 5 % citronella essential oil, 5 % linalool and 5 % geraniol candles were compared to negative controls (paraffin) in an indoor environment. Compared to paraffin, linalool and geraniol reduced human biting rates by 71 % and 86 %, respectively, while citronella oil had less pronounced effects reducing biting rates by 29 %.

One of the most promising and extensively studied natural candidates is catnip, *Nepeta cataria* (Lamiaceae). Nepetalactone, the major component of catnip oil, was reported to be repellent to 13 different insect families (Eisner, 1964), cockroaches (Peterson et al., 2002), mosquitoes (Peterson, 2001) and stable flies (Zhu et al., 2010). Its spatial efficacy against *Ae. aegypti* has been evaluated in several laboratory assays. Triple-cage olfactometer trials indicated that catnip was more effective in inhibiting *Ae. aegypti* attraction to a synthetic blend (lactic acid and acetone) or human odors than deet (N,N-diethyl-m-toluamide) (Bernier et al., 2005). Peterson and Coats (2011) tested the effect of catnip oil and its nepetalactone isomers in a static air chamber. The set-up consisted of a glass tube with a central opening for the introduction of the test mosquitoes and lids to cover the ends. Test compounds were dissolved in acetone and applied to filter paper disks. One end of the chamber received a treated disk while the other end remained repellent-free and was provided with a solvent treated disk. Test mosquitoes´ distribution inside the chamber was documented 15 minutes after they had been liberated. All test compounds showed significant spatial activity against *Ae. aegypti.* Catnip oil repelled up to 59 % of the test mosquitoes from the treatment site, while the isomers deterred 56 % (*E,Z*-nepetalactone) and 50 % (*Z,E*-nepetalactone). When deet was applied to the filter paper, only 10 % of the test mosquitoes avoided the treated site.

Field data on the spatial repellency of catnip or other natural compounds are scarce. Chauhan et al. (2012) suggested a field bioassay to evaluate spatial effects by monitoring trap catches in the presence and absence of different repellents. A standard miniature light trap (by J.W. Hock Company) supplemented with additional CO_2 was surrounded by a 4 x 4 m horizontal frame, which held a total of 16 repellent receptacles (1.5 ml PE tubes, 4 per side). The spatial repellent potential of cypermethrin, vetiver oil, catnip oil, deet, and *E,Z*-dihydronepetalactone was evaluated against local mosquito species in Beltsville, Maryland, USA. Dihydronepetalactone is a minor component of nepetalactone-rich catnip oils and has been reported to be highly repellent to mosquitoes and blackflies (Spero et al., 2008). In Chauhan´s field assays, deet and *E,Z*-dihydronepetalactone were the only compounds that showed spatial effects and were able to reduce trap catch rates by 37 % (deet) and 25 % (*E,Z*-dihydronepetalactone).

Catnip also showed spatial activity against the stable fly, *Stomoxys calcitrans* L. More than 70 % of the tested flies were repelled from the treatment port in olfactometer trials. Catnip´s spatial efficacy was further evaluated in greenhouses where flies were released. In these trials, one half of the green house received treatment (catnip oil on filter paper) while the other half received solvent only (hexane). *Stomoxys calcitrans* movement patterns were documented every hour and the atmospheric concentration of catnip was determined by solid phase micro extraction (SPME). After 4 hours, 50 % of the flies were repelled from the treated site and the catnip atmospheric concentration had reached a level that was 6 fold higher compared to the start of the tests. A slow release formulation using 10 % catnip oil in wax pellets showed promising but short lived effects in the field. In the first 3 hours after the application, the abundance of stable flies was reduced by more than 95 % in the treated areas, however, the spatial effects soon dissipated. After 3 hours, the catnip atmospheric concentration was reduced by 50 % compared to the start of the tests, which may explain the loss of the spatial repellent impact (Zhu et al., 2010).

2.5 Spatial Repellents and Their Use in Push-Pull Control Systems

The idea of push-pull goes back to late 1980s, when Pyke et al. (1987) presented their control strategy for cotton moths that had become resistant to standard insecticides. Push-pull was suggested as a means of integrated pest management, an alternative approach to combat growing resistance by using non-toxic, sustainable and cost saving components to affect the abundance of an insect pest.

The establishment of push-pull strategies in vector control is a subject of great interest. A strong spatial repellent that affects host-seeking mosquitoes at a distance is of great importance for such a strategy and crucial to the success of the system.

Sublethal doses of common insecticides have been discussed as push components for an *Ae. aegypti* control strategy (Manda et al., 2011; Paz-Soldan et al., 2011; Achee et al., 2012ab; Salazar et al., 2012b; Manda et al., 2013). Other studies have examined non-insecticidal spatial repellents, such as catnip (Bernier et al. 2005; Zhu et al., 2010; Obermayr et al., 2012), linalool (Kline et al., 2003), commercial repellents containing 15% deet (Kitau et al., 2010), or outdoor plantings of mosquito repelling plants (Mngongo et al., 2011).

As discussed earlier, sublethal doses of insecticides can deter mosquitoes away from their source of release. This deterrence, however, could also be elicited by neurotoxic effects causing mosquitoes to rest and seek shelter. The behavior modifying effects of pyrethroids were investigated by pre-exposing *Ae. aegypti* mosquitoes to three common insecticides, DDT, transfluthrin, and metofluthrin, and subsequently monitoring BGS trap catch rates in a semi-field environment (Salazar et al., 2012b). After having been exposed to standardized sublethal doses of the chemicals for 6 hours, mosquitoes were introduced to the trapping set-up immediately or with a delay of 12 hours. DDT and metofluthrin had no impact on the recapture rate of *Ae. aegypti* compared to contact trials. In immediately following trials, transfluthrin significantly reduced recapture rates whereas delayed trials showed no significant changes in BGS trap catches.

The success of a push-pull system for vector control relies on a strong spatial repellent that affects host-seeking mosquitoes in a way that they are deterred from their preferred host but are still attracted to alternative target traps. There are indications that some commonly used insecticides, like allethrin and transfluthrin, do interfere with host-seeking and cause the mosquito to seek shelter, thereby reducing the effectiveness of the attractant trap.

2.6 Conclusion

The phenomenon of spatial repellency has been extensively studied over the past decades. The range of methods available today allows us to highlight almost any aspect of repellent-mosquito interaction: laboratory systems help us to understand the different impacts of new and known chemicals on mosquito behavior, field trials provide valuable insight into the real world situation and modern air sampling techniques give us the opportunity to estimate doses that repel but do not kill the target vector. Sublethal doses could play an important role in new control approaches like push-pull systems. In order to achieve success in a push-pull system, repellent compounds will be required that do not paralyze the mosquito but allow it to seek alternative attractant sources, resulting in increased trap catches and decreased human-vector contact. Even though we have gained great insight into mosquito behavioral reactions, we still need to learn more about the physiological basis of repellency caused by a chemical. Expanding our knowledge will broaden the spectrum of available application techniques and lead to the development of new and improved vector control strategies.

3. Laboratory Screening of Candidate Materials for Their Spatial Repellency Against *Ae. aegypti*

This chapter has been updated and shortened. It was originally published as **Obermayr, U., Ruther, J., Bernier, U., Rose, A. and Geier M. Laboratory Evaluation Techniques to Investigate the Spatial Potential of Repellents for Push-pull Mosquito Control Systems.** Journal of Medical Entomology, 2012. DOI: http//dx.doi.org/10.1603/ME12084

Author contributions: U.O., J.R., M.G. and A.R. designed the study; U.O. performed all experiments; M.G., A.R. (BGS traps, BG Lure); U.B. (1-methylpiperazine, homopiperazine) provided material; U.O., J.R., and U.B. wrote the manuscript.

A protocol has been developed for the indoor evaluation of candidate spatial repellents intended for use in push-pull systems. Single treatments (catnip oil (CN), 1-methylpiperazine (MP) and homopiperazine (HP)) and a mixture of catnip oil and homopiperazine (CN-HP) were tested with yellow-fever mosquitoes (*Ae. aegypti*) in Y-tube olfactometers to determine (a) if these compounds inhibited mosquito host-seeking at short distances and (b) if results obtained in olfactometer tests can be correlated with a larger scale room test set-up. All test materials significantly decreased the ability of mosquitoes to find host odors (from a human finger) by up to 96.7 % (CN-HP). Similar effects could be observed within a new room test set-up, which involved a repellent dispensing system and an attractive trap (BG-Sentinel). Mosquitoes captured by the BGS trap had to fly through a treatment-containing air curtain created by the dispensing system. Compared with the use of a control (ethanol solvent (EtOH) without candidate repellent), trap catch rates were significantly reduced when CN, MP or HP was dispensed. HP produced the greatest level of host-seeking inhibition with a 95 % reduction in the trap catches. The experimental set-up was modified to test the viability of those technologies in a simple push-pull situation. The combination of BGS-trap and CN-HP helped to reduce human landing rates by up to 44.2 % with a volunteer sitting behind the curtain and the trap installed in front of the curtain.

3.1 Introduction

Mosquito-borne diseases are a major threat to human health. Half of the world´s population is at risk of malaria, which caused an estimated 655,000 deaths in 2010 (WHO, 2012) and around 2.5 billion people in more than 100 countries are at risk of dengue fever (DF). In contrast to malaria, no treatment and no vaccine are yet widely available against DF; therefore control of this disease depends primarily on measures taken against the vectors (WHO, 1997, Horstick et al., 2010), *Ae. aegypti* and *Ae. albopictus*. Traditional control methods like adulticidal fogging are frequently inadequate because the adult mosquito rests in secluded sites (Matthews, 1996). Furthermore, indiscriminate or inefficient application has led to increased development of insecticide resistance (Fonseca-Gonzales et al., 2010; Polson et al., 2011). An alternative could be push-pull, which has been reported as a strategy for integrated pest management (IPM) (Pyke et al., 1987). The approach of this seminal work used a combination of repelling and attracting stimuli to control the distribution of insecticide-resistant cotton moths (genus *Heliothis*). Through the use of both deterring and attracting stimuli, the abundance of insect pests can be changed in a given area by interfering with the ability of the target pest to find their resource ("push") and luring them to an alternative source where they are trapped and killed ("pull"). Currently, most successful push-pull techniques are used in crop pest management, but similar strategies may improve the control of mosquitoes and other disease vectors (Cook et al., 2007).

Our approach involves the BGS trap as the pull component, because it is a superior trap for *Aedes* species, such as *Ae. albopictus*, *Ae. aegpyti* and *Ae. polyniensis,* even without the use of CO_2 (Meeraus et al., 2008; Schmaedick et al., 2008; Farajollahi et al., 2009; Azil et al., 2011). The BGS trap attracts host-seeking females by mimicking convection currents produced by a human body, through visual cues and by emitting artificial host odors from a synthetic attractant dispenser, the BG-Mesh Lure (BG ML, Kröckel et al., 2006). The synthetic lure is composed of lactic acid, caproic acid and ammonia. These compounds are present on human skin and are known to play an important role in the host finding process of *Ae. aegypti* (Geier & Boeckh, 1999).

Numerous substances have been identified to act as mosquito repellents but in contrast to its Latin origins ("repellere" = to repulse, to drive away), some common mosquito repellents do not mediate a targeted movement away from their source (that would result in contact prevention) but rather work at a short distance or through direct contact and instead result in bite prevention (Bernier et al., 2007). Only a few substances with spatial repellent properties have been discovered; among these catnip (*Nepeta cataria*) essential oil and linalool have been reported to show promising effects against *Ae. aegypti* in olfactometer bioassays (Kline et al., 2003; Bernier et al., 2005). Preliminary screening trials in our y-tube olfactometers confirmed the spatial repellent potential of catnip, however, linalool did not show a comparable effect and was therefore excluded from future experiments (data not shown).

In more recent research, 1-methylpiperazine and homopiperazine were reported as compounds that interfere with host-seeking ability and therefore act as attraction-inhibitors of kairomones (Bernier et al., 2012). In olfactometer bioassays, these compounds reduced the attraction of *Ae. aegypti* and *An. albimanus* towards a synthetic human odor blend from 92.7 % to 12.8 % and from 67.5 % to 8.2 %, respectively.

To date, those compounds have only been evaluated in olfactometers and their performance under more realistic conditions, for example, in a room, or outdoors in a field setting is unknown. The use of a potent spatial repellent is crucial to the success of a push-pull control system. To address the issue of scaling up this technology for field use we have developed a room test protocol that involves the use of a simple repellent dispensing system in combination with an attractive BGS mosquito trap and human bait to investigate how trap catch rates and human landing rates are altered in the presence of test repellent compounds. Results from conventional olfactometer bioassays will be compared with room tests to determine how well results from laboratory olfactometers correlate with results from larger scaled room tests.

3.2 Material and Methods

3.2.1 Test Materials

MP and HP were purchased from Sigma-Aldrich (Taufkirchen, Germany), pure CN and thyme (*Thymus vulgaris*) (THY) essential oils were acquired from Aromaland (Röttingen, Germany). All single compounds were diluted vol:vol in ethanol (96 %, p.a.) to final concentrations of 2.5 % and 5 %. In addition, 5 % CN and 5 % HP were mixed at a 1:1 ratio to obtain a 2.5 % ethanolic formulation of the two compounds. In y-tube olfactometer trials, 30 µl (0.75 mg - 1.5 mg active ingredients) were used per individual trial.

The 10 % formulation for room tests was obtained by mixing 20 % CN and 20 % HP at a 1:1 ratio. In room tests, doses of 500 µl were used per individual trial, corresponding to approximately 25 mg (5 % formulations) and 50 mg (10 % formulation) of active ingredients.

A proprietary repellent formulation (Autan Protection Plus, SC Johnson GmbH, Erkrath, Germany) containing 20 % picaridin (PIC) (hydroxyethyl isobutyl piperidine carboxylate) was acquired from a local drugstore. PIC was used at quantities of 6 mg in olfactometer trials and 100 mg in room tests.

3.2.2 Test Mosquitoes

Aedes aegypti females aged 10-21d were used for all tests. Preliminary olfactometer tests (data not shown) revealed that our colony shows a comparable susceptibility for repellent volatiles at days 6-20 after emergence while responses to a finger or repellent volatiles show greater variations at a younger age (1-5d). The colony was obtained originally from BAYER AG, Monheim, Germany and has been maintained in our facilities over the past 17 years. Mosquitoes were reared at 26 ± 1°C and 60 ± 5 % RH under a photoperiod of 12:12 (L:D) h. After hatching of the eggs, larvae were kept in a water basin (30 cm × 30 cm × 10 cm) filled with a 1:1 mixture of tap water and deionized water and fed with Tetramin® fishfood flakes (Tetra GmbH, Melle, Germany). Pupae were transferred into breeding cages (40 cm × 30 cm × 20 cm). Adult mosquitoes were provided with a 10% glucose solution on filter paper.

Behavioral tests were performed with host-seeking females, which were lured out of their breeding cages at least 10min before the start of the tests. The breeding cages contained a circular opening covered by fine mosquito netting in the left wall, while the right wall was fitted with a port and rotating door, where a transfer container could be attached. The transfer container consisted of a perspex cylinder with a rotating door on one end and a cover made from fine mosquito netting at the other end. A fan running at 7.5 V was connected to the opening in the left wall of the breeding cage to pull air into the cage, while a human hand was held to the transfer container on the opposite side of the cage and rotating doors were opened. Female mosquitoes that were seeking a blood meal flew upwind into the transfer container, attracted to the skin odors.

3.2.3 Y-tube Olfactometer Assays

Olfactometer tests were performed according to Geier and Boeckh (1999). In total, four y-tubes, identical in construction (Fig. 2.3, p. 20) were used to measure the behavioral responses of host-seeking *Ae. aegypti* females towards CN, MP, HP, THY, a mix of CN-HP, and PIC. Each olfactometer consisted of a transparent plexiglas base leg, followed by a decision chamber and two branches which terminated into attached Teflon chambers, where the test stimuli were introduced (Fig. 2.3). A constant air stream from the institute´s pressurized air system was purified with a filter of activated charcoal, heated up to 26 ± 1°C and humidified to a relative humidity of 70 ± 5 % before it was transported through the tube system and into the base leg, with wind velocities of 0.4 m/s in the branches and 0.2 m/s in the base leg. Rotating doors in both branches, as well as at the downwind end of the base leg, allowed the release and entrapment of the test mosquitoes. Cohorts of 15-21 mosquito females were attached to the apparatus at its downwind end.

Test procedure: Before the start of a test, 30 µl of an ethanolic test formulation were applied to a 1 cm × 3 cm filter paper strip (Schleicher & Schuell Microscience GmbH, Dassel, Germany), which was attached to a tempered metal wire and suspended into one of the Teflon chambers. The door of the base leg remained closed for 15 s to keep the test mosquitoes in the airstreams containing repellent. A forefinger was then inserted into the Teflon chamber behind the paper strip and the rotating door of the base leg was opened. Mosquitoes were allowed 15s to fly upwind and decide between the test branch with volatile stimuli and the control branch with pure air. The rotating doors were closed and the number of mosquitoes that migrated from the release cage (=active), the number of mosquitoes inside the test cage (where the stimuli were applied), and the number of mosquitoes in control cage (with filtered air) were documented. At the conclusion of a test, the airflow in the apparatus was inverted and mosquitoes were lured back into the release cage by the palm of the hand and the next of four y-tubes was used for testing. Treatments were tested in randomized order, and after each run, the control branch and test branch were changed to avoid position or adaptation effects. There were 10 replicates of each single compound or mix. All treatments were tested against a control of the forefinger and a paper strip treated with 96% ethanol. The spatial properties of CN, THY, MP, and HP were evaluated in experiment 1. Experiment 2 included the evaluation of PIC in comparison to THY and Y-tube experiment 3 compared the effects of CN, HP, CN-HP and MP.

Data Analysis Olfactometer Assays: For each treatment, mean percentages of active test mosquitoes, mosquitoes inside test chamber and control chamber were calculated, as well as corresponding standard errors. Data were subjected to an arcsine transformation and then compared using a one-way analysis of variance (ANOVA) and Tukey´s honestly significant difference (HSD) test as a post-hoc test to verify significant differences between single treatments. A P value ≤ 0.05 was regarded as statistically significant. All statistical tests were performed using PAST version 3.04 (Hammer, 2001).

3.2.4 Room Tests with Repellent Evaporating System and BGS-Trap

In experiment 4, treatments of CN, MP, HP, THY, and a CN-HP mix were investigated in a novel experimental set-up to simulate conditions of real world usage. Tests were performed in an air-conditioned 40.25 m^3 windowless room (4.6 m × 3.5 m × 2.5 m) with artificial light from two fluorescent tubes (350 Lux). The temperature and relative humidity of the air in the room were 25 ± 1° C and 60 ± 5 %, respectively. The ventilation from the air conditioner entered the room through an opening in the ceiling and exited the room through a second opening 4.5 m apart on the far side of the ceiling. A tent structure comprised of cotton fabric was built around the air entry with bottom edges held on the floor by wooden bars. The tent had three sides and contained a volume of 5.2 m^3 (1.2 m × 1.2 m × 2.5 m) with the open side that was 2.2 m^2 (1.2 m × 1.8 m). A repellent dispensing system was placed at the top of the open side of the tent (Fig. 3.1). The dispensing system consisted of a polyethylene (PE) tube (length 1 m, diameter 0.5 mm; Festo AG & Co. KG, Esslingen, Germany) attached to a tripod, a 500 ml fritted gas wash bottle, flow meter and compressed air connection. The tube served as a dispensing device and contained fine holes (diameter 0.2 mm, distance between holes 2 cm) at the rear side to release the test volatiles. For each test, 500 µl of the ethanolic treatment formulations were dropped onto round filter papers (Schleicher & Schuell Microscience GmbH, Dassel, Germany) at the bottom of the fritted wash bottle. In control tests, 500 µl ethanol was used. Pressurized air was passed through the bottle at a flow rate of 15 L/min, then loaded with treatment as it continued to flow into the PE tube attached to the tripod. In this way, a treatment enriched air curtain was released at the top of the tent window. To avoid a mixing of the treatments within the dispensing system, dedicated PE tubes and wash bottles were used for each treatment.

Room Test: A BGS trap fitted with BG lure dispenser (L-lactic acid, caproic acid, ammonia) was placed inside the tent to attract host-seeking *Ae. aegypti* to fly through the curtain for potential capture. For each test, 10 mosquitoes were released into the room at the side furthest from the tent. After release, mosquitoes were allowed to respond for 15 min. At the end of the test time, the catch rate of the trap was documented and free-flying mosquitoes were removed with a modified hand-held vacuum cleaner. Mosquitoes that did not approach the investigator or that were still sitting inside the transport cage were recorded as inactive. There were 10 replicates conducted for each single compound or mix. Results were compared with the control (BGS trap and a repellent-free air curtain). Additional control tests without air curtain were performed to determine if air movement alone was a physical barrier that prevented mosquitoes from flying into the tent. Treatments were tested in a randomized order. To avoid an accumulation or mixing of the volatile stimuli, the room was aerated for at least 30 min between experiments.

Fig. 3.1: Room test set-up with repellent-dispensing system and BG-Sentinel trap.

3.2.5 Room Tests with Repellent Evaporating System and Human Bait

In experiment 5, a volunteer sat behind the air curtain in the middle of the tent and provided attractive odor cues in comparative tests of CN-HP, PIC, and repellent-free ethanol controls. The test procedure was consistent with that described above (experiment 4), apart from the following modifications: Mosquitoes that flew through the air curtain and landed on the volunteer were collected with a modified hand-held vacuum cleaner. The total number of mosquitoes entering the tent within 15 min as well as the times of landing were documented.

3.2.6 Room Test with Repellent Evaporating System, Human Bait and BGS-Trap

A simple push-pull situation was evaluated in experiment 6. A BGS trap was positioned on the left side of the tent opening and fitted with a BG lure dispenser. Two independent trials were conducted. The first included the regular tent opening from all former tests (experiments 4 and 5). In the second trial, the size of the opening was reduced to 80 cm × 80 cm and centered in the front tent wall. In both set-ups (regular and reduced tent opening), a CN-HP mix was compared with PIC and repellent-free ethanol controls. The test procedure was consistent with that described for experiment 4, apart from the following modifications: Mosquitoes which flew through the air curtain and landed on the volunteer were collected with a modified hand vacuum cleaner. The total number entering within 15 min and the times of landing were documented. At the end of the test, the catch rate of the BGS trap was recorded and free flying mosquitoes were removed.

Data Analysis Room Tests: For each treatment, mean percentages and standard errors of active test mosquitoes and mosquitoes caught by BGS trap or volunteer were calculated. Data were subjected to an arcsine transformation and then compared using a one-way analysis of variance (ANOVA) and Tukey´s HSD test as a post-hoc test to compare differences between treatments. In experiments 5 and 6, times of entry of the test mosquitoes were of a normal distribution, thus mean times of entry as well as corresponding standard errors were calculated and compared using one-way analysis of variance (ANOVA) and Tukey´s HSD test as a post-hoc test. A P value ≤ 0.05 was regarded as statistically significant. All statistical tests were performed using PAST version 3.04 (Hammer, 2001).

3.3 Results

3.3.1 Olfactometer Assays

Experiment 1 consisted of the evaluation of CN, THY, HP and MP at a quantity of 1.5mg per individual test. THY essential oil was selected as a negative control because it was reported to have weak spatial effects on *Ae. aegypti* in olfactometer tests (Drapeau et al., 2009). The flight activity was high when odors from the finger were tested in combination with ethanol (Fig. 3.2 A, B). The proportion of mosquitoes that left the release cage and flew into the test cage averaged 65.9 to 72.6%. Compared with the ethanol controls, all test compounds had an inhibitory effect on the test mosquitoes. With an average reduction of up to 45% in tests with MP, the overall flight activity was significantly reduced when CN, THY, HP, and MP volatiles were present in the olfactometer (F = 16.91; df = 4; $P \leq 0.001$). The proportion of mosquitoes reaching the test cage close to the stimulus source was also significantly reduced during compound tests (F = 68.93; df = 4; $P \leq 0.001$); however, CN, MP, and HP produced a significantly greater reduction than THY ($P \leq 0.038$; Tukey´s HSD test).

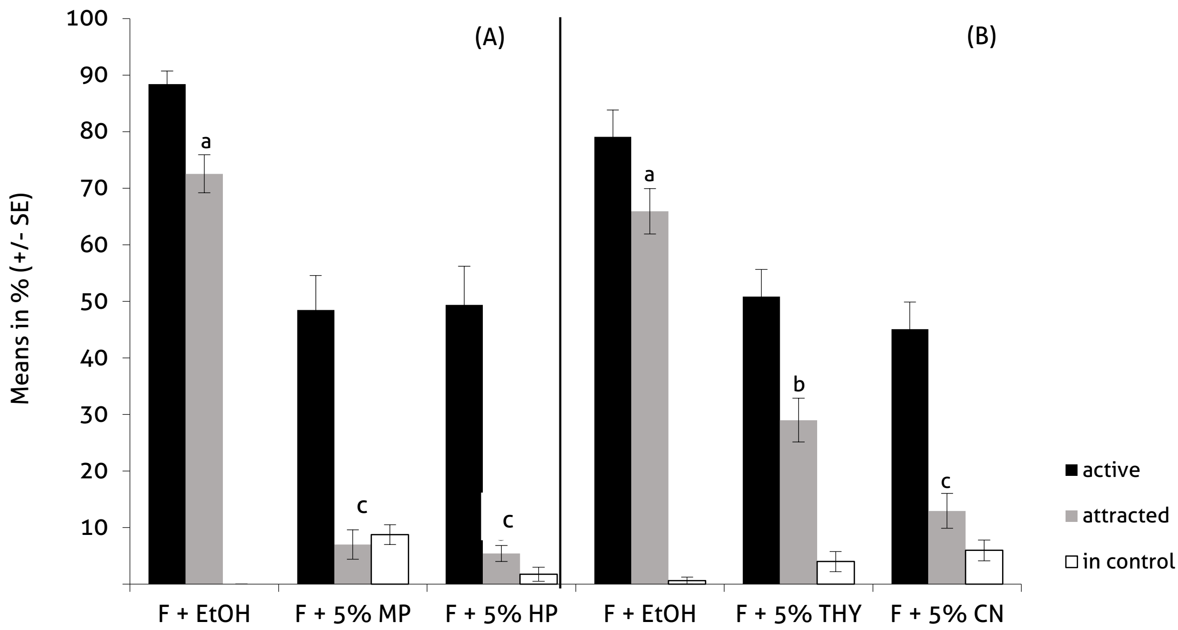

Fig. 3.2: Y-tube olfactometer experiment 1. Mean percentages and standard error (SE) of active mosquitoes, mosquitoes inside test cage (= attracted) and inside control cage. Treatments: Finger (F) plus: ethanol (EtOH) (control); 1-methylpiperazine (MP); homopiperazine (HP); thyme oil (THY) and catnip oil (CN). Test formulations were used at a concentration of 5 %, per trial approximately 1.5 mg of active ingredient were applied. Treatments were evaluated in two experimental blocks (A and B), each treatment was tested in 10 repetitions with *Ae. aegypti*. Different letters indicate significant differences between the proportions of attracted mosquitoes ($P < 0.05$; Tukey´s HSD test).

Experiment 2 evaluated PIC as a negative control. The selection of PIC was based on observations made during repellent efficacy tests of this substance in cages. Shortly after PIC application, *Ae. aegypti* tends to land on the treated skin but then immediately takes off again, indicating that repellency requires direct contact at the normally applied topical concentration. The repellency of 6 mg PIC was compared with ethanol controls and 0.75 mg THY. Flight activity was high and reached an average of 81.4 % in control tests. At the end of the tests an average of 69.3 % of the test mosquitoes were found in the test cage. No inhibition of attraction was observed when PIC was released and the proportion of activated mosquitoes (85.6 %) and mosquitoes inside the test cage (72.5 %) were not significantly different from ethanol controls ($P = 0.97$; Tukey´s HSD test). However, THY produced a significant reduction in the proportion of activated mosquitoes ($F = 10.3$; df = 2; $P \leq 0.001$) and the proportion of mosquitoes that reached the stimulus source ($F = 17.48$; df = 2; $P \leq 0.001$) with average reductions of 28.9 % and 56 %, respectively.
In experiment 3, the effects of 0.75mg of CN, HP, CN-HP, and 1.5mg MP were compared with ethanol controls (Fig. 3.3). All single compounds and the mixture significantly reduced the

proportion of activated mosquitoes (F = 13.01; df = 4; $P \leq 0.001$) and the proportion of mosquitoes attracted to the treatment stimuli (F = 28.52; df = 4; $P \leq 0.001$). CN-HP reduced the proportion of mosquitoes that flew into the test cage by 96.7 %; this was the highest level of reduction recorded for these tests within the olfactometer.

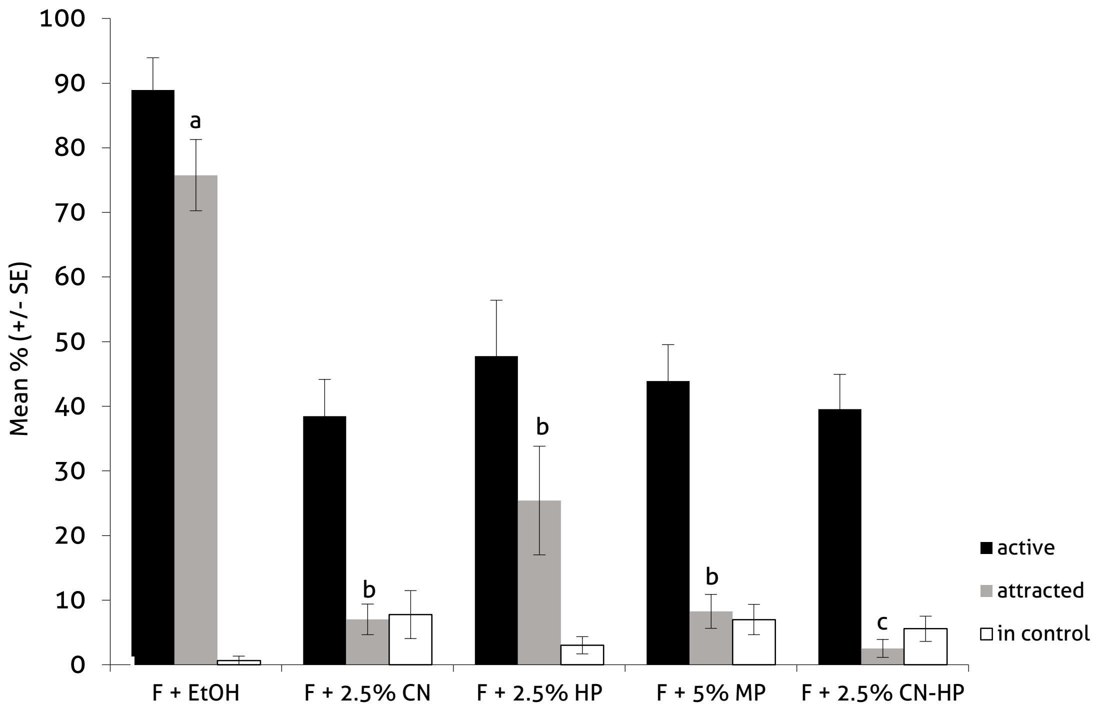

Fig. 3.3: Y-tube olfactometer experiment 3. Mean percentages and standard error (SE) of active mosquitoes, mosquitoes inside test cage (= attracted) and inside control cage. Treatments: Finger (F) plus: ethanol (EtOH) (control); catnip oil (CN); homopiperazine (HP); 1-methylpiperazine (MP) and a mix of catnip oil and homopiperazine (CH-HP). Test formulations were used at concentrations of 2.5 % and 5 %, approximately 0.75 mg to 1.5 mg of active ingredient(s) were applied per individual trial. Each treatment was tested in 10 repetitions with *Ae. aegypti*. Different letters indicate significant differences between the proportions of attracted mosquitoes (P < 0.05; Tukey´s HSD test).

3.3.2 Room Tests with Repellent Evaporating System and BGS-Trap

Experiment 4 was a novel test designed to evaluate the spatial effects of CN, MP, HP, and a CN-HP mix in a room by measuring BGS trap catch rates compared with THY and ethanol controls (Fig. 3.4). Two controls of the BGS trap were recorded with the evaporating system either switched on or off. Mean control catch rates showed no significant differences between the two test conditions (F = 0.80; df = 18; P = 0.42). All of the test compounds significantly reduced the catch rates of the trap (*F* = 37.46; df = 6; *P* ≤ 0.001) but no differences in the general activity of the test mosquitoes were observed (*F* = 0.40; df = 6; *P* = 0.875). Compared with control tests with a repellent-free air curtain, 25mg THY produced the weakest effect with a decreased average trap catch rate of 30 %. Greater reductions in the trap catch rates were observed when 25 mg of MP, CN-HP, and HP were dispensed into the room (*P* ≤ 0.001; Tukey´s HSD test), the highest with a reduction of 95.3 % in tests with HP.

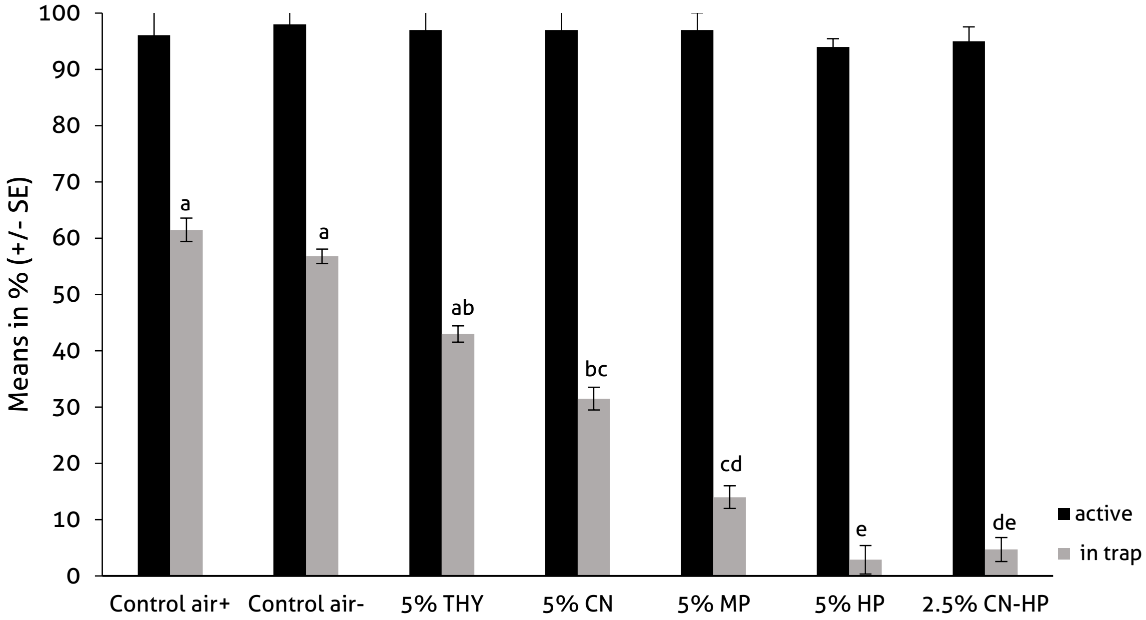

Fig. 3.4: Room test experiment 4. Mean percentages and standard error (SE) of active mosquitoes and proportion of active mosquitoes caught by the BGS trap within 15 min. Each treatment was tested in 10 repetitions with *Ae. aegypti*. Control air+: control tests with air curtain, control air-: control tests without air curtain. Repellent trials: BG-S air+ dispensing thyme oil (THY); catnip oil (CN); 1-methylpiperazine (MP); homopiperazine (HP) and a mixture of catnip oil and homopiperazine (CN-HP). Test formulations were used at concentrations of 2.5 % and 5 %, approximately 12.5 mg or 25 mg of active ingredient(s) were applied per individual trial. Different letters indicate significant differences between the proportions of captured mosquitoes (*P* < 0.05; Tukey´s HSD test).

3.3.3 Room Tests with Repellent Evaporating System and Human Bait

In experiment 5, a volunteer sat inside the tent and documented the number of mosquitoes entering and the times of landing when treatments of either ethanol, 100 mg PIC or 50 mg CN-HP were dispensed at the tent opening. None of the treatments prevented test mosquitoes from entering the tent. All of the mosquitoes that were released reached the volunteer in control tests and tests with PIC and an average of 97.2 % were caught when CN-HP was dispensed. The mean landing/catch rates were not significantly different (F = 2.184; df = 2; P = 0.132), however, the mean times when mosquitoes entered and landed on the volunteer were significantly delayed when CN-HP was released (F = 15.25; df = 2; $P \leq 0.001$) (Fig. 3.5).

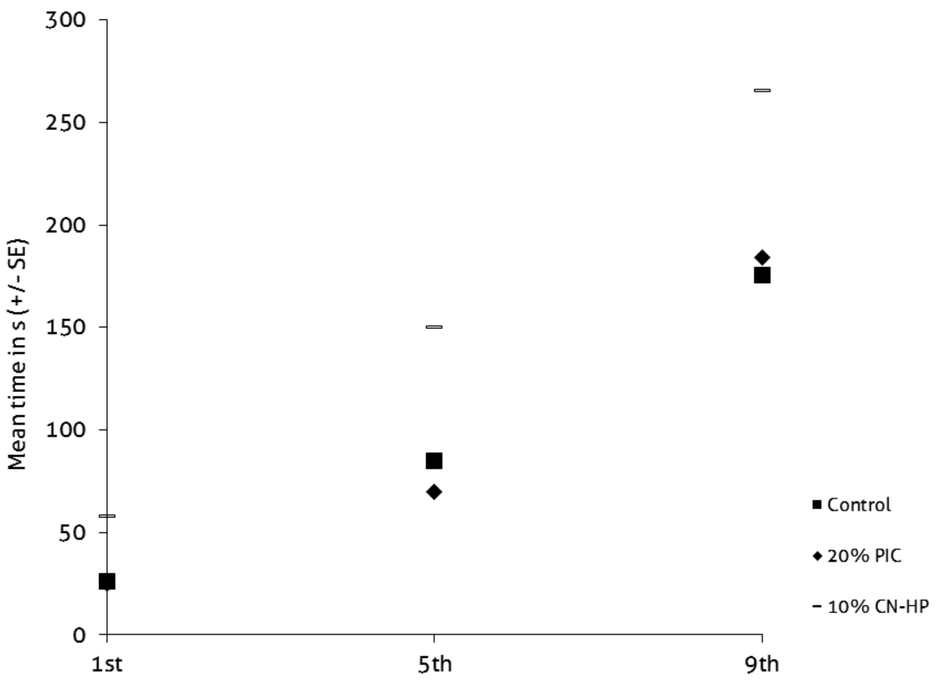

Fig. 3.5: Room test experiment 5. Mean times of the first, fifth, and ninth mosquito landing on the volunteer. Means were generated from 10 replicates per treatment. Tests were performed with one volunteer and *Ae. aegypti*. Treatment formulations were used at concentrations of 10 % and 20 %, a total of 50 mg or 100 mg of active ingredient(s) were used per individual trial.

3.3.4 Room Tests with Repellent Evaporating System, Human Bait and BGS-Trap

We next investigated whether the presence of a BGS trap adds to a reduction of mosquitoes entering the tent and landing on the volunteer. In experiment 6, CN-HP and PIC were used in combination with the BGS trap and evaluated in two settings: 10 replicates with the regular tent opening and 10 replicates using a window opening on the front side. During tests with the regular tent opening, 91.0 % and 82.1 % of the test mosquitoes reached the volunteer during ethanol control tests and tests using 100 mg PIC, respectively. Trap catches averaged 9 % for controls and 17.9 % for PIC. The number of entering and landing mosquitoes was significantly reduced when 50 mg of CN-HP were dispensed (F = 16.95; df = 2; $P \leq 0.001$) while the number of mosquitoes caught by the BGS trap increased (F = 13.37; df = 2; $P \leq 0.001$). Compared with control tests, the proportion of mosquitoes landing on the volunteer was reduced by 42.4 % when CN-HP was used in combination with the BGS trap (F = 6.42; df = 2; $P \leq 0.001$). During control tests, the first mosquito was caught after an average of 31.7 ± 4.6s compared with an average of 82.2 ± 12.6s when CN-HP was present.

The second room test set-up used an 80 cm × 80 cm window opening in the front side of the tent. Compared to trials with the regular opening, the mean catch rates of the BGS trap increased in tests with the control and PIC; however the majority of the released mosquitoes still landed on the volunteer (Fig. 3.6). In contrast, the proportion of mosquitoes that landed on the volunteer was significantly decreased when CN-HP was dispensed (F = 4.53; df = 2; P = 0.02) while the proportion caught by the BGS trap increased. At the end of the test time, an average of 37.8 ± 6.5 % had reached the volunteer while an average of 46.6 ± 5.7 % were caught by the BGS trap. This equates to a 44.3 % reduction in landing rates and a 150 % increase in trap catch rates. The mean landing times of mosquitoes were again significantly delayed during tests of CN-HP (F = 13.21; df = 2; $P \leq 0.001$). The first mosquito was caught after an average of 37.3 ± 5.6s in control tests; compared with an average of 150.8 ± 21.7s for CN-HP.

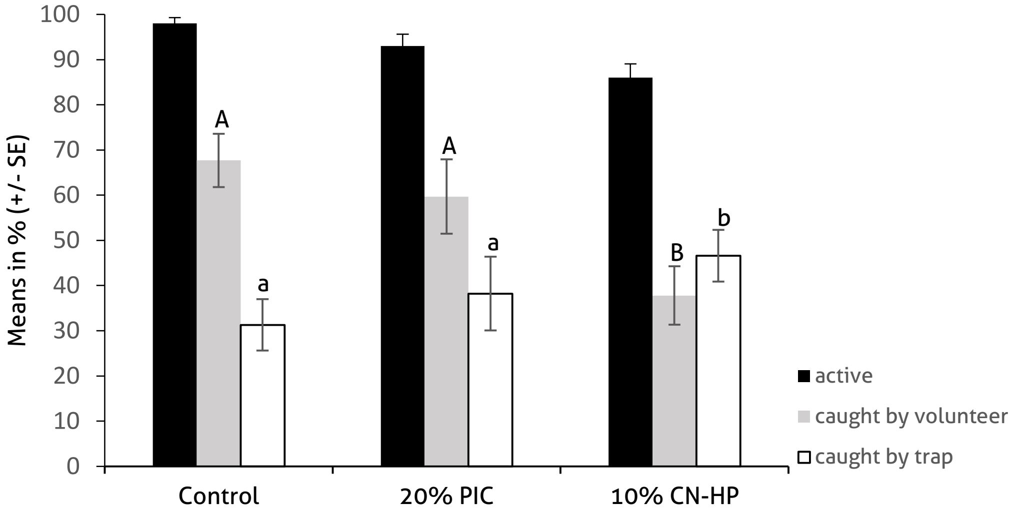

Fig. 3.6: Room test experiment 6 with centered window opening. Mean percentages and standard error (SE) of active test mosquitoes and mosquitoes caught by the BGS trap and volunteer within a testing time of 15 min. Each treatment was tested in 10 repetitions with *Ae. aegypti*. Control: no repellent. Repellent trials included picaridin (PIC) and a mixture of catnip oil and homopiperazine (CN-HP). Test formulations were used at concentrations of 10 % and 20 %, approximately 50 mg (CN-HP) and 100 mg (PIC) of active ingredient(s) were used per individual trial. Different letters indicate significant differences between the proportions of captured mosquitoes ($P < 0.05$; Tukey´s HSD test).

3.4 Discussion

This is the first study to describe a laboratory test set-up which allows the evaluation of mosquito repellents and inhibitors under standardized conditions, while providing insight into the correlation between results obtained in olfactometer assays and in larger scaled tests. Our results also provide evidence that the combination of a suitable spatial repellent or attraction-inhibitor with an attractive trapping system such as the BGS trap can yield a significant reduction of human landing rates (Fig. 3.7).

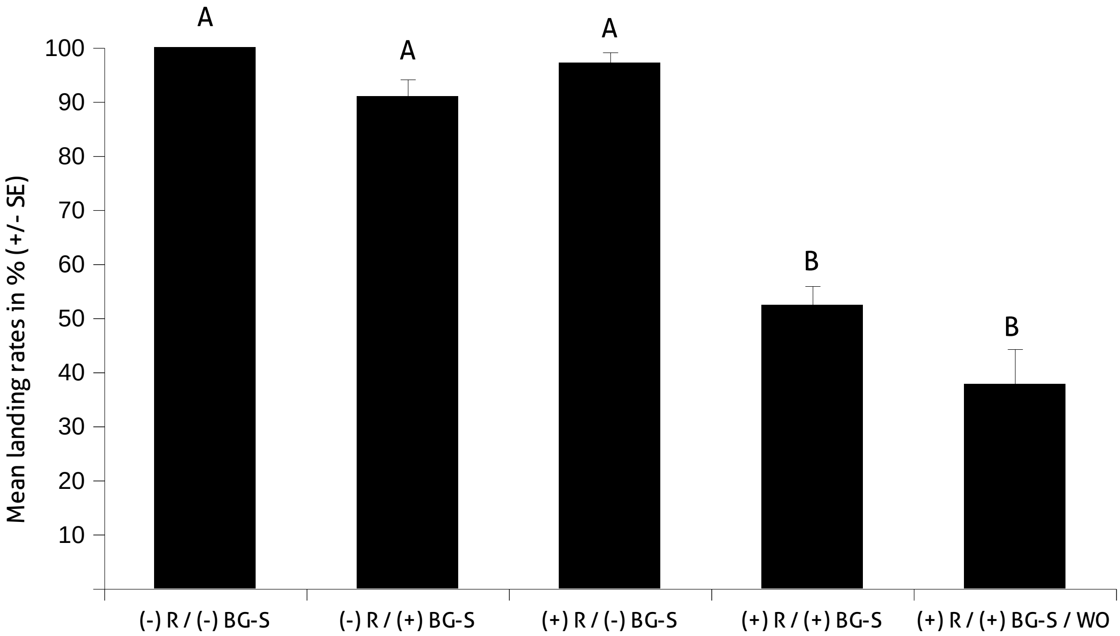

Fig. 3.7: Summary of room test experiments with 10 % CN-HP. Mean mosquito landing rates (± standard error, SE) on the volunteer during different treatments: (-) R / (-) BG-S: no repellent, no trap; (-) R / (+) BG-S: no repellent, trap installed; (+) R / (-) BG-S: repellent dispensed in regular tent opening, no trap; (+) R / (+) BG-S: repellent dispensed in regular tent opening, trap installed; (+) R / (+) BG-S/WO: repellent dispensed in centered window opening, trap installed. Each treatment was tested in 10 repetitions with *Ae. aegypti*. Different letters indicate significant differences between mean mosquito landing rates ($P < 0.05$; Tukey´s HSD test).

3.4.1 Y-Tube Olfactometer Assays

Olfactometer tests are a quick and efficient way to evaluate the behavioral responses of mosquitoes toward volatile stimuli, however they sometimes overestimate efficacy because of the restrictions related to a confined volume and short distances from the point source release of the odors. Thus, results obtained from olfactometer assays may not correlate well with field results obtained with the same test chemicals. To address this issue, we screened potentially non-spatial or contact repellents to find out if the chosen experimental set-ups can provide reliable results for (contact) repellents, as well as spatial repellents that function by inhibiting the ability of mosquitoes to detect and find the source of attractive odors.

Results from olfactometer assays with PIC supported its action as a contact repellent whereas THY exhibited inhibition of the test mosquitoes to find the attractive odor source, even though it was not as potent as effects produced by the release of CN, MP, HP, and CN-HP. Thus, PIC is better suited than THY to serve as a negative control in these spatial efficacy evaluations.

The potential of CN as a spatial repellent has been reported previously (Bernier et al., 2005) and results presented here support those findings. The attraction-inhibitors MP and HP showed a significant reduction of the test mosquitoes' response towards human odors. These inhibitors were reported as superior to CN in recent studies (Bernier et al., 2012). However, the use of MP as a push component is less favorable because it has an unpleasant and obtrusive odor. We decided to focus on HP that also has a distinct but less perceivable smell. The experiments with the formulated CN-HP mixture exhibited the strongest inhibition of *Ae. aegypti* host-seeking, with an average reduction in the attraction of 96.7 %.

3.4.2 Room Tests

A novel test was used to investigate the efficacy of the candidate materials on a larger scale (in a room) by measuring and comparing BGS trap catch rates in the presence and absence of the experimental treatments. In contrast to the olfactometer assays, CN had a weaker effect on mosquitoes in room tests. The best results were obtained from use of HP, which was as efficient in reducing the BGS trap catches as was the CN-HP combination. Room test experiment 4 was designed to investigate if mosquito host-finding can be decreased via the use of an attraction-inhibitor. Only three treatments were evaluated to reduce bites received by the volunteer. Even though CN-HP held great promise based on results from olfactometer assays, it did not provide adequate protection within room tests with landing rates on the volunteer of nearly 100 %. In contrast to olfactometer tests, additional cues like CO_2, body heat, and vision contribute to the test mosquito attraction toward the volunteer and these may override the ability of the attraction-inhibitors to block the detection of other host-produced kairomones. The discrepancy between our results from y-tube and room tests also demonstrates that although olfactometer tests are suited to discriminate between spatial and contact repellent properties (and thereby represent a quick and efficient way to screen a large number of interesting candidate compounds in a short period of time), they do not provide a reliable indication on the magnitude and quality of effects in a larger area. Room test experiment 5 involved a simple push-pull situation to determine if the combination of an attractive trap and an inhibitor leads to a reduction in human-mosquito contact. The push-pull system led to human landing rates that were reduced by 42.4 % and trap catches increased fivefold when 10% CN-HP was dispensed and tested in combination with the BGS trap.

Smoke experiments with ammonium chloride mist revealed that the air curtain had greatest density at the top of the tent opening where it was released and that the density was decreased in the lower half. The air curtain also thinned out towards the sides of the opening and towards the floor. Based on these findings, a modification of the set-up was introduced that used a window opening on the front side of the tent to create a defined area with high repellent density. Even though the window opening restricted the ability of mosquitoes to fly into the tent, the majority of the test mosquitoes still reached the volunteer in control experiments. However, use of the 10 % CN-HP mixture led to an inversion of the catch ratio between volunteer and BGS trap. For the first time in our experiments, more mosquitoes were found in the trap while less than 40 % reached the volunteer (Fig. 3.5). An optimization of the volatile release device might further increase the efficacy of the system.

The establishment of push-pull strategies in vector control is a subject of great interest. Recently, new promising research on behavior modifying chemicals has been published on a mosquito odorant co-receptor-agonist which could disrupt olfactory-mediated behaviors, such as host-seeking (Jones et al., 2011). However, results of this work are based on electrophysiological assays and further testing under realistic conditions is required to verify and confirm the promising effects. Turner et al. (2011) identified odors which inhibit CO_2-sensitive neurons or evoke a CO_2-like activity and their use as more powerful tools in repelling and trapping mosquitoes was suggested. Semi-field tests in experimental greenhouses involving CO_2-emitting counter-flow traps revealed that trap catch rates could be decreased by about 25 % when CO_2-response-modifying odors were dispensed. These findings, however, were not confirmed in tests with human odors as some of the described agonists and antagonists may have undesirable safety profiles at higher concentrations which could disqualify them for human use. In 2010, semi-field tests evaluated the bite-reducing efficacy of the Mosquito Magnet trap (MM trap, American Biophysics Corporation, currently owned by Woodstream Corporation, Lititz, PA, USA) in combination with conventional & commercially available repellents (Kitau et al., 2010). The MM trap ran on propane gas that was catalytically converted to CO_2, heat and water vapor and was equipped with a dispenser that emitted L-lactic acid and ammonia. The use of the trap and a skin repellent in a confined outdoor sphere led to a significant decrease in the human biting rates but further modes of application of the repellent component need to be investigated to reduce the personal effort within the presented approach. Recently, an initial assessment study on the acceptability of a push-pull control strategy which involved common household insecticides in combination with the BGS trap as an outdoor trapping tool was published (Paz-Soldan et al., 2011). Results indicated that the chosen concept could be well accepted by the communities, but it implies the use of insecticides which produce increased resistance in a target mosquito population when used inefficiently or indiscriminately (Fonseca-Gonzales et al., 2010; Polson et al., 2011) and does not comply with the general idea of push-pull as a nontoxic means of pest control (Cook et al., 2007). In addition, depending on the concentration used, insecticides can intoxicate the target mosquito and cause it to rest or seek shelter instead of being attracted to a trap and get caught, a behavior which was also observed during the semi-field tests by Kitau et al. (2010). Our work therefore focused on evaluating non- to low-insecticidal compounds which mediate distance effects without killing or intoxicating the target mosquitoes. In a similar study, repellent plants like wild sage, neem and lemongrass in outdoor plantings were studied for their effect on mosquito house entry in rural tropical areas (Mngongo et al., 2011). When *Lantana camara* was planted outdoors, up to 83 % fewer *An. funestus* were collected indoors compared to control houses. The project aimed to identify affordable means of mosquito control for developing countries and did not imply research on pull components.

All these publications indicate that push-pull could be a viable concept for mosquito control; however, there is still a great need for (1) standardized methods to assess and evaluate spatial effects of both repelling and attracting compounds and (2) elaborate techniques to apply single components in the most efficient yet easy way in a natural setting. Even though our experimental design represents a simple and basic approach, our findings from laboratory tests indicate that a push-pull system based on an attractive trapping tool and volatile inhibitors is capable of reducing human-vector contact in a confined area. Field tests involving experimental huts or tents need to be the next step and will help to investigate if distance effects persist under realistic conditions. Future studies should also include modifications of the repellent dispensing system, to create long-lasting effects as well as the use of multiple traps and various ways of trap arrangement in order to find out if human landing rates can be further decreased.

4. The Potential of Carbon Dioxide in Mosquito Host Attraction

Successful interference with the host-seeking process of anthropophilic mosquitoes depends on the inhibition of both, CO_2 and skin odor perception. In 2012, our group screened several compounds for their attraction-inhibiting potential for skin odors in y-tube olfactometers. We introduced a two-component blend, a mixture of homopiperazine and catnip oil (CN-HP) that reduced *Ae. aegypti* attraction to skin odors by more than 95 %, however, when tested in room tests involving a human volunteer, inhibitory effects could no longer be detected (Obermayr et al., 2012). We hypothesized that the presence of CO_2 rendered the attraction inhibition to skin odors ineffective.

In the present study, we tested the potential of a CO_2 blocking blend consisting of 2,3-butanedione, 1-butanal, 1-pentanal and 1-hexanol as reported by Turner et al. (2011) on *Ae. aegypti* in y-tube olfactometer trials and room tests. Results confirm the strong impact of CO_2 on behavioral assays, and our experiments showed that mosquito host attraction could not be interrupted even when a combination of CN-HP and the CO_2 blocking blend were used.

4.1 Introduction

Mosquitoes are attracted to their blood-hosts through a variety of cues, including heat, humidity, movement and most importantly host-derived odorants like exhaled CO_2 and skin odors (Daykin et al., 1965; Day, 2005; Cardé, 2015). Host location primarily relies on olfactory cues that are perceived by specific receptors located inside cuticular sensory hairs (*sensilla*) on the antennae and maxillary palps (McIver, 1982; Davis, 1984).

Mosquito olfaction is driven by olfactory receptor neurons (ORNs) embedded in the sensilla. Here, chemical signals are transduced into electric outputs that impart information to higher brain centers for the regulation of many behaviors (Galizia & Roessler, 2010; Kaupp, 2010; Bohbot & Dickens, 2012). Detection of specific odorants is accomplished by receptor proteins localized in the dendritic membrane of the ORNs. These receptor proteins belong to one of three classes: odorant receptors (OR), ionotropic receptors (IR) or CO_2-sensing gustatory receptors (GR) (Hansson & Stensmyr, 2010; Bohbot & Pitts, 2015). ORs have been best studied in insects. A functional OR is a heteromeric complex that consists of an odorant-binding subunit and a universal co-receptor called *Orco* (Sato et al., 2008; Kaupp, 2010; Jones et al., 2011, Bohbot & Pitts, 2015). Orco plays a key role in dendrite signal trafficking and orco knockout mutant mosquitoes showed a reduced response to human odors in the absence of CO_2 (DeGennaro et al., 2013). In the presence of CO_2, attraction to host odors was retained but the preference for human odors was impaired in *Ae. aegypti* orco mutants. It was speculated that OR/Orco pathways provide information about the identity of a host and specific ORs may mediate preference for humans in anthropophilic mosquitoes (DeGennaro et al., 2013). While the exact composition of the OR/Orco complex is still unknown, there are strong indications that both units interact to form a functional, ligand-activated ion channel (Sato et al., 2008). Early electrophysiological studies classified ORs as "generalists" (responding to host- or plant odors) and "specialists" (responding to pheromones) (Boeckh et al, 1965). The "weak shape theory" postulates that generalists recognize only part of an odorant and therefore can accommodate a variety of odorants provided that they share common chemical features (Rinaldi, 2007; Bohbot & Pitts 2015). Recent studies on OR functions in *An. gambiae* support the generalist definition for most ORs (Hallem & Carlson, 2006). However, a growing number of "specialized generalist" ORs have been identified that show high ligand specificity (Hughes et al., 2010; Pelletier et al., 2010), enantiomer selectivity (Bohbot & Dickens, 2012) or specifically respond to insect repellents (Pellegrino et al., 2011) or other synthetic compounds (Jones et al, 2011). These findings indicate that narrow tuning is not an exclusive attribute of specialist ORs and that the *combinatorial coding theory* which proposes that a general odorant is detected by a unique set of generalist ORs while a pheromone is specifically recognized by a specialist OR needs to be revisited (Bohbot & Pitts, 2015). Newer models suggest an evolutionarily driven adaption of orthosteric sites (ligand binding sites) on the receptor that allow the specific recognition of low concentrations of semiochemicals (narrow-tuning) while broad-tuning is caused by high concentrations of chemicals that interact with orthosteric and allosteric sites (non-ligand binding sites) (Bohbot & Dickens, 2012, Bohbot & Pitts, 2015). With a limited set of 110 ORs in *Ae. aegypti* (Bohbot et al., 2007), detection and distinction of evolutionary meaningful chemicals may also be based on the ecological context, e.g. mate, host or oviposition site, and not so much on the pharmacological properties (Bohbot & Pitts, 2015, McBride, 2016).

Expanding our knowledge on olfactory receptors involved in host-location could be useful for the development of novel insect control strategies, e.g. masking agents that inhibit the response to attractive human odors or CO_2 (Carey & Carlson, 2011; Ray 2015). Carbon dioxide is detected by cpA neurons housed in the capitate peg sensilla on the maxillary palps. These particular ORNs express three conserved members of the Gr gene family (Gr1, Gr2 and Gr3) that form the CO_2 receptor (Robertson & Kent, 2009). In 2011, Turner et al. investigated volatile odorants for their ability to elicit an unusual, ultra-prolonged activation or inhibition of the cpA neuron, thereby compromising the test mosquitoes´ ability to detect CO_2. After pre-exposure to a four-component blend, *Ae. aegypti* females showed strongly reduced upwind flight behavior towards CO_2 in wind tunnel experiments. Compared to controls, their attraction to 1 vol% of CO_2 was reduced by 90 % after pre-exposure to a 0.1 % formulation of 2,3-butanedione, 1-butanal, 1-pentanal and 1-hexanol. When the same four-component blend was used in CO_2-baited traps in a semi-field environment, trap catch rates for *Cx. quinquefasciatus* were reduced by 60 % compared to controls. Carbon dioxide is a very potent mosquito attractant. In wind tunnel experiments, *Ae. aegypti* responded with a far greater sensitivity to dilutions of human-emitted levels of CO_2 compared to dilutions of skin odors. Source finding rapidly decreased when skin odors were diluted, however the test mosquitoes´ ability to locate the odor source was restored as soon as CO_2 was added (Dekker et al., 2005).

In a previous study, our group reported a significant reduction in the attraction to skin odors when *Ae. aegypti* was exposed to CN-HP in y-tube olfactometers (Obermayr et al., 2012). On average, test mosquitoes' responses to the attractive odor source were reduced by more than 95 %. However, when tested on a larger scale with a human volunteer emitting the full spectrum of attractive cues including CO_2, such an effect could no longer be documented. Nearly 100 % of the test mosquitoes located the volunteer within a room test set-up, indicating that the attraction reduction to skin odors provided by the two component blend was rendered ineffective in the presence of CO_2.

In the present chapter, we further investigated the impact of CO_2 on spatial repellency and tested if the additional use of potential CO_2 blocking agents can help to reestablish the attraction reduction to human body odors. We used the four-component blend described by Turner et al. (2011) for pre-exposure experiments with *Ae. aegypti* prior to y-tube olfactometer and room tests and investigated its impact on test mosquitoes' responses towards CO_2 and body odors.

4.2 Materials and Methods

4.2.1 Test Materials

Homopiperazine (HP), 2,3-butanedione, 1-butanal, 1-pentanal and 1-hexanol were purchased from Sigma-Aldrich (Taufkirchen, Germany), pure catnip (CN) (*Nepeta cataria*) essential oil was acquired from Aromaland (Röttingen, Germany). CN and HP were diluted vol:vol in ethanol (96 %, p.a.) to final concentrations of 10 % and 5 %. Both compounds were also diluted w:w in paraffin oil (laboratory grade) to a final concentration of 1 %. Following the protocol of Turner et al. (2011) all remaining compounds were diluted w:w in paraffin oil to final concentrations of 1 %.

The kairomone-attraction inhibiting mixture was prepared by diluting 5 % CN and 5 % HP at a 1:1 ratio to obtain a 2.5 % formulation in ethanol. In previous olfactometer trials this mixture has shown strong attraction-inhibiting effects when used at doses of 30 µl per individual test (representing approximately 0.75 mg per active ingredient). Following the methodology of Turner et al. (2011) the CO_2 blocking blend ("Turner blend", TB) was prepared by diluting 1 % 2,3-butanedione, 1 % butanal, 1 % 1-pentanal and 1 % 1-hexanol at a 1:10 ratio in paraffin to obtain a 0.1 % formulation. In olfactometer trials, 30 µl (0.03 mg per active ingredient) of TB were used per single test. In room tests with pre-exposure, mosquitoes inside transport cages were transferred to an upended metal bowl to be exposed to 100 µl of 0.1 % TB or 0.1 % CN-HP (0.1 mg per active ingredient) for 3 min, similar to the procedure described by Turner et al. (2011). The 0.1 % CN-HP formulation was obtained by diluting 1 % CN and 1 % HP at a ratio of 1:10 in paraffin oil.

Spatial repellent room test trials involved a 10 % formulation of CN and HP that was obtained by mixing 20 % CN and 20 % HP at a 1:1 ratio in ethanol. Following the protocol from previous room tests (3.2.4, p. 31) 500 µl of the 10 % mixture (representing approximately 50 mg per active ingredient) were used per individual test.

4.2.2 Test Mosquitoes

Aedes aegypti females (originally obtained from BAYER AG, Monheim, Germany) aged 10-16 days were used for all tests. Preliminary olfactometer tests revealed that our colony shows a comparable susceptibility for repellent volatiles at days 6-20 after emergence while responses to a finger or repellent volatiles show greater variations at a younger age (1-5 d). Mosquitoes were reared according to the procedure described in chapter 3 (3.2.2, p. 29). Behavioral tests were performed with host-seeking females, which were lured out of their breeding cages at least 10 min before the start of the tests. The selection procedure followed the one described in chapter 3 (3.2.2, p. 30).

4.2.3 Y-Tube Olfactometer Assays

Olfactometer tests were performed according to Geier and Boeckh (1999). The test apparatus was as described in chapter 2 (2.2.2, p. 20). In total, four Y-tubes, identical in construction, were used to measure the behavioral responses of host-seeking *Ae. aegypti* females towards a mix of CN-HP and TB. Carbon dioxide (99.9% purity) was taken from a 10 kg gas cylinder (Linde, Germany) and applied at a flow rate of 300 ml/min. This particular flow rate produces a concentration of approximately 0.2 vol% of CO_2 inside the airstream of the olfactometer. Strong activation and upwind flight responses have been reported for *Ae. aegypti* when CO_2 was used of at concentrations between 0.1 and 5 vol% in the olfactometer (Geier et al., 1999b).

Test procedure: the attraction-inhibition to skin odors was measured according to the procedure described in chapter 3 (3.2.3, p. 30). In trials involving the CO_2-inhibiting blend (TB), a modification of the apparatus was made by positioning a perspex ring between the release cage and downwind end of the base leg (Fig. 4.1). The ring had a 2 cm × 0.5 cm opening that was used to introduce a 1 cm × 3 cm filter paper strip that had been treated with 30 µl of 0.1 % TB or 0.1 % CN-HP. The rotating door of the release cage remained closed and mosquitoes were exposed to the volatiles for 3min. Afterwards the filter paper was removed, the attracting stimulus (CO_2 at 300 ml/min) was added and mosquitoes were allowed to respond for 30s. All treatments were tested in a randomized order, and after each run, the control branch and test branch were changed to avoid position or adaptation effects.

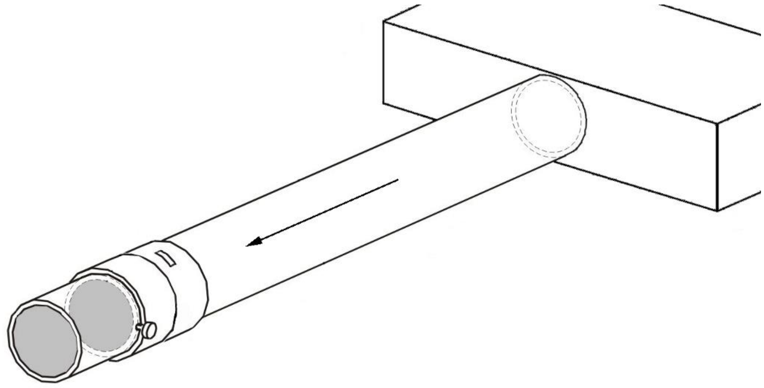

Fig. 4.1: Modification of the y-tube olfactometer base leg to test the effects of potential CO_2 blocking agents. A perspex ring with a 0.5 cm × 2 cm opening is inserted between release cage and base leg for the introduction of treated filter paper strips.

Within each experimental block, 10 replicates were performed per treatment. In experiment 1, the kairomone attraction reduction potential of CN-HP was tested against two controls; (1) a forefinger and a paper strip treated with 96 % ethanol and (2) a forefinger, a paper strip treated with 96 % ethanol and 300 ml/min CO_2. In experiment 2, test mosquitoes responses towards CO_2 were tested after a 3min pre-exposure to paraffin (control), TB or CN-HP.

Data Analysis Olfactometer Assays: For each treatment, mean percentages of active test mosquitoes, mosquitoes inside test chamber, and control chamber were calculated, as well as corresponding standard errors. Data were subjected to an arcsine transformation and then compared using one-way analysis of variance (ANOVA) and Tukey´s honestly significant difference (HSD) test as a post-hoc test to verify significant differences between single treatments. A P value ≤ 0.05 was regarded as statistically significant. All statistical tests were performed using PAST version 3.04 (Hammer, 2001).

4.2.4 Room Tests with Repellent Evaporating System and Human Bait

The test set-up used was as described in chapter 3 (3.2.4, p. 31). A volunteer sat inside the tent to attract host-seeking *Ae. aegypti* to fly through the curtain for potential capture. For each test, 10 mosquitoes were released into the room at the side furthest from the tent. After release, mosquitoes were allowed to respond for 15min. Mosquitoes that flew through the air curtain and landed on the volunteer were collected with a modified hand-held vacuum cleaner. The total numbers of mosquitoes entering the tent within 15min as well as the times of landing were documented. In trials with pre-exposure, mosquitoes were held inside an upended metal bowl for 3min. At the end of the exposure period, they were quickly transferred (less than 10s) to the test room and released. In room test experiment 3, a total of four treatments were tested; (1) no pre-exposure to TB, no dispersal of CN-HP (= control); (2) pre-exposure to TB, no dispersal of CN-HP; (3) pre-exposure to CN-HP, no dispersal of CN-HP and (4) pre-exposure to TB, dispersal of CN-HP. Each treatment was tested in 10 replicates in a randomized order. To avoid an accumulation of the volatile stimuli, the room was aerated for at least 30min before the next test was conducted.

Data Analysis Room Tests: For each treatment, mean percentages and standard errors of mosquitoes caught by the volunteer were calculated. Times of entry of the test mosquitoes were of a normal distribution, thus mean times of entry were compared using one-way analysis of variance (ANOVA) and Tukey´s HSD test as a post-hoc test. A P value ≤ 0.05 was regarded as statistically significant. All statistical tests were performed using PAST version 3.04 (Hammer, 2001).

4.3 Results

4.3.1 Olfactometer Assays

Experiment 1 compared the kairomone attraction inhibiting potential of CN-HP on skin odors (alone) and skin odors in combination with CO_2. Flight activity and attraction to the source were high when skin odors were presented (Fig. 4.2). In controls using the forefinger, an average of 79.8 % left the release cage and an average of 75.2 % was attracted to the stimulus source. The addition of CO_2 significantly increased the test mosquitoes´ response to the positive stimuli (F = 18.9; df = 3; P < 0.004 (upwind flight) / F = 31.6; df = 3; P < 0.001 (attraction to the source)). In trials using the forefinger in combination with CO_2, an average of 98.6 % left the release cage and an average of 90.8 % reached the stimulus source.

Compared to these controls, the addition of CN-HP had an inhibitory effect and caused a significant reduction in the proportion of test mosquitoes reaching the stimulus source (F = 31.6; df = 3; P < 0.001). When CN-HP was added to skin odors, the attraction reduction averaged 72 % compared to controls. In the presence of CO_2, the average attraction reduction was 27 % compared to controls. Regarding the level of inhibition through CN-HP, significantly more mosquitoes reached the stimulus source when CO_2 was present compared to tests of skin odors only (F = 31.6; df = 3; P < 0.001).

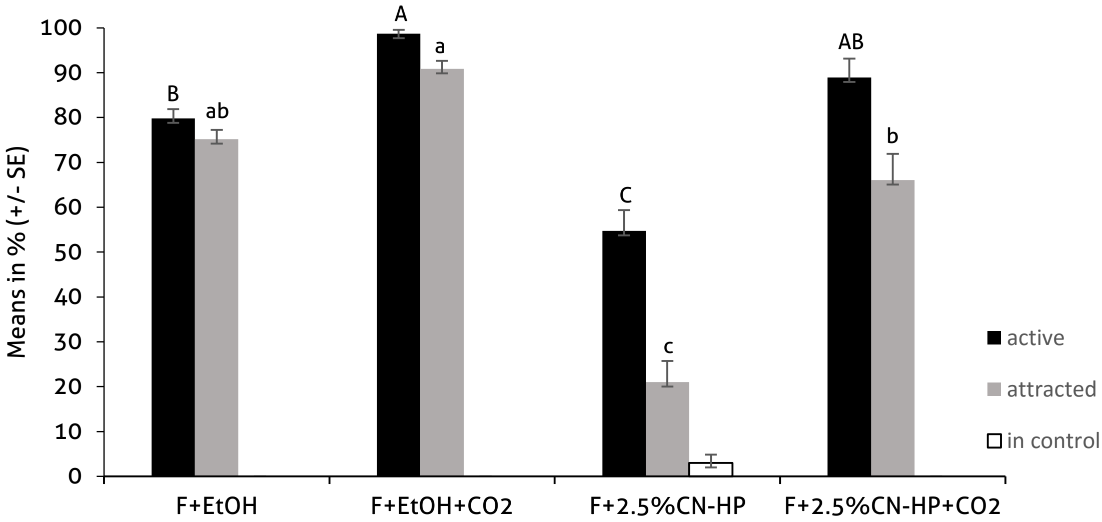

Fig. 4.2: Y-tube olfactometer experiment 1. Mean percentages and standard error (SE) of active mosquitoes, mosquitoes inside test cage (=attracted) and inside control cage. Treatments: Finger (F) plus ethanol (EtOH) / plus CO_2 in comparison to finger plus repellent (catnip and homopiperazine, CN-HP). CN-HP was used at a concentration of 2.5 %, approximately 0.75 mg per active ingredients were applied per individual trial. Each treatment was tested in 10 repetitions with *Ae. aegypti*. Different letters indicate significant differences between the proportions of active and attracted mosquitoes (P < 0.05; Tukey´s HSD test).

In experiment 2, mosquitoes were pre-exposed to volatiles of TB or CN-HP and their behavioral response towards CO_2 was compared to controls using paraffin oil (Fig. 4.3). In control experiments, 80.4 % were activated and showed upwind flight behavior; an average of 43 % reached the test cage by the end of the testing time. Compared to controls, the pre-exposure to TB had no significant inhibitory effect on the test mosquitoes´ response to CO_2 (F = 6.425; df = 2; P = 0.078), their attraction was reduced by an average of 24 %. The inhibitory effect was significantly greater after the pre-exposure to CN-HP (F = 6.425; df = 2; P = 0.004), now the proportion reaching the stimulus source was reduced by an average of 37 %.

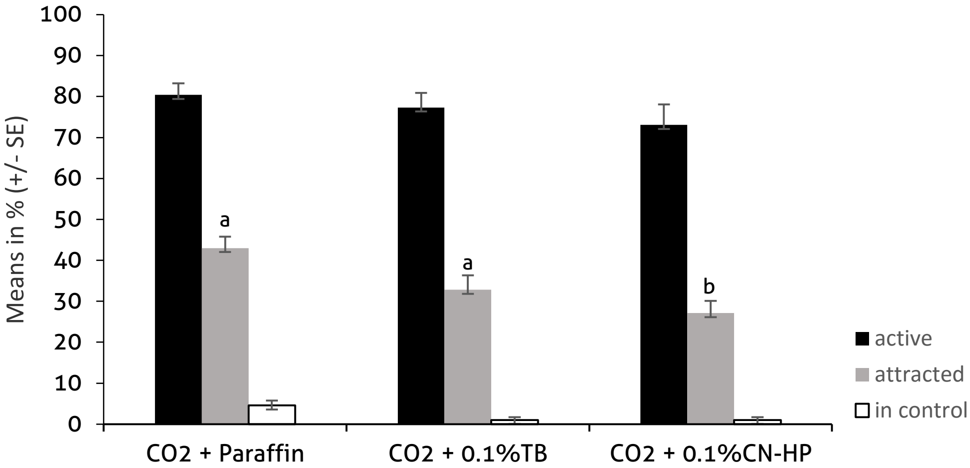

Fig. 4.3: Y-tube olfactometer experiment 2. Mean percentages and standard error (SE) of active mosquitoes, mosquitoes inside test cage (=attracted) and inside control cage. Treatment formulations were used at a concentration of 0.1 %, representing approximately 0.03 mg of active ingredient(s) per individual trial. In controls, mosquitoes were pre-exposed to paraffin oil; in CO_2 blocking trials mosquitoes were pre-exposed to the Turner blend (TB) or catnip oil and homopiperazine mix (CN-HP). Each treatment was tested in 10 repetitions with *Ae. aegypti*. Different letters indicate significant differences between the proportions of active and attracted mosquitoes ($P < 0.05$; Tukey´s HSD test).

4.3.2 Room Tests with Repellent Evaporating System and Human Bait

In experiment 3, a volunteer sat inside the tent and documented the number of mosquitoes entering and their times of landing when treatments of either ethanol (control) or CN-HP were dispensed at the tent opening and test mosquitoes had been pre-exposed to paraffin (control), TB or CN-HP. None of the treatments had an inhibitory effect and prevented test mosquitoes from entering into the tent, between 97 % and 100 % were recaptured by the volunteer. The mean times when mosquitoes landed on the volunteer also showed no significant differences between control and treatment trials ($F = 1.581$; df = 3; $P = 0.211$) (Fig. 4.4).

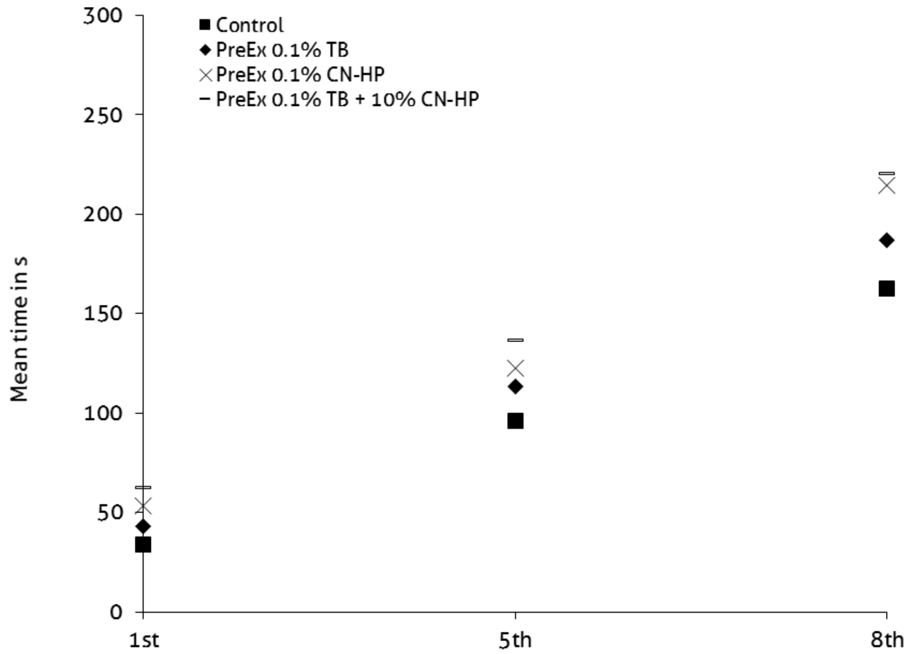

Fig. 4.4.: Room test experiment 2. Mean times of the first, fifth, and eighth mosquito landing on the volunteer. Treatment formulations were used at concentrations of 0.1 % or 10 %. In pre-exposure experiments (PreEx), mosquitoes were exposed to 0.1 mg of Turner blend (TB) or catnip and homopiperazine (CN-HP). In room tests, approximately 50 mg CN-HP were dispensed. Each treatment was tested in 10 repetitions with *Ae. aegypti*.

4.4 Discussion

The identification and development of volatile chemicals that interfere with mosquitoes´ ability to locate a host has been a challenge to the research community for decades. Mosquitoes possess a highly sensitive olfactory system that allows them to decode complex odor blends emitted from human skin and perceive minute changes in background CO_2 levels; thus any effective interruption of mosquito host perception requires blocking of both, skin odor- and CO_2 perception. In 2012, our group presented a two-component blend (CN-HP) that reduced mosquito attraction to skin odors in y-tube olfactometers by more than 95%; however when tested on a larger scale, in the presence of a volunteer emitting the full spectrum of attractive odors, attraction reduction was no longer observed (Obermayr et al., 2012).

When CO_2 was added to skin odors in olfactometer trials mosquito attraction to the positive stimuli was significantly stronger in the presence of CN-HP compared to tests of skin odors alone (F = 31.6; df = 3; P < 0.001), demonstrating the powerful impact of CO_2. We hypothesized that the addition of CO_2 blocking agents to CN-HP might restore the inhibitory effects and this is the first study to investigate the effect of such a CO_2 blocking blend (Turner blend, TB) in the presence of a human volunteer.

When Turner et al. (2011) introduced their four-component blend they used wind tunnels to investigate its impact on CO_2 perception. Mosquitoes were pre-exposed to the blend for 1min, transferred to the wind tunnel and allowed to respond to 1 vol% of CO_2. In control experiments, *Ae. aegypti* showed 100 % upwind flight behavior and 100 % source finding, while pre-exposure to the four-component blend led to a 90 % reduction in upwind flight and a 95 % reduction in source finding.

In contrast to the original wind tunnel protocol, our study used y-tube olfactometers and exposed mosquitoes to the same blend at the downwind end of the base leg before they were allowed to respond to 0.2 vol% of CO_2. Preliminary experiments using an upended bowl and a 1min pre-exposure revealed that mosquitoes were not responding to positive stimuli shortly after being attached to the apparatus, even in paraffin controls (data not shown). Since their behavioral responses to CO_2 needed to be measured within seconds after the pre-exposure, we decided to expose mosquitoes to the blend at the downwind end of the olfactometer. We increased pre-exposure time from 1min to 3min since mosquitoes were now held in a constant air-stream compared to stagnant air in the upended bowl set-up.

In control tests, mosquitoes responded with strong activation and upwind flight behavior, approximately half of the activated mosquitoes reached the CO_2 source. This response is typical for CO_2 in wind tunnels, it elicits orientation behavior and high flight velocities while source finding is usually reduced in the absence of skin odors (Dekker et al., 2005). In our experiments, a change in the test mosquitoes´ response to CO_2 could not be detected after pre-exposure to TB; upwind flight activity remained high and 33 % reached the stimulus source (compared to 43 % in paraffin controls). Interestingly, CN-HP had a significantly greater inhibitory effect compared to TB (F = 6.425; df = 2; P = 0.004), here only 27 % reached the CO_2 source (Fig. 4.3).

It is possible that the poor inhibitory effects of TB were related to our modified olfactometer set-up, in particular to the pre-exposure inside an air-stream that could lead to an increased evacuation of volatiles. Subsequently, we conducted room tests with a human volunteer and used mosquitoes that had been pre-exposed to TB inside an upended bowl, following the original protocol by Turner et al. (2011). Preliminary experiments with a 1min exposure showed no behavioral changes in the test mosquitoes´ responses (data not shown) thus pre-exposure was set to 3min. Again, no inhibitory effects were observed. Mosquitoes were highly attracted to the volunteer after being pre-exposed to paraffin, TB or CN-HP and recapture rates ranged between 99 % and 100 %. When 10 % CN-HP was dispensed into the test room as an additional spatial repellent, landing times were only slightly delayed when mosquitoes had been pre-exposed to TB (Fig. 4.4).

Currently, there are very few other studies available that investigate the inhibitory effects of newly discovered compounds or mixtures in the presence of humans. Jones et al. (2011) presented VUAA1 [=2-(4-ethyl-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-ylthio)-N-(4-ethylphenyl)acetamide], a molecule of relatively low volatility that was found to be an agonist to Orco in *An. gambiae* (AgOrco) and was suggested to interfere with the discrimination of odors in mosquitoes. Their results were derived from small-molecule screens against human embryonic kidney cells expressing AgOrco. However, up to now there are no data on the impact of VUAA1 on mosquito behavior available. Recently, an extensive computational screening of over 400,000 chemicals for their potential to act as ORN ligands led to the discovery of two compounds that inhibited the cpA neuron, which is responsible for CO_2 detection in mosquitoes. The impact of these compounds (ethyl pyruvate, methyl pyruvate) has not yet been tested on a larger scale in the semi-field or field. When ethyl pyruvate was used in cage tests with *Ae. aegypti*, attraction to human skin was significantly reduced, indicating that this compound might also interfere with the perception of skin odors (Tauxe et al. 2013).

A better understanding of the underlying neuronal mechanisms of repellent-receptor interactions could help to develop new generations of substances that interfere with mosquito host attraction in a more specific and powerful way. Effort has been put into the screening for such compounds (Ray, 2015; Tauxe et al., 2013) but behavioral data are scarce. Our results demonstrate the strong impact of host cues, especially CO_2, on the outcome of spatial repellent assays; indicating that behavioral tests in the presence of human volunteers will be essential to fully assess the inhibitory potential of newly discovered compounds.

5. Using Catnip Essential Oil as a Paradigm to Search for New Spatial Repellents for *Ae. aegypti*

Catnip (*Nepeta cataria*) (CN) essential oil shows spatial repellent properties against a variety of insects including mosquitoes and this activity can be attributed to nepetalactone, the main constituent of the oil. Nepetalactone is a bicyclic monoterpene, a substance class that can be found in a large number of plant families, but also in the defensive secretions of insects. The following chapter investigates whether other structurally related compounds, like iridomyrmecin (IM) and menthalactone (ML), or other defensive secretions like salicylaldehyde (SA) show comparable spatial repellent effects. We also included another essential oil that is rich in mono- and sesquiterpenes (*Valeriana officinalis* L.). When tested in y-tube olfactometers against *Ae. aegypti*, CN, IM, ML and valerian oil (VAL) significantly reduced test mosquitoes´ attraction to skin odors by an average of 70% to 88% (P <0.001, Tukey´s HSD test).

5.1 Introduction

Plants produce a variety of chemical compounds (*phytochemicals*) that play important roles in plant-plant, plant-pathogen and plant-insect interactions. Among these, terpenoids are the most abundant and structurally diverse group of secondary metabolites (McGarvey & Croteau, 1995; Cheng, 2007). Terpenoids are derived from five-carbon isoprene units and depending on the number of units, are classified into different groups, like monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), tetraterpenes (C40) and poly-terpenes (Paluch et al., 2009a). Many mono- and sesquiterpenes have been characterized to act as mosquito repellents (Paluch et al., 2009ab) and, depending on their volatility, they exhibit either contact or spatial repellent properties. At lower vapor pressures, the residual repellency on a treated surface is stronger, whereas at higher vapor pressures greater evaporation and spatial repellent effects occur (Schultz et al., 2006).

The main constituent (70-98%) of catnip oil is nepetalactone, an *iridoid* monoterpene that occurs in two isomers, *Z,E*- and *E,Z*-nepetalactone (Fig. 5.1) (Bates and Sigel, 1963). Iridoids form a large group of bicyclic monoterpenes found in a number of plant families. Their name is derived from iridomyrmecin, iridolactone and iridodial, compounds that were first isolated from defensive secretions of ants belonging to the genus *Iridomyrmex* (Cavill et al., 1956; El-Naggar & Beal, 1980).

Nepetalactone has diverse effects and is, for instance, repellent to members of at least 13 families of insects (Eisner, 1964), a feline attractant (Waller et al., 1969), a component of the defensive secretions of coconut stick insects, *Graeffea crouani* Le Guillou (Smith et al., 1979) and part of the sex pheromone of the damson-hop aphid, *Phorodon* *humuli* Schrank (Campbell et al., 1990). In mosquitoes, catnip oil has been reported to act as a spatial repellent (Bernier et al., 2005; Polsomboon et al., 2008; Peterson & Coats, 2011; Obermayr et al., 2012). When catnip essential oil and purified nepetalactone isomers were compared for their spatial repellent impact on *Ae. aegypti* in a static air chamber, no significant differences in the individual efficacy were reported (Peterson & Coats, 2011). This demonstrates that the spatial repellent impact of catnip oil is mainly attributed to nepetalactone and that both isomers are equally effective against *Ae. aegypti.* Using nepetalactone as a template, a search for related molecules and mixtures was initiated to identify other potential spatial repellents that might be even more powerful than our catnip oil standard. This chapter investigates the attraction-inhibiting potential of three interesting groups: [1] monoterpenes that are structurally related to nepetalactone, [2] substances that are found in the defensive secretions of certain insects and [3] an essential oil rich in mono- and sesquiterpenes. Iridomyrmecin is structurally related to nepetalactone (Fig. 5.1) and as mentioned earlier, is part of the defensive secretion of ants (Cavill et al., 1956; El-Naggar & Beal, 1980). Menthalactone is also structurally related to nepetalactone (Fig. 5.1). In nature, this monoterpene can be found in a variety of aromatic plants belonging to the genus *Mentha* (Villasenor & Sanchez, 2009) and other *Lamiaceae* that give off minty odors, e.g. *Micromeria* (Radulovic & Blagojevic, 2012; Al-Hamwi et al., 2011). Another defensive secretion included in our experiments is salicylaldehyde (Fig. 5.1), which is produced by some chrysomelid larvae that feed on willows and poplars (*Salicaceae*). Salicylaldehyde is sequestered from a plant precursor, salicin, a characteristic compound found in the leaves and bark (Barbosa & Letourneau, 1988). Valerian essential oil was chosen because it is rich in mono- and sesquiterpenes (Bos et al., 2000; Pavlovic et al., 2004; Wang et al., 2010). We used y-tube olfactometer assays to evaluate the spatial repellent potential of these three groups in comparison to catnip oil. Control experiments involved picaridin (Fig. 5.1), a known contact repellent (Obermayr et al., 2012).

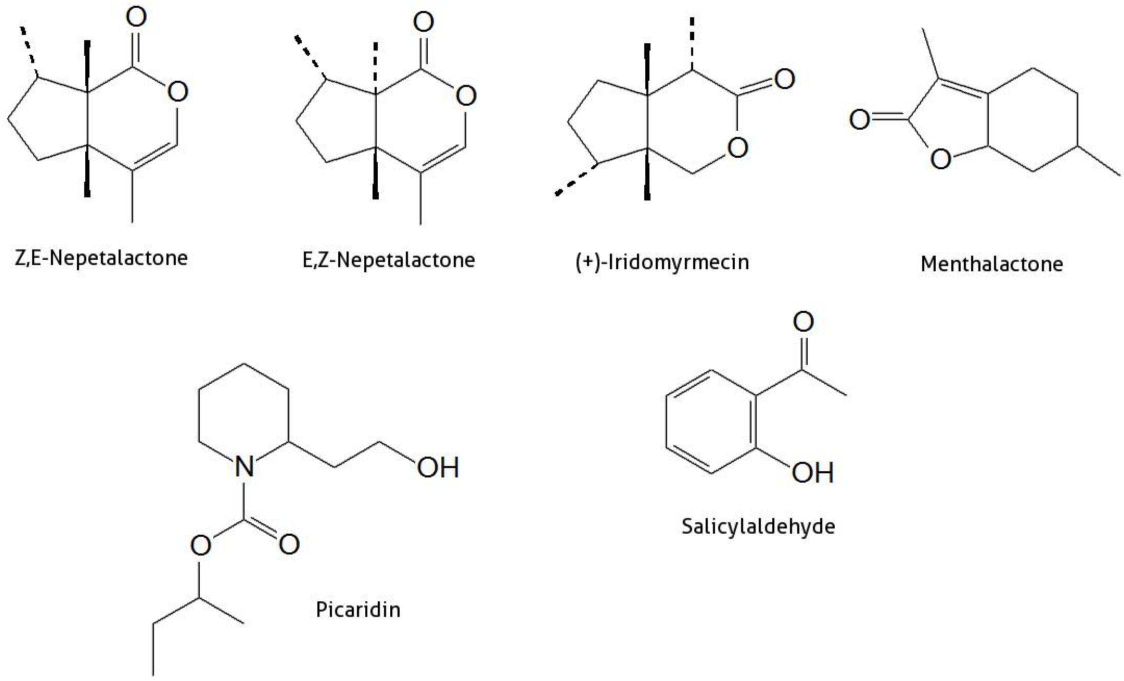

Fig. 5.1: Chemical structures of some repellent molecules used in spatial repellent activity tests with *Ae. aegypti.*

5.2 Material and Methods

5.2.1 Test Materials

ML (unknown stereochemistry) and SA were purchased from Sigma-Aldrich (Taufkirchen, Germany), CN and VAL essential oils were acquired from Aromaland (Röttingen, Germany). For a sound comparison, CN, VAL, ML and SA were diluted vol:vol in ethanol (EtOH; 96 %, p.a.) to a final concentration of 1 %. Only (+)-IM (synthesized by Dr. J. Hofferberth, Kenyon College, Gambier, Ohio) was provided at a concentration of 1% in dichloromethane (DCM). A proprietary repellent formulation (Autan Protection Plus, SC Johnson GmbH, Erkrath, Germany) containing 20 % picaridin (PIC) (hydroxyethyl isobutyl piperidine carboxylate) was purchased from a local drugstore. Per single test, 30 µl (approximately 0.3 mg CN, ML, SA, IM and VAL and 6 mg PIC) of a test formulation were dropped onto a filter paper and held into the air-stream of the olfactometer.

5.2.2 Test Mosquitoes

Aedes aegypti females aged 10-21 d were used for all tests. Preliminary olfactometer tests revealed that our colony shows a comparable susceptibility for repellent volatiles at days 6-20 after emergence while responses to a finger or repellent volatiles show greater variations at a younger age (1-5 d). The colony was obtained originally from BAYER AG, Monheim, Germany, and has been maintained in our facilities over the past 15 years. Mosquitoes were reared according to the procedure describe in chapter 3 (3.2.2, p. 29). Behavioral tests were performed with host-seeking females, which were lured out of their breeding cages at least 10 min before the start of the tests. The handling procedure was as described in chapter 3 (3.2.2, p. 30).

5.2.3 Y-tube Olfactometer Assays

Olfactometer tests were performed according to Geier and Boeckh (1999). The apparatus and testing conditions were as described in chapter 3 (3.2.3, p. 30). In total, four y-tubes, identical in construction, were used to measure the behavioral responses of host-seeking *Ae. aegypti* females towards CN, VAL, ML, IM, SA and PIC. Spatial repellency assays were divided into three different test blocks: block 1 included CN and IM; block 2 included CN, ML and VAL and block 3 included SA and PIC. Treatments were tested in randomized order within each block and spatial repellent effects were compared to solvent controls (EtOH and DCM in block 1, EtOH in blocks 2 and 3). Ten replicates were performed per treatment.

Data Analysis Olfactometer Assays: For each treatment, mean percentages of active test mosquitoes and mosquitoes inside the test and control chambers were calculated as well as corresponding standard errors. Data were subjected to an arcsine transformation and then compared using a one-way analysis of variance (ANOVA) and Tukey´s honestly significant difference (HSD) test as a post-hoc test to verify significant differences between single treatments. A P value ≤ 0.05 was regarded as statistically significant. Ethanol controls of block 1, 2, and 3 as well as data from CN in blocks 1 and 2 showed no significant differences regarding the activation and attraction of the test mosquitoes (EtOH (activation): $F = 0.97$; df = 2; $P = 0.39$ / (attraction): $F = 0.45$; df = 2; $P = 0.64$; CN (activation): $F = 2.1$; df = 1; $P = 0.17$ / (attraction): $F = 0.07$; df = 1; $P = 0.79$), thus data from all blocks were pooled for the statistical analysis. All statistical tests were performed using PAST version 3.04 (Hammer, 2001).

5.3 Results from Olfactometer Assays

In all experimental blocks, flight activity was high when skin odors were tested in combination with EtOH or DCM (Fig. 5.2). The proportion of mosquitoes that left the release cage and flew into the test cage averaged 86 % and 74 % for EtOH and 83 % and 70.4 % for DCM. Compared to the solvent controls, CN, IM, ML and VAL had an inhibitory effect on upwind flight activity and finding the stimulus source. Such an effect was not observed during tests of SA and PIC, here overall flight activity and attraction to the stimulus source were not significantly different from the solvent controls (SA: P = 0.77 (activity); P = 0.71 (attraction) / PIC: P = 0.98 (activity); P = 1.0 (attraction), Tukey´s HSD test). Significant reductions in flight activity were observed in the presence of VAL, IM, CN and ML (F = 20.34; df = 7; P < 0.001). The proportion of mosquitoes reaching the stimulus source was also significantly reduced in the presence of VAL CN, IM and ML (F = 55.58; df = 7; P < 0.001), with an average reduction of up to 88 % in the presence of VAL.

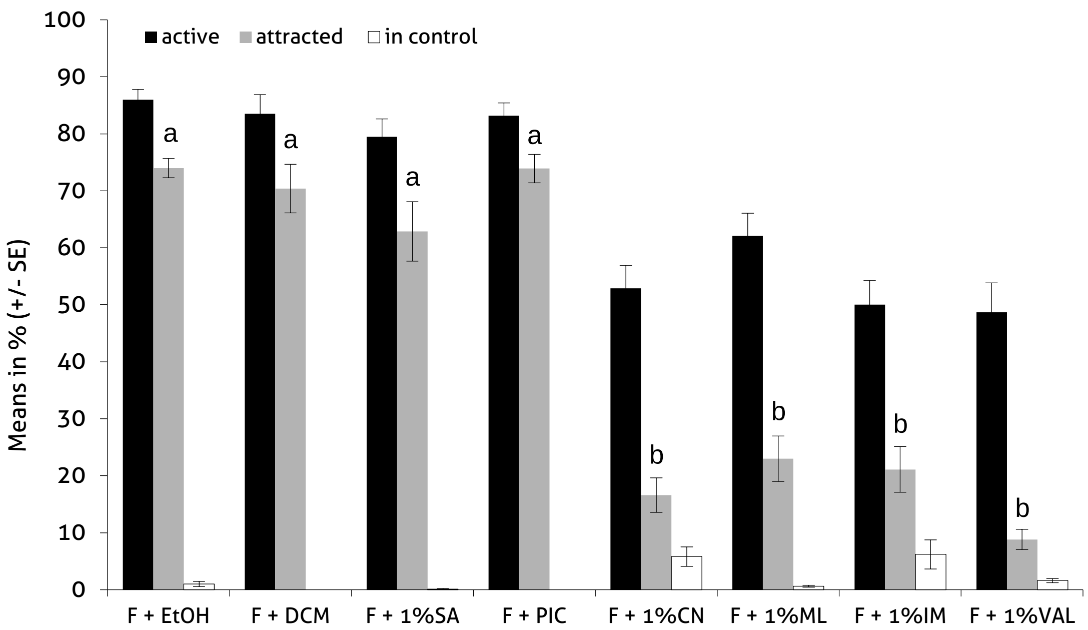

Fig. 5.2: Y-tube olfactometer blocks 1 to 3. Mean percentages and standard error (SE) of active mosquitoes, mosquitoes inside test cage (= attracted) and inside control cage. Treatments: Finger (F) plus ethanol (EtOH) and dichloromethane (DCM) in comparison to finger plus repellent: salicylaldehyde (SA), picaridin (PIC), catnip oil (CN), menthalactone (ML), iridomyrmecin (IM) and valerian oil (VAL). SA, CN, ML, IM and VAL were used at a concentration of 1 %, approximately 0.3 mg of active ingredient(s) were applied per individual trial. PIC was used with 6 mg per trial. Ethanol was tested in 30, CN in 20 and all other treatments in 10 repetitions with *Ae. aegypti*. Different letters indicate significant differences in the mean proportions of attracted mosquitoes at P < 0.001 (Tukey´s HSD test).

5.4 Discussion

This is the first study to investigate the attraction-inhibiting potential of (+)-Iridomyrmecin, menthalactone, salicylaldehyde and valerian essential oil on *Ae. aegypti*.

(+)-Iridomyrmecin, menthalactone and valerian oil showed spatial repellent activity in y-tube olfactometer assays; in their presence, test mosquitoes´ attraction to human skin odors was significantly reduced (P < 0.001, Tukey´s HSD test) and spatial effects were comparable to the ones elicited by catnip oil. Salicylaldehyde did not impact the test mosquitoes attraction to skin odors and results were not statistically different from controls (P = 0.7; Tukey´s HSD test) or tests of picaridin (P = 0.7; Tukey´s HSD test).

Even though attraction reduction was not significantly different between trials using (+)-iridomyrmecin, menthalactone, valerian and catnip essential oil (P = 0.8; Tukey´s HSD test), it should be noted that valerian oil yielded the highest attraction reduction from all samples tested, with an average of 88 % compared to repellent-free controls.

Essential oils have been characterized and used as mosquito repellents for decades, including eucalyptus and cinnamon (Zhu et al., 2006), amyris and siam wood (Paluch et al., 2009), lemongrass, cedar, pine or patchouli essential oils (Maia & Moore, 2011). Until now, valerian essential oil has not been studied for its spatial repellent activity against mosquitoes. Depending upon the geographical region, season and cultivation type, between 20 and 62 components have been identified in *V. officinalis* by GC-MS analysis, with the most abundant compounds being the monoterpenoids alpha-pinene and bornyl acetate and the sesquiterpenoids patchoulol, valerenal, valerianol and valeranone (Bos et al., 1999; Pavlovic et al., 2001; Wang et al., 2010). It was also reported that *V. officinalis* contains *actinidine* (Torssell & Wahlberg, 1967; Janot et al., 1979), a compound structurally related to nepetalactone. The chemical composition of the essential oil used in our studies is unknown but some of the compounds within the oil clearly exhibited spatial repellent activity against *Ae. aegypti* as attraction reduction reached almost 90 %.

The molecular structure of repellent receptors and the mechanisms that lead to olfactory repellency are unknown, although there has been an increased effort to identify olfactory receptors and neurons that are involved with repellent detection and triggering the avoidance reaction (Bohbot & Dickens, 2010; Bohbot et al., 2011; DeGennaro et al., 2013; Ray, 2015). The development of the mosquito repellent picaridin is a significant example of the correlation between chemical structure and repellent activity. Picaridin was developed by the Bayer Company through *molecular modeling,* a computational technique that was used to create a general structural framework from a set of known repellents (Boeckh et al., 1996). This framework was used to postulate structural motifs relevant for repellent action and after the screening of hundreds of compounds, a new repellent candidate was discovered – KBR 3023 or picaridin. Its repellent efficacy against mosquitoes was later confirmed in behavioral tests (Boeckh et al., 1996). Paluch et al. (2009) established a similar framework for sesquiterpenes, a so-called "quantitative structure – activity relationship" (QSAR) model, that was used to predict spatial or contact repellent activity. Their model was based on 12 sesquiterpenes of known repellent action (contact or spatial) and prediction parameters were correlated with vapor pressure (volatility) as well as electronic and electrotopological properties of certain carbons. QSAR was also used to predict the repellent activity of 200 acylpiperidines, of which 34 promising candidates were identified, some of them providing protection times more than three times longer compared to deet (Katritzky et al., 2008). All presented models identified structures that seem to play a role in receptor-ligand interaction and allowed prediction of repellent activity to some extent.

Our studies verified the spatial repellent activity of catnip oil, rich in nepetalactone. Structurally related compounds showed comparable spatial effects, indicating that these effects might be correlated with their specific monoterpene structure. Salicylaldehyde did not exert such an effect even though it is, for instance, a known repellent to ants (Pasteels et al., 1983; Matsuda & Sugawara, 1980), the cabbage fly *Delia radicum* L. (Den Ouden & Bulsink, 2009) and flower thrips *Frankliniella occidentalis* Pergande (Koschier et al., 2007). Compared to nepetalactone, salicylaldehyde is more volatile, with a vapor pressure of 90 Pa at 25°C (MSDS by Merck AG, 2013) while nepetalactone isomers have a vapor pressure of 0.9 Pa (Z,E) and 1.2 Pa (E,Z) at 25°C (Simmons et al, 2015). It is possible that salicylaldehyde evaporated too quickly to have an effect; then again, salicylaldehyde might simply not be repellent to mosquitoes.

Compared to catnip oil, isolation or synthesis of menthalactone and (+)-iridomyrmecin is costly and time-consuming, likely disqualifying both compounds as alternative spatial repellents. Valerian oil on the other hand is more cost-efficient compared to catnip oil and should be further investigated in larger scaled set-ups like room or semi-field tests to verify the promising outcome of the y-tube olfactometer assays. It will also be interesting to compare oils from different suppliers or regions to learn more about which components of the oil can be correlated with the spatial repellent effect.

6. Laboratory and Semi-Field Evaluation of a Novel Push-Pull Approach

This chapter has been updated and shortened. It was originally published as **Obermayr, U., Ruther, J., Bernier, U., Rose, A. And Geier, M. Evaluation of a Push-Pull Approach for *Aedes aegypti* (L.) Using a Novel Dispensing System for Spatial Repellents in the Laboratory and in a Semi-Field Environment.** PLoS ONE 10(6): e0129878. doi: 10.1371/journal.pone.0129878, June 26, 2015.

Author contributions: U.O., J.R., U.B. and M.G. designed the study; U.O. performed all experiments; M.G., A.R. provided material (BGS traps, BG Lure); U.O., J.R. and M.G. wrote the manuscript.

The increase in insecticide-resistant mosquito populations necessitates the exploration of novel vector control intervention measures. Push-pull strategies for insect control have been successful when used in integrated crop pest management. Through the combinatory use of deterring and attracting stimuli, the abundance of insect pests can be changed in a given area. A push-pull strategy might also significantly reduce human-vector contacts and augment existing mosquito control strategies, *e.g.* through the combination of an attractive trapping system and a potent spatial repellent. Our approach includes the BG-Sentinel (BGS) trap in combination with catnip oil (*Nepeta cataria*), a known spatial repellent for *Ae. aegypti*. To impart a deterrent effect on mosquitoes at a distance, a homogenous and continuous dispersal of volatile repellent compounds is crucial. We have developed a repellent dispensing system that is easy to use and provides a homogenous dispersal of repellent in an air curtain. The use of five 9V fans and custom-made repellent sachets containing 10% catnip essential oil created a repellent loaded air curtain that provided coverage of an area of 2 m^2 (1.2 m × 1.65 m). Air was sampled at four different heights in the curtain and analyzed via thermal desorption (TD) and consecutive gas chromatography - mass spectrometry (GC-MS). Nepetalactone, the main constituent of the oil, was detected in air at a concentration range of 80 to 100 $\mu g/m^3$ and the amounts were comparable at all four sampling positions. When a human volunteer was sitting behind the repellent curtain and a BGS trap was installed in front of the curtain in laboratory push-pull trials, *Ae. aegypti* landing collections decreased significantly by 50 % compared to repellent-free controls. However, in a semi-field environment, comparable protective effects could not be achieved and further research on suitable repellent concentrations for outdoor implementation will be required.

6.1 Introduction

Vector-borne viral diseases like dengue, dengue hemorrhagic fever and chikungunya present a major threat to human health. They are transmitted by *Ae. aegypti* and *Ae. albopictus*, two widely distributed and very competent vector mosquitoes that mate, feed and oviposit close to human dwellings. Breaking the transmission cycle depends primarily on eliminating or reducing the vector population; however, control measures are often inefficiently applied (Morrison et al., 2008). Intervention strategies mostly rely on the use of insecticides (Horstick et al., 2010), but traditional methods such as adulticidal fogging can be inadequate to target *Aedes* mosquitoes, as they tend to rest in secluded sites (Matthews, 1996). Indiscriminate or inefficient insecticide application also has led to an increased development of insecticide resistance (Fonseca-Gonzáles et al., 1996; Polson et al., 2011; Aponte et al., 2013). A recent study on the susceptibility status of eight *Ae. albopictus* populations collected in the United States revealed DDT resistance in 3 strains (Marcombe et al., 2014). According to the authors, continuous monitoring of the insecticide resistance status is absolutely essential since "underlying DDT resistance often results in pyrethroid resistance". The same group emphasized the serious threat of insecticide resistance to dengue vector control programs in Southeast Asia, South America and the Caribbean where high levels of resistance have been reported (Marcombe et al., 2011). Insecticide resistance in wild mosquito populations necessitates exploration of novel intervention measures. Current vector control measures need to be augmented or replaced by alternative strategies to contend with the growing numbers of resistant populations. A successful strategy used in integrated crop pest management is push-pull (Pyke et al., 1987; Cook et al., 2007). Through the combinatory use of deterring and attracting stimuli, the abundance of an insect pest can be changed in a given area by interfering with the ability of the target pest to locate a resource ("push") and luring it to an alternative source where it is trapped and removed ("pull"). In mosquito control, push-pull strategies have generated great interest over the past few years as they may provide useful techniques to help improve existing control measures. Push components, such as spatial repellents, are used to keep mosquitoes away from human dwellings and trapping systems baited with attractant lures can be used to remove mosquitoes from the intervention area. A recent study from Kenya provides evidence that such a strategy could help to reduce human-mosquito contact (Menger et al., 2015).

A spatial repellent is defined as a chemical that deters mosquitoes at a distance and inhibits their ability to locate a host (Gouck et al., 1967; Nolen, 2002). The term has also been used to describe the action of vaporized insecticides that cause knock-down, mortality, or inhibition of feeding. Sublethal doses of highly volatile pyrethroids, like metofluthrin, transfluthrin or allethrin have therefore been suggested to be implemented as spatial repellents in push-pull control strategies (Paz-Soldan et al., 2012; Salazar et al., 2012; Achee et al., 2012ab). Over the past few years there have also been increased efforts to investigate plant-derived, non-insecticidal spatial repellent candidates and a few have been identified, like linalool and dehydrolinalool (Kline et al., 2003), geraniol (Müller et al., 2008) and catnip oil (*Nepeta cataria)* (Peterson, 2001), the most promising one. The major constituent of catnip oil, nepetalactone, is repellent to plant hoppers, ants, caddisflies, beetles (Eisner, 1964), cockroaches (Peterson et al., 2002), mosquitoes (Peterson, 2001) and stable flies (Zhu et al., 2010). Several laboratory studies have indicated spatial effects of catnip against *Ae. aegpyti.* Catnip was more effective than deet in inhibiting *Ae. aegypti* attraction to human odors in triple-cage olfactometers (Bernier et al., 2005), reduced mosquito attraction to a human finger by more than 70 % in y-tube olfactometer assays (Obermayr et al., 2012), caused 60 % of a test mosquito population to fly from a repellent treated chamber to a repellent-free chamber in trials without human odors (Peterson & Coats, 2011) and elicited an 80 % escape rate in contact trials within excitorepellency test chambers (Polsomboon et al., 2008).

The host finding process of *Ae. aegypti* has been extensively studied and is well understood. Several compounds naturally found on human skin play an important role for mosquito-host attraction, like L-lactic acid (Acree et al., 1968; Geier et al., 1996), ammonia (Geier et al., 1999b), fatty acids (Bosch et al., 2000), acetone and dimethyl sulfide (Bernier et al., 2003). In combination with traps, synthetic kairomone blends can help to increase catching efficacy or enhance target species selectivity (Bernier et al., 2007). The BG lure (Biogents AG, Regensburg), a commercially available kairomone dispenser, has been especially designed for *Aedes* species, combining three synthetic compounds that are highly attractive to *Ae. aegypti* and *Ae. albopictus:* L-lactic acid, hexanoic acid and ammonia. The BG lure is commonly used in combination with the BG-Sentinel (BGS) trap, currently the most successful trapping tool to target *Aedes* species (Maciel-de-Freitas et al., 2006; Schmaedick et al., 2008). Even in the absence of CO_2, BGS traps equipped with a BG lure dispenser caught significantly more *Ae. aegypti* than CO_2-baited EVS traps (Williams et al., 2006) and significantly more female *Ae. albopictus* than CO_2-baited CDC traps (Meeraus et al., 2008). Based on its superiority in capturing *Aedes* species compared to other standard trapping systems, the BGS has been suggested to serve as a pull component in *Ae. aegypti* push-pull control strategies (Paz-Soldan et al., 2011; Salazar et al., 2012, Obermayr et al., 2012).

In contrast to our extensive knowledge on mosquito host attraction and trapping technologies, finding a powerful spatial repellent that is nonhazardous, long lasting and releases an unobtrusive odor is by far the greater challenge. Some groups investigated the potential of low-dose insecticides to serve as spatial repellents (Achee et al., 2012ab; Ogoma et al., 2014) while others focused on identifying non-toxic plant-derived compounds that reduce mosquito host attraction (Kline et al., 2003; Müller et al., 2008; Mng´ong´o et al., 2011).

In a recent study, our group presented a simple repellent dispensing device for the indoor evaluation of candidate spatial repellents to be used in push-pull systems (Obermayr et al., 2012). The device consisted of a perforated polyethylene (PE) tube and compressed air connection. Repellent formulations were released through fine holes in the PE tube, creating a repellent loaded air curtain. Test mosquitoes had to fly through this curtain to reach a BGS trap that served as the attracting stimulus. In these tests, the most successful materials, catnip oil (*Nepeta cataria*) and a mix of catnip oil and homopiperazine, reduced trap catches by 50 % to 90 %. However, the system had its drawbacks: the created air curtain was heterogeneous and contained gaps that allowed easy access for the mosquitoes leading to a great variation in the obtained results. A homogenous repellent dispersal was determined to be crucial for the success of a push-pull system and there was a need for a more reliable device that could easily be implemented within more realistic settings. In the present study, we developed and compared two new experimental set-ups for repellent dispersion, the "shower head" (SHS) and "five fan system" (FFS). Results obtained with the new systems were compared to determine which of the two set-ups provided a more homogenous repellent-loaded air curtain. In semi-field tests, the efficacy and the applicability of the system were investigated under more realistic outdoor conditions.

6.2 Material and Methods

6.2.1 Chemicals

Pure catnip (CN) (*Nepeta cataria*) essential oil was purchased from Aromaland (Röttingen, Germany). For SHS and olfactometer trials, CN was diluted with ethanol (96 %, p.a.) to final concentrations of 2.5 % and 10 % (w:w). In y-tube olfactometer trials, 30 µl of a 2.5 % test formulation were used (0.75mg active ingredients). In room tests with the SHS, 3 × 500 µl of a 10 % test formulation were applied to filter papers (150 mg active ingredients). For trials using the FFS, CN was diluted in paraffin oil to a final concentration of 10 % (w:w). Menthalactone (ML) (≥ 99 % purity) used as internal standard for the chemical analysis was obtained from Sigma-Aldrich (Taufkirchen, Germany).

6.2.2 Repellent Sachets

FFS trials utilized repellent sachets to disperse volatile active ingredients. Each sachet consisted of a 7.5 cm × 100 cm piece of Stericlin tube (Vereinigte Papierwarenfabrik GmbH, Feuchtwangen, Germany) filled with 100 g polymer Ingeo 4043D granules (NatureWorks LLC, Minnesota, USA) that had been loaded with either 10 g of a 10 % CN in paraffin formulation or 10 g of paraffin only (controls). One surface of the Stericlin tube is a non-permeable, transparent foil while the other consists of a Tyvek membrane that is permeable for gases but not for liquids.

During trials, dispensers were hung above the fans inside the FFS. In the laboratory, one trial lasted up to 15min, in the semi-field dispensers were operated for 1h. In between trials, dispensers were coiled and stored at room temperature inside a hermetically sealed PE box (14 cm × 10 cm × 7 cm). The loss of volatile ingredients from the sachets was measured gravimetrically after each experiment. Paraffin dispensers remained at the same mass in laboratory trials, but in the semi-field the loss reached an average of 0.009 g/h. Based on these findings, the mass loss in repellent dispensers was attributed to the evaporation of CN from the polymer granules. In laboratory trials, CN evaporated at approximately 0.06 g/h; in the semi-field the weight of CN dispensers decreased by approximately 0.05 g/h. Repellent dispensers contained an initial amount of 1 g CN (dissolved in 9 g paraffin); they were replaced as soon as their mass had declined by 0.5 g to ensure that sufficient amounts of active volatiles were still present in the dispenser. In the laboratory, CN sachets could be used for at least 8h, in the semi-field situation dispensers lasted for an average of 10h.

6.2.3 Test Mosquitoes

Six to 20-d-old *Ae. aegypti* females were used for all laboratory and semi-field tests. Preliminary behavioral assays in y-tube olfactometers with our lab colony demonstrated comparable susceptibility for spatial repellents at 6-20 days after emergence while responses showed greater variability when mosquitoes were at a younger age (1-5 d). The colony was originally obtained from BAYER AG (Monheim, Germany) in 1998 and has been maintained in our facilities over the past 17 years.

Mosquitoes were reared according to the procedure described in chapter 3 (3.2.2, p.29). Behavioral tests were performed with host-seeking females, which were lured out of their breeding cages at least 10 min before the start of the tests. The selection procedure followed the one described in chapter 3 (3.2.2, p. 30).

In semi-field tests, *Ae. aegypti* females from the Orlando strain were used. Previous olfactometer trials verified the positive spatial repellent activity of catnip oil against this strain (Bernier et al., 2005). The colony has been maintained since 1952 at the facilities of the United States Department of Agriculture, Agricultural Research Service, Center for Medical, Agricultural and Veterinary Entomology (USDA-ARS-CMAVE) in Gainesville, Florida, following a similar protocol. In the morning of each test day, host-seeking mosquitoes were lured out of the breeding cages into a collection trap by natural host stimuli and then immobilized at 4°C for 30 min. Mosquitoes were counted into batches of 100 females on a cooled tray, placed into holding containers, provided with 10 % sugar water and kept at 26 ± 1°C and 70 ± 5% until the start of the tests. A maximum of 6 tests were performed per day. In order to be able to distinguish mosquitoes from different rounds they were labeled with different luminous powders (BioQuip Products Co., Gardena, CA, USA) inside their holding containers.

6.2.4 Room Tests with Repellent Dispensing Systems and BGS Trap

Tests followed the procedure described previously (3.2.4, p. 31). Tests were performed in an air-conditioned 40.25 m^3 windowless room (4.6 m × 3.5 m × 2.5 m) with artificial light from two fluorescent tubes (350 Lux). The temperature and humidity of the air in the room were set to 25 ± 1°C and 60 ± 5% RH, respectively. Results from previous olfactometer trials at 28 ± 1°C and 80 ± 5% RH indicated that the spatial activity of CN was not impacted at this higher temperature and humidity combination (unpublished data). Clean, warm and humid air entered the room through an opening in the ceiling and exited the room through a second opening 4.5 m distant on the far side. A tent structure comprised of cotton fabric was built around the air entry with bottom edges held on the floor by wooden bars. The tent measured 1.2 m × 1.2 m × 2.5 m (L × W × H) and had three closed sides and an open entrance (1.2 m × 1.8 m) on the forth side. The repellent dispensing systems were installed at the top of the tent entrance.

6.2.4.1 Experiment 1 - Shower head system (SHS)

The SHS consisted of three conventional shower heads (Mixomat LED Handbrause, BAHAG AG, Mannheim Germany). Each shower head was connected to a poly propylene (PP) container (12 cm × 12 cm × 8 cm) by PE tubing (Supplemental Information, Fig. S1). Containers included a second opening for the introduction of pressurized air. For each test, three repellent treated round filter papers (Schleicher & Schuell BioScience GmbH, Dassel, Germany) were enclosed in the PP containers. Pressurized air was passed through the containers to pick up the repellent volatiles evaporating from the filter paper. Smoke experiments (data not shown) indicated a greater density of the curtain between 0.3 m and 1.45 m above ground, gaps were noticed between the three shower heads and the curtain appeared to thin out towards the ground (Supplemental Information, Fig. S2). The volume of the created air curtain was estimated at approximately 0.18 m^3 (1.65 m × 1.2 m × 0.09 m). The speed of the repellent-loaded air that left the shower heads was measured with an anemometer, it reached 0.1-0.2 m/s at a distance of 1 cm from the nozzles. This air flow corresponds to the one measured in previous trials with the PE tube system (Obermayr et al., 2012). In control trials, new shower heads were used and filter papers were treated with ethanol only. In all experiments, air flow was switched on 5min before test mosquitoes were released into the room.

6.2.4.2 Experiment 2 - Five fans system (FFS)

The FFS consisted of a 120 cm × 15 cm × 30 cm wooden frame into which five 12 V DC fans were mounted equidistantly with the down flow facing the tent opening (Fig. 6.1). The fans could be operated at 3 V, 4.5 V, 6 V, 7.5 V, 9 V and 12V, with each voltage creating different air speeds in the tent opening (Supplemental Information, Tab. S1). Control tests were used to identify the speed that did not generate a mechanical barrier to the mosquitoes. Mosquitoes were able to overcome the air curtain at all tested speeds (Supplemental Information, Fig. S3), even at 0.8 – 2.0 m/s which were measured in the center of the opening when fans were operated at 12V. The lowest variation was found at 9V, when wind speeds in the center of the opening reached 0.8 – 1.4 m/s. Based on these findings, all consecutive trials were conducted with fans operating at 9V. Smoke experiments (data not shown) indicated that the entire tent opening was uniformly covered (Supplemental Information, Fig. F4), the volume of the generated air curtain was estimated at approximately 0.24 m^3 (1.7 m × 1.2 m × 0.12 m)

Fig. 6.1: Laboratory test set-up showing the tent structure, BGS trap and five fan system (FFS) (front view).

Prior to the start of a test, a repellent sachet was hung in the frame with its permeable Tyvek side facing the row of fans (Fig. 6.2). Mosquitoes were released immediately after the system was switched on. In all experiments, a BGS trap fitted with a BG-Lure dispenser was used as a proxy for a human target and placed inside the tent to attract host-seeking *Ae. aegypti.* Those mosquitoes that passed the air curtain were captured by the trap. For each individual test, 10 mosquitoes were released into the room at the side furthest away from the tent and allowed to respond for 15min. Preliminary room tests had revealed that this was the maximum time period needed for all mosquitoes to be caught by the BGS trap and/or volunteer. At the end of the test time, the investigator entered the room and documented the trap catch rate. Mosquitoes still free-flying were aspirated with a modified hand-held vacuum cleaner. Mosquitoes that did not approach the investigator or that were still sitting inside the transport cage were recorded as inactive. For each dispensing system, 10 replicates were conducted per treatment (repellent and control). Treatments were tested in a randomized order. To avoid an accumulation of the volatile stimuli, the room was aerated for 30min before the next test was conducted.

Fig. 6.2: Stericlin repellent sachet attached to the FFS with the permeable side facing the fans.

6.2.4.3 Laboratory Push Pull Set-Ups

In experiment 3, two BGS traps were used with the FFS to simulate a push-pull situation. One trap served as an attracting stimulus inside the tent (BGS I) while the second one was used as an alternative target on the outside (BGS O). Capture rates of BGS I and BGS O were measured in the presence of catnip and compared to control trials with paraffin.

In experiment 4, BGS I was replaced by a human bait to determine if the combinatory effects of a BGS trap plus repellent curtain can decrease human landing rates. Human landing collections were performed in [a] absence of BGS O and absence of repellent, [b] presence of BGS O and absence of repellent, [c] absence of BGS O and presence of repellent and [d] presence of BGS O and presence of repellent (= push-pull situation). In both experiments, a total of 10 replicates were performed per set-up.

6.2.5 Quantification of Nepetalactone

Nepetalactone is the main component of catnip oil (Bates & Sigel, 1963) and constituted 84 % of the sample used in our experiments (data not shown). Therefore, we quantified the concentration of this compound in the air curtain of the SHS and the FFS set-ups and for comparison also in the Y-tube olfactometer used in our previous study, in which catnip oil was found to be highly efficient against *Ae. aegypti* (Obermayr et al., 2012). For quantification, we performed headspace analyses by thermal desorption gas chromatography coupled to mass spectrometry (TD-GC-MS). Volatile sampling was performed by aspiration of the volatile laden air for 30s at a flow rate of 200 ml/min through pre-packed thermal desorption filters filled with a combined Tenax-TA/Carboxen adsorbent (Sigma-Aldrich, Taufkirchen, Germany). After volatile sampling, 5 ng of the internal standard menthalactone (dissolved in 2 µl methanol) were applied to each adsorbent tube and the solvent was removed by purging the filter for 5min in a stream of nitrogen at a flow rate of 60 ml/min. Filters were thermally desorbed for 8min at 250°C using an automated Shimadzu TD20 thermal desorption system. The desorber was connected to a Shimadzu 2010 plus GC-MS system (Shimadzu, Duisburg, Germany) equipped with a non-polar BPX-5 column (30 m length, 0.32 mm i.d., 0.25 µm film thickness, SGE Analytical Science, Milton Keynes, UK). Helium was used as carrier gas at a linear velocity of 50 cm/s. The GC program started at 40°C and was ramped at a rate of 3°C/min to 163°C and then at 10°C/min to 280°C (final hold 6min). The MS was operated in electron impact (EI) mode at 70eV and a scan range from 35-600/mz. A calibration curve was generated by applying 1 µl aliquots of catnip oil dilutions in methanol representing 0.84 ng – 168 ng nepetalactone and 5 ng of the internal standard to the adsorption tubes. The solvent was removed as described above and before the standard samples were analyzed with the same TD-GC-MS method.

Air samples were collected at four different heights (position 1= 137 cm (above ground); position 2= 107 cm; position 3= 77 cm and position 4= 44 cm) and at four different points in time after switching on the SH or FF dispensing systems (at 0min, 5min, 10min and 15min). Prior to volatile collection in the y-tube olfactometer, 30 µl of a 2.5 % ethanolic catnip solution were dropped onto filter papers and held into the air stream of the apparatus after the solvent had evaporated (30s). This treatment has been shown to be highly efficient in repelling *Ae. aegypti* in previous studies (see 3.3.1, p. 33). Volatile sampling (n=10 replicates) was done as described above whereby the sample tube was positioned at the bottom center of the base leg.

6.2.6 Semi Field Test

Semi field experiments of the FFS were conducted at the USDA in Gainesville, Florida, between September 18 and 30, 2014. All experiments were performed inside a large outdoor cage (9.1 m wide × 18.3 m long × 4.9 m high, gabled to 6.1 m) covered with mosquito screen to allow entry of precipitation and wind. The cage contained vegetation and was equipped with a 12 – 14 personnel tent (HDT Base-X Model 305 Shelter, HDT Global, Solon, Ohio, USA; 5.5 m wide × 7.6 m long × 2.5m high). The FFS set-up was installed at the top of a tent opening (2 m high × 1.3 m wide). All mosquitoes used in semi field tests came from the USDA colony (see above).

Due to the limited time outdoor test facilities were available, two experimental set-ups were used in semi field trials: [1] A BGS trap fitted with a BG lure dispenser was installed inside the tent as an attracting stimulus (Fig. 6.3). Trap catch rates were documented in the presence of catnip and compared to control trials with paraffin oil only. [2] A human volunteer sat inside the tent to attract mosquitoes to fly through the air curtain while one BGS trap was installed outside (= push-pull set-up). Compared to laboratory trials, the greater space provided by the semi-field set-up necessitated a longer testing period. Mosquitoes were released at the far end of the cage and allowed for 1h to respond to the test stimuli, following the USDA standard testing procedure for semi-field trials involving traps (Daniel L. Kline, personal communication). Mosquitoes approaching the volunteer were aspirated into collection tubes attached to a modified hand-held vacuum cleaner. At the end of a test, BGS catch bags and collection tubes were removed and stored at -20° for later counting. A total of 10 replicates were performed per treatment (repellent and control).

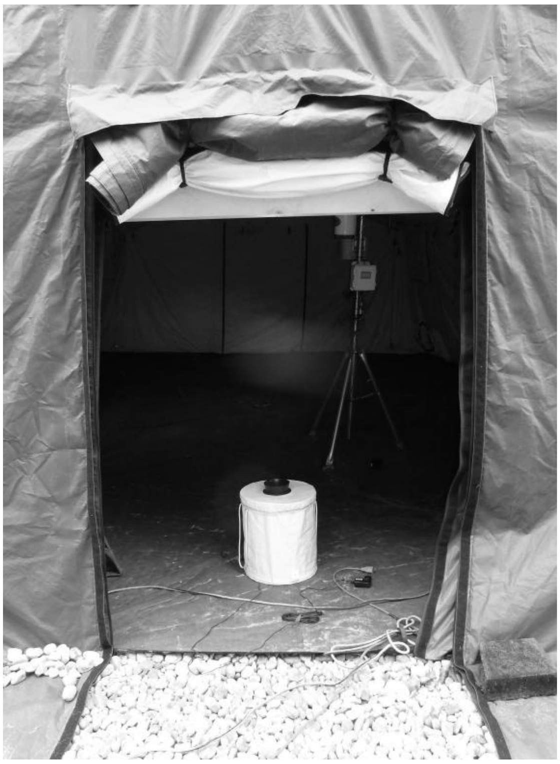

Fig. 6.3: Semi-field set-up showing the tent inside the outdoor cage, BGS trap and FFS.

6.2.7 Ethics Statement

The volunteer in this study provided written informed consent to conduct human landing accounts as described in the section on "volunteering for the semi-field test," which is part of the Institutional Review Board (IRB) Study #636-2005, approved by the University of Florida IRB-01.

6.2.8 Data Analysis

For both, laboratory and semi-field tests, mean percentages and corresponding standard deviations of mosquitoes caught by the BGS trap and volunteer were calculated from CN and control trials. Data were subjected to an arcsine transformation prior to the statistical analysis. Means from laboratory experiments 1, 2, 3, and semi-field were compared independently by non-parametric Mann-Whitney-*U*-test. Mean human landing collections from experiment 4 were compared using Kruskal-Wallis followed by pairwise Mann-Whitney-*U*-test with Bonferroni corrected *P*-values to look for significant differences between the four test scenarios.

For nepetalactone quantification, mean quantities and corresponding standard deviations of each sampling position and point in time were calculated. Mean quantities were compared using two-way analysis of variance (ANOVA) with Tukey´s honest significant difference (HSD) test as a post hoc test in order to examine if position and time had an influence on nepetalactone quantities. A P value ≤ 0.05 was regarded as statistically significant. All statistical tests were performed using PAST version 3.04 (Hammer, 2001).

6.3 Results

6.3.1 Room tests of the SHS and FFS

The first experiment evaluated the new SHS set-up. Compared to control trials, BGS capture rates were reduced significantly in the presence of catnip (U = 0.00; Z = −2.8271; P = 0.0047) (Fig. 6.4). When CN was dispensed, the average BGS catch rates dropped by 38 %. Experiment 2 involved the FFS in combination with Stericlin sachets and BGS trap. In the presence of catnip, BGS catch rates were reduced significantly by 70 % (U = 0.00; Z = −3.7489; P ≤ 0.001) (Fig. 6.4).

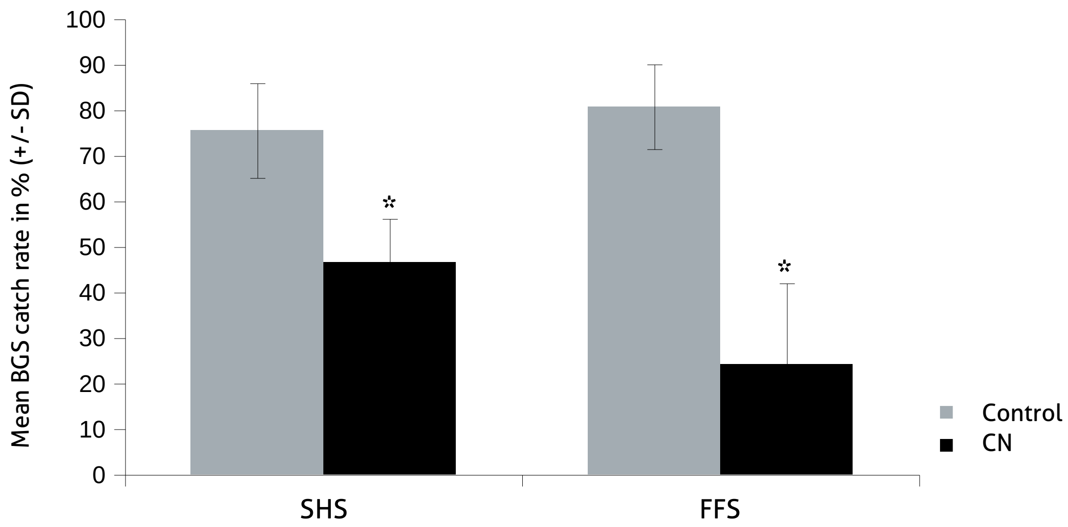

Fig. 6.4: BGS trap catch rates (means ± standard deviation, SD) of *Ae. aegypti* in control (grey) and catnip (CN) (black) trials of the shower head system (SHS) (experiment 1) and five fan system (FFS) (experiment 2). Asterisks indicate significant differences in mean BGS catch rates in tests of the SHS (P = 0.0015, Mann-Whitney-U-test, n=10) and FFS (P < 0.001, Mann-Whitney-U-test, n=10).

A simple push-pull set-up using the FFS with two BGS traps was tested in experiment 3 (Fig. 6.5). In the presence of CN, BGS I catch rates significantly decreased by more than 70 % (U = 19; Z = −2.3916; P = 0.017) whereas mean BGS O catches did not significantly differ from control trials (U = 39; Z = −0.7988; P = 0.424).

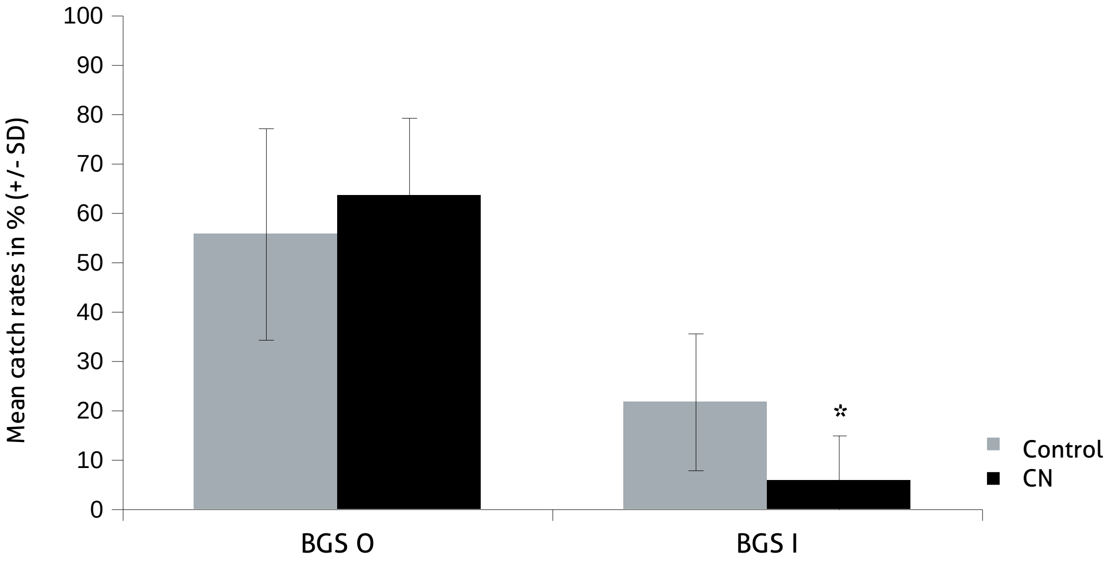

Fig. 6.5: *Ae. aegypti* recapture rates (means ± standard deviation) of BGS outside (O) and BGS inside (I) in control (grey) and catnip (CN) trials (black) of the FFS. The asterisk indicates significant differences at P = 0.017 (Mann-Whitney-U-test, n=10).

BGS I was replaced by a human volunteer in experiment 4, representing a more realistic push-pull set-up (Fig. 6.6). Human landing collections showed significant differences between the 4 scenarios (H = 25.79; P < 0.001): in test scenarios [a], [b] and [c] the majority of the released mosquitoes reached and landed on the volunteer, resulting in human landing collections of 92.9 ± 7.9 %. When CN was used in combination with BGS O in test scenario [d], human landing collections decreased significantly by 45 % to 50 % compared to test scenarios [a], [b] and [c] (P < 0.001) while BGS trap catches significantly increased (U = 3.5; Z = −3.5418; P < 0.001).

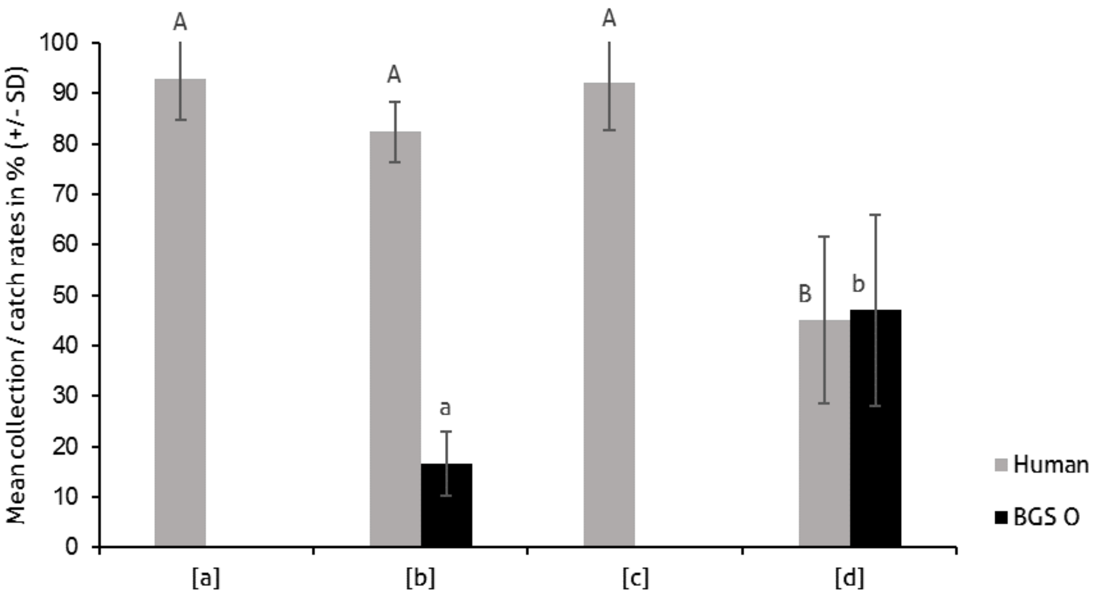

Fig. 6.6: Human collection rates (grey) and BGS catch rates (black) (means ± standard deviation, SD) of *Ae. aegypti* in laboratory room tests of the FFS in combination with Stericlin dispensers for catnip oil dispersal (experiment 4). Test scenarios: [a] no repellent, BGS outside (O) absent; [b] no repellent, BGS O present; [c] repellent, BGS O absent and [d] repellent, BGS O present (= push-pull situation). Different letters indicate significant differences in mean BGS trap catch rates (uppercase) at $P < 0.001$ (Mann-Whitney-*U*-test, n=10) or mean human landing rates (lowercase) at $P < 0.001$ (Mann-Whitney-*U*-test, n=10).

6.3.2 Quantification of nepetalactone

Volatile collections with the SHS revealed that both position of the adsorption tubes (F = 19.3; df = 3; $P < 0.001$) and sampling time (F = 3.088; df = 3; P = 0.029) significantly influenced nepetalactone quantities in the air curtain (Tab. 6.1). There was also a significant interaction between positions and point in time (F = 2.045; df = 9; P = 0.039). Mean nepetalactone quantities fluctuated over time, at positions 1 and 4 greater quantities were found after 15min compared to 0min. At 5min, 10min and 15min mean quantities collected at position 1 were greater than the ones obtained at positions 2, 3, and 4.

Compared to the SHS, quantification data of the FFS indicated a more homogenous and constant nepetalactone dispersal (Table 6.1). Mean nepetalactone quantities were not significantly different between the four positions (F = 1.336; df = 3; P = 0.27) and quantities did not significantly change over time (F = 1.84; df = 3; P = 0.149). A significant interaction between the positions and point in time was not detected (F = 0.522; df = 9; P = 0.853).
Nepetalactone quantities were greater in samples taken from the FFS: mean quantities collected from all positions at 0min (81.4 ± 37.5 µg/m^3) were significantly greater than mean olfactometer collections (54.9 ± 13.3 µg/m^3) (U = 43; Z = −2.363; P = 0.018). Overall, mean nepetalactone quantities (pooled from all positions and every point in time) were

significantly greater in FFS collections (91.0 ± 26.3 µg/m^3) compared to collections from the SHS (57.9 ± 65.0 µg/m^3) (U = 2237; Z = −7.3949; P < 0.001). At sampling point 0min, mean nepetalactone quantities of the SHS (40.0 ± 77.9 µg/m^3) were also significantly lower than mean olfactometer collections (U = 57; Z = −3,2079; P < 0.001).

Table 6.1: Concentration of nepetalactone (mean ± SD) as determined in the air curtains of two dispensing systems (shower head (SH) and five fan (FF)) and y-tube olfactometer by thermal desorption headspace gas chromatography-mass spectrometry (TD-GC-MS). Nepetalactone quantities are given in relation to the distance of the sampling point and the time passed after the start of the experiment. Position 1: 137 cm above ground, position 2: 107 cm, position 3: 77 cm and position 4: 44 cm. Different lowercase letters indicate significant differences between rows for each set-up at P < 0.03 (Tukey´s HSD test, n=9), capital letters indicate significant differences inside columns at P < 0.007 (Tukey´s HSD test, n=9).

	Mean nepetalactone quantities (µg/m^3) ± SD			
	SH dispensing system (n=9)			
Position	**0min**	**5min**	**10min**	**15min**
1	48.3 ± 25.4 a	108.3 ± 56.7 ab/A	135.5 ± 57.8 b/A	164.3 ± 89.9 b/A
2	21.5 ± 15.2	22.4 ± 14.6 B	24.9 ± 11.6 B	28.7 ± 17.2 B
3	62.1 ± 14.3	33.1 ± 16.1 B	42.9 ± 20.6 B	45.1 ± 17.1 B
4	25.2 ± 27.1 a	49.0 ± 30.1 ab/B	44.5 ± 18.8 ab/B	68.0 ± 29.9 b/B
	FF dispensing system (n=5)			
1	64.4 ± 10.9	88.9 ± 18.4	96.6 ± 10.4	89.2 ± 20.3
2	94.0 ± 41.9	98.1 ± 11.4	110.4 ± 24.3	110.1 ± 8.1
3	97.4 ± 41.8	80.4 ± 13.1	97.2 ± 29.6	101.8 ± 21.5
4	66. ± 26.8	83.1 ± 19.7	94.7 ± 16.0	82.2 ± 16.0
	Y-tube olfactometer (n=10)			
	54.9 ± 12.6	-	-	-

6.3.3 Semi field trials with the FFS set-up

When the BGS trap was used as attracting stimulus in set-up [1], recapture rates in control and catnip trials were not significantly different (U = 45; Z = −0.3402; P = 0.734) (Fig. 6.7). In trials involving the push-pull set-up [2] human landing collections were slightly reduced by 15 % in the presence of CN but showed no significant differences to control trials (U = 39; Z = −0.7943; P = 0.427). BGS trap catch rates increased by 30 % in the presence of CN and were significantly higher compared to control trials (U = 22; Z = −2.082; P = 0.037) (Fig. 6.7).

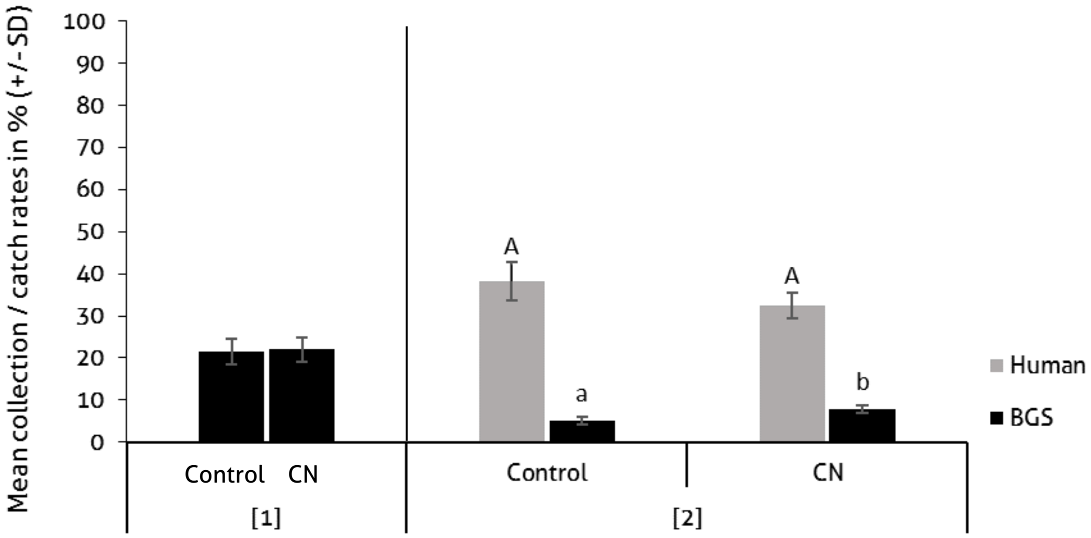

Fig. 6.7: Human collection & BGS catch rates (means ± standard deviation, SD) of *Ae. aegypti* in semi-field trials of the five fan system (FFS) in combination with catnip (CN). The x-axis shows the test scenarios: [1] BGS as attracting stimulus inside the tent and [2] Human volunteer inside the tent plus BGS outside (O) (= push-pull situation). Different letters indicate significant differences at P < 0.05 (Mann-Whitney-U-test, n=10).

6.4 Discussion

The establishment of push-pull strategies in mosquito control is a subject of great interest but the successful implementation of such a strategy has not been reported yet. One critical aspect is finding the proper spatial repellent and an effective means of dispensing it. With the FFS, we have developed an application-oriented, easy-to-use spatial repellent dispensing system that facilitates a homogenous dispersal of repellent, a crucial parameter for the successful implementation of push-pull strategies. Indoor volatile collections from the FFS and consecutive nepetalactone quantification via TD-GC-MS showed constant and comparable amounts of active ingredient at each of the four sampling positions and throughout the entire sampling period. Smoke experiments used to visualize the air movement inside the curtain supported the assumption of a homogenous dispersal within the FFS. Further research on the SHS was discontinued, as both smoke experiments and nepetalactone quantification indicated gaps or areas of lower repellent density within the air curtain that most likely provided easy access for the mosquitoes. In addition, the dependence on pressurized air also impeded the overall applicability of this technology in an outdoor setting. The FFS is an easy-to-use alternative, however the fans require (battery) power and this could also be an impediment to using this technology in disease endemic settings.

A combination of the FFS and catnip essential oil plus BGS trap provided promising effects in a confined space. Within the laboratory setting, human landing collections were reduced by 50 %; however when transferred to a semi-field environment, protective effects were not as distinct. Although the BGS O catch rates increased significantly in the presence of catnip odors, human landing collections were only reduced slightly. Future research needs to investigate if these limitations can be overcome, *e.g.* through (1) the use of dispensers that emit greater quantities of the essential oil, (2) the implementation of CO_2 as additional trapping cue and (3) the use of multiple BGS traps.

When tested in a different semi-field set-up, catnip was reported to also work as a spatial repellent against *An. gambiae* (Menger et al., 2014). Mosquito Magnet X (MM-X) traps baited with CO_2 and odor blends were used to follow mosquito house entry. When catnip was dispensed outdoors at the four corners of an experimental hut, indoor trap catches were reduced by 50% but spatial effects were not examined in the presence of a human volunteer. Our work demonstrates that human-vector contact can be reduced by catnip in a confined area but for a successful outdoor implementation of push-pull we still need to extend our knowledge on the characteristics, capacities and limitations of spatial repellents.

The deterrence elicited by a neurotoxic compound can cause mosquitoes to rest and seek shelter, a behavior that was observed in semi-field studies in Tanzania (Kitau et al., 2010). In allethrin trials, Mosquito Magnet trap catch rates decreased in treated areas compared to insecticide free controls and mosquitoes were found to rest on walls and vegetation inside the experimental area without showing any host-seeking behavior. When *Ae. aegypti* mosquitoes were pre-exposed to common insecticides and subsequently introduced into recapture trials with BGS traps, pre-exposure to transfluthrin significantly reduced BGS trap catch rates in trials immediately after exposure (Salazar et al., 2012b). These findings emphasize that the success of a push-pull control strategy strongly depends on the characteristics of the push component; the target mosquito needs to be repelled but still be attracted to an alternative host and such a reaction might be impeded by the neurotoxic action of pyrethroids. Not only for these reasons, but also to avoid human exposure to the potential hazards of pyrethroids, there have been increased efforts to discover alternative, non-toxic spatial repellents and some groups suggested to use plant-derived chemicals as a safe alternative to pyrethroids for indoor personal protection (Revay et al., 2013). Field data on the impact of plant-derived compounds on human landing rates are scarce and up to now the protective effects of catnip on humans have never been examined under outdoor conditions.

In general, the impact of a spatial repellent seems to be restricted to short distances and minimal air movement (Revay et al., 2013) and effects are greater in the presence of homogenous „bubbles" compared to a point-source release of active ingredient (Ogoma et al., 2014). With the FFS, we describe here a repellent dispensing system that created a homogenous repellent air curtain in an indoor set-up. In close proximity to a human host, mosquito-host attraction significantly decreased in the presence of a catnip enriched air curtain and a BGS trap. In our laboratory experiments, part of the test mosquitoes were found to hover in front of the repellent curtain, a behavior that could be described as "repellent-initiated hesitation", and eventually some of them got caught by the BGS trap. This hesitation behavior was apparently elicited by nepetalactone concentrations that were sufficient to deter or confuse the test mosquitoes but did not inhibit attraction as some mosquitoes still flew through the curtain while others were attracted to the BGS trap.

When *Ae. albopictus* was exposed to 0.013 $\mu g/cm^3$ vapors of geraniol or anisaldehyde, no noticeable changes in their host-seeking ability were observed. After being exposed to higher doses (0.25 $\mu g/cm^3$) of the same compounds, host-seeking ability decreased by 70-80%, indicating a dose-dependent inhibition in the host seeking behavior (Hao et al., 2008). Compared to these physiologically critical doses, mosquitoes were exposed to far lower concentrations of nepetalactone (0.08 – 0.1 ng/cm^3) in our FFS experiments and within this range, host-seeking was not inhibited but mosquito host finding was slightly delayed. However, such an effect could not be observed when the FFS was evaluated under outdoor conditions. Experiments were conducted in September 2014, at an average ambient temperature of 25.9 °C, 77.5 % relative humidity and wind speeds ranging between 4 and 16 km/h. Air movement most likely had a great impact on the integrity of the repellent curtain. In contrast to laboratory trials with nearly static conditions, wind could pass through the outdoor cage and thereby dissipate the repellent curtain at the tent entrance. On the other hand it is also quite possible that greater nepetalactone concentrations will be required to obtain effects that are comparable to the indoor performance of the system. Another important aspect that might have had a great impact on the outcome of the semi-field tests was the color and contrast conditions of the test site: while the BGS trap or a human volunteer was presented in front of a white background in laboratory trials, mosquitoes were exposed to a dark tent opening in semi-field tests (Figs. 2 and 3). It has long been known that *Ae. aegypti* is highly attracted to dark colors (Howlett, 1910; Brett, 1938; Gjullin, 1947) and the black interior of the tent could have diminished the impact of the push component.

A recent study investigated the spatial effect of catnip against stable flies in an outdoor situation (Zhu et al., 2010). Wax pellets containing 10 % of the essential oil were dispersed in known stable fly resting areas and atmospheric concentrations of catnip oil volatiles were measured using solid-phase micro extraction (SPME). Right at the start of the experiment approximately 160 ng/min of catnip oil volatiles were detected after a three minute exposure of the SPME fiber to the volatile laden headspace, correlating with a significant reduction in stable fly landing rates. The effect on the insects vanished after 24 hours and at this point in time the recovery of catnip oil volatiles had diminished to approximately 60 ng/min. Unfortunately, a cross-comparison of SPME and our purge & trap sampling method is not possible, but results of the cited study indicate that catnip oil can achieve spatial repellent effects against insects also in an outdoor setting, provided that a particular, critical threshold is reached. Future studies of the FFS should therefore include the evaluation of dispensers with higher catnip oil loading. In laboratory weighing experiments, catnip sachets lost around 0.06 g/h of catnip oil (representing approximately 13 mg of nepetalactone within a 15min sampling period). Volatile sampling and TD-GC-MS analysis revealed nepetalactone quantities between 80 – 100 µg/m^3 inside the air curtain generated by the FFS, which had an estimated volume of 0.25 m^3. With an average wind speed of 0.9 m/s passing through an area of 0.14 m^2 in 15min, a total air volume of 117 m^3 was generated which took up between 9 to 12 mg of catnip oil volatiles (80 – 100 µg/m^3 × 117 m^3), thus quantification data correlate well with results from gravimetric mass loss measurements of the catnip dispensers. Future studies could investigate if dispensers filled with polymer granules holding higher doses of catnip oil have a greater spatial impact in semi-field trials. Likewise, using CO_2 in an outdoor setting might boost BGS trap catch rates in the presence of a human volunteer and should be implemented in future studies.

7. General Discussion

The integration of push-pull strategies in mosquito vector control continues to be a challenging task. Major drawbacks are the lack of powerful, non-toxic spatial repellents, effective application tools and experience from larger scale field trials on how such a system needs to be implemented to impact the vector population.

This thesis introduces novel tools for the improved evaluation of spatial repellent effects. Volatile chemicals can be investigated under more realistic yet laboratory conditions, which benefit from defined and reproducible settings. The presented room test procedure will not supersede field tests but it allows for a sound and time-efficient identification of promising candidates before extensive field studies are initiated. Our results show that non-pyrethroid, plant-derived repellents are able to elicit spatial repellent responses in *Ae. aegypti*, provided that repellent volatiles are dispensed homogenously and continuously within a confined space. This thesis also demonstrates that the combination of catnip essential oil and the BG-Sentinel trap is significantly more successful in reducing human landing collections than pull-only and push-only approaches. When tested in the semi-field, however, prominent effects on human landing rates could not be observed. For the further optimization of our push-pull system, future research should focus on testing higher spatial repellent concentrations, different numbers of traps and additional attracting cues.

Most research on spatial repellent effects has been conducted with volatile pyrethroids, including allethrin, transfluthrin and metofluthrin (Kawada et al., 2006; Lucas et al., 2007; Achee et al., 2012ab; Ogoma et al., 2012ab; Salazar et al., 2012b; Manda et al., 2013). Using the term "spatial repellent" to define their mode of action however is a little misleading, as all of the substances listed above are neurotoxic insecticides. The literature research summarized in chapter 2 demonstrates the inconsistent use of terms to describe mosquitoes´ avoidance reactions to chemicals. Interestingly, the origin of the term "spatial repellency" is closely intermeshed with dichlorodiphenyltrichloroethane (DDT), the former silver bullet of insecticide research. After discovering DDT´s superior insecticidal properties, the initial and main focus of DDT research was on finding practical applications. However, in the mid-1940s its mode of action and effects on mosquitoes were studied more closely, which led to the discovery of its ability to induce excitatory and repellent responses (Buxton, 1945; Gahan, 1945; Metcalf, 1945). In the field, fewer mosquitoes were found in DDT treated huts (Hocking, 1947) and a greater proportion seemed to be able to escape unharmed (Davidson, 1953). The reduced number of mosquitoes inside spray-treated huts was not only due to an increased exiting behavior elicited through the contact-irritant properties of DDT but mosquitoes were also deterred from entering treated houses (De Zulueta & Cullen, 1963; Smith & Webley, 1969). Busvine (1964) stated that DDT was able to elicit the following reactions: (1) repellency at a distance or spatial repellency, that keeps mosquitoes from entering a structure and (2) contact repellency, that either elicits greater activity (*excitorepellency* or *irritancy*) or increased responsiveness to light, allowing mosquitoes to escape easily from a structure. These early studies helped to recognize additional, non-lethal effects of insecticides that still led to a reduction in human-vector contact. This also explains why the mode of action of volatile pyrethroids is often described as spatial repellency (Ogoma et al., 2012), even though their impact on mosquitoes is most likely neurotoxic and not olfactory mediated (Wagman et al., 2015).

Kitau and colleagues (2010) were the first ones to investigate volatile pyrethroids as push-components in combination with a commercial mosquito trap (Mosquito Magnet® Liberty Plus, using CO_2, L-lactic acid and ammonia as attractants). They measured human landing- and trap catch rates in the presence and absence of their candidate repellent materials in a semi-field environment. Even though allethrin yielded a significant reduction in human landing rates, trap catch rates did not increase. Test mosquitoes were found to rest and seek shelter after having made contact with the insecticide, a behavior that explains the low trap catch rate. A similar observation was reported by Wagman (2015) who evaluated a push-pull approach against two local *Anopheles* species in Northern Belize, Central America. His set-up was based on a combination of transfluthrin and CDC miniature light traps. Even though hut entry by *An. albimanus* was significantly reduced in the presence of transfluthrin, outdoor catch rates did not increase. In 2012, Salazar and colleagues investigated the paralyzing effects of common insecticides and their impact on mosquito attraction to trapping systems. Trap capture rates were significantly lower after mosquitoes had been pre-exposed to transfluthrin but attraction was re-established after a recovery period of 12 hours.

There is a strong indication that some volatile pyrethroids impede mosquitoes´ attraction to trapping systems, thereby rendering the pull component ineffective (Kitau et al., 2010). Including volatile pyrethroids in a push-pull strategy for vector mosquitoes is tempting as most active ingredients have been on the market as household pesticides for a long time and consumers are familiar with their use (Paz-Soldan et al., 2011). However, their neurotoxic action even at sublethal doses, the possibility of increased resistance in the target population and, most importantly, human exposure to irritant chemicals make them less favorable (Revay et al., 2013). Thus, research on alternative active ingredients continues and is accompanied by a necessary shift in current screening protocols to better assess spatial repellent activity and find compounds that do not interfere with trapping systems (pull component). It was suggested to develop a screening cascade that allows the step-wise identification of candidate compounds, first in the laboratory and later on in the semi-field and field (Achee et al., 2012a).

Laboratory assays offer certain advantages, e.g. they can be performed at any time under constant, standardized and defined conditions. Common methods to evaluate spatial repellent effects involve static air chambers (Grieco et al., 2005; Peterson & Coats, 2011) and y-tube olfactometers (Kline et al., 2003; Bernier et al., 2005 & 2012; Obermayr et al., 2012; WHO, 2013). These set-ups use a rather small and confined space to expose mosquitoes to volatile chemicals. They enable rapid testing of a great number of samples and are well-suited for screening purposes. However, they sometimes overestimate spatial effects as the air of the test environment can contain high levels of active ingredients which may cause greater avoidance reactions compared to larger set-ups. Promising results from small-scaled laboratory trials therefore need to be verified in more realistic settings, ideally in the semi-field or field (Ogoma et al., 2012b). Compared to laboratory trials, field tests are time-consuming and costly and influenced by a variety of biotic and abiotic factors, e.g. density, activity and physiological state of the local mosquito population, light, temperature, humidity and wind (Barnard et al., 2007). Thus, data sets may suffer from greater variation compared to laboratory tests.

To address these issues we have developed a novel procedure that compensates for the aforementioned limitations of small set-ups and allows the laboratory evaluation of spatial repellent effects under more realistic yet standardized conditions. Chapter 3 summarizes the experiments that led to the development of the novel room test procedure and introduces active ingredients and mixtures that showed a significant spatial potential with regard to their ability to reduce test mosquitoes' attraction to human skin odors. Initially, we performed y-tube olfactometer assays to screen candidate materials as they allow the evaluation in the presence of human odors, while skin exposure to both mosquito bites and test chemicals is excluded. Instead of using human skin odors, some protocols suggested attractive mixtures as positive stimuli (Kline et al., 2003; Bernier et al., 2005; Bernier et al., 2012), e.g. blends consisting of L-lactic acid, acetone and dimethyl disulfide, to compensate for potential differences in the attractiveness of individual experimenters. Mosquito attraction to synthetic blends, however, is generally lower compared to human odors (Bernier et al., 2005), i.e. it is quite likely that the attraction reduction to a synthetic blend is achieved more easily. Catnip essential oil for instance was reported to reduce *Ae. aegypti*´s attraction to a synthetic blend by 63 % while the attraction to human hand odors was only reduced by 25 % (Bernier et al., 2012). Since y-tube olfactometer assays tend to overestimate efficacy, we decided to create more rigorous testing conditions by using human skin odors as attracting stimuli throughout all tests. To compensate for potential variations in individual attractiveness, all experiments presented in this thesis were conducted by the same volunteer. Test mosquitoes' attraction to the volunteer was high, baseline measurements recorded an average flight activity (upwind flight behavior) of 84 ± 3.7 % (n = 190), while an average of 71 ± 4.5 % (n = 190) was attracted to the stimulus source. Mosquito host odor attraction was monitored in every experimental block and only data from trials showing values that were comparable to the baseline measurements (or higher) were used to evaluate the potential of spatial repellent candidates.

To verify that our y-tube olfactometer set-up was suited to detect spatial effects, the first trials involved the evaluation of materials that have previously been described to act as spatial repellents or kairomone attraction-inhibitors. Results from our assays confirmed the inhibitory effects of 1-methylpiperazine, homopiperazine (Bernier et al., 2012) and catnip essential oil (Bernier et al., 2005). Average attraction reduction to skin odors was greater than 90% in trials involving the piperazine compounds and greater than 80 % in trials using catnip essential oil. Effects observed in our trials were slightly stronger compared to other studies, which reported an average attraction reduction of 75 % to skin odors in the presence of 1-methylpiperazine and only 25 % in the presence of catnip essential oil (Bernier et al., 2012). The discrepancy in the results with catnip could be due to variations in the composition of the oils used as the nepetalactone isomers show seasonal variations that impact the repellent potential of the oil. Generally, the *E,Z*-isomer is more repellent than the *Z,E*-isomer (Schultz et al., 2004).

Our experiments also revealed that y-tube olfactometers are suited to differentiate spatial repellent- from contact repellent-properties (Obermayr et al., 2012). Picaridin, an active ingredient in a variety of commercial repellents including Autan® (Stiftung Warentest, 2013), provides contact-mediated bite protection when applied to the skin. In y-tube olfactometer trials, picaridin did not impede test mosquitoes' attraction to skin odors, compared to repellent-free controls and flight activity and attraction to the stimulus source remained high. These findings allow the important conclusion that y-tube olfactometer assays are an efficient screening tool for spatial repellents before their effectiveness is verified on a larger scale.

The olfactometer procedure was used as a template for the development of the novel room test. The main objective was to create a dispensing system that allowed the establishment of a repellent-enriched air space inside the test room. This barrier would have to be overcome by the test mosquitoes in order to reach an attractive source located behind. The starting point for the new set-up was a 5 m^3 tent inside a 40 m^3 test room. The tent had an open side facing the room and repellents were dispensed along the top of this opening. A BGS trap was placed inside the tent to attract mosquitoes to fly through the opening (=push-only trial).

Initial tests involved passive emanation from strips of disposable paper towels which were hung at the top of the tent opening. No decrease in trap catch rates could be documented in the presence of 1-methylpiperazine, homopiperazine, catnip essential oil and a mix of homopiperazine and catnip oil (data not shown), even when relatively high quantities were used. While olfactometer assays revealed strong inhibitory effects at quantities of 0.75 mg to 1.5 mg of active ingredients per individual test, no spatial effect was noticed when repellent materials were applied at 100 mg per trial in room tests. Apparently, passive emanation was either not sufficient to build up effective volatile concentrations or achieve coverage of a greater area. These findings led to the development of the *perforated tube system* using pressurized air. Repellents were dropped onto filter papers inside a fritted gas wash bottle and air was led through the bottle to pick up active ingredients before it was dispensed at the tent opening. With this modification, we were able to measure spatial repellent effects at lower quantities compared to the previous passive emanation trials. Significant trap catch reductions were documented in push-only trials when using 25 mg of active ingredients: the piperazine compounds caused an average reduction of 77 % (1-methylpiperazine) and 95 % (homopiperazine) while catnip essential oil reduced trap catches by an average of 50 %.

Data on the performance of 1-methylpiperazine, homopiperazine and catnip essential oil in set-ups other than y-tube olfactometers are scarce. Chauhan and colleagues (2012) investigated the impact of catnip oil on catch rates of CDC light traps in a field bioassay. Their method involved the passive emanation of the oil from polypropylene tubes that were attached to a cubical frame around a trap. When applied at quantities of 640 mg to 2,560 mg, catnip oil yielded average trap catch reductions of 30 %. When Menger and colleagues (2014) investigated the spatial potential of catnip oil in a semi-field environment, 4.0 g of catnip oil were dispensed by fans around an experimental hut per individual trial. Compared to controls, catch rates of a lure baited trap located inside the hut were reduced by an average of 55 %. These findings support the spatial repellent properties of catnip oil but they also indicate that considerably higher quantities need to be applied in a field setting which would have a great impact on costs.

The positive outcome of the push-only trials led to the next set of room tests, which involved a human volunteer as attracting stimulus instead of the BGS trap. None of the previously successful repellent materials had a protective effect on the volunteer in push-only trials, human landing collections were greater than 95 % and did not differ from repellent-free controls. A comparable outcome was also observed in pull-only trials, where a BGS trap was placed outside the tent in the absence of volatile repellents. Again, more than 90 % of the test mosquitoes reached the volunteer while the trap caught only a small proportion of 9 %. In contrast to previous room tests using the BGS trap as an attracting stimulus or y-tube olfactometer assays, the full spectrum of host attractants including CO_2 and body heat contributed to the test mosquitoes' attraction to the human volunteer. Results demonstrate that the use of either a spatial repellent or BGS trap alone is insufficient to counteract the overpowering effects of a natural host. Another important conclusion from these early laboratory trials is that even though y-tube olfactometers are well suited for a fast screening of potential candidate materials, they do not provide a reliable indication of the quality and magnitude of spatial effects in a larger setting.

Little is known about the specific mode of action of insect repellents on mosquito sensory structures. From currently available active ingredients, deet is one of the best studied insect repellents; however, there is no consensus among researchers on its molecular action and contradictory theories have evolved over the past years. While inhibition of lactic-acid sensitive neurons was attributed to deet in early works (Davis &

Sokolove, 1976), more recent studies suggested that deet inhibits the response of the OR8/Orco complex to the attractant 1-octen-3-ol in *An. gambiae* (Ditzen et al., 2008). This hypothesis was later contradicted by a study in *Cx. quinquefasciatus* which supports a "masking effect" of deet on the release of attractive odors rather than an inhibitory effect on reception (Syed & Leal, 2008). Studies on OR responses to different repellents (including deet, picaridin and IR3535) in the presence and absence of attractive odorants indicated that the majority of repellent compounds acted as agonists or antagonists, thereby modulating OR activity (Bohbot & Dickens, 2010). "Repellents belonging to structurally diverse chemical classes modulate the function of mosquito ORs through multiple mechanisms" (Bohbot et al., 2011). The aforementioned studies mainly focused on the responses of mosquito OR8 which is tuned to 1-octen-3-ol, an odorant that does not seem to be a strong attractant for *Ae. aegypti* (Russell, 2004). Interference with CO_2 reception, on the other hand, is of great interest, since our laboratory studies already provided us with promising compounds that lowered the attraction to skin derived odors. Carbon dioxide is a very potent olfactory cue for the host-seeking female, as it works at a distance and increases the attraction to skin odors and body heat (Dekker et al., 2005). In the environment, exhaled CO_2 is diluted against a background of atmospheric CO_2 and mosquito females use these fluctuating levels to orient towards their host. In *Ae. aegypti,* minute changes in CO_2 levels cause a strong activation, upwind orientation and increased flight speeds. (Dekker et al., 2005; Cardé & Gibson, 2011; Dekker & Cardé, 2011). Thus, the poor protection of the human volunteer in room tests is most likely correlated with the presence of CO_2. Consequently, our approach required adjustment and we decided to include potential CO_2 blocking agents in subsequent experiments.

Recently, a few compounds have been described that either cause (1) an ultraprolonged activation of the cpA neuron which compromises the mosquitoes´ ability to detect CO_2 or (2) act as inhibitors of the CO_2-sensing unit (Turner et al., 2011; Tauxe et al., 2013; Ray, 2015). We decided to test the efficacy of a potential inhibiting blend consisting of 2,3-butanedione, 1-butanal, 1-pentanal and 1-hexanol and incorporated the procedure described by Turner et al. (2011) into our room test experiments.

Again, test mosquitoes´ attraction to the natural host was unaffected by the test chemicals. After the pre-exposure to the Turner blend, a procedure that was previously shown to inhibit test mosquitoes' response to CO_2, human landing rates reached 100 %. The combinatory use of CO_2 inhibitors and spatial repellents (catnip oil and homopiperazine) to target both, attraction to skin odors and exhaled breath, also had no protective effect on the volunteer and landing rates reached 95 %. Results from these room test experiments again demonstrate the strong attraction of *Ae. aegypti* to its host that overrides the impact of potential inhibiting / blocking chemicals. They also emphasize that promising results obtained from laboratory tests in the absence of a human host, e.g. the wind tunnel studies conducted by Turner et al. (2011), need to be considered with great care as they may not correlate well with results obtained under field conditions.

However, an interesting observation was consistently made in push-only trials with catnip oil and homopiperazine (CN-HP): before passing the repellent-loaded air curtain, mosquitoes were found to be hovering in front of the tent opening, as if they were hesitating to fly through. Indeed, landing times on the volunteer were significantly delayed in the presence of CN-HP compared to repellent-free controls. This short hesitation period proved to be beneficial for push-pull trials, when for the first time a significant reduction in human landing rates could be observed. When a mixture of 50 mg catnip oil and 50 mg homopiperazine was dispensed in combination with a BGS trap outside the tent, human landing rates significantly decreased by 40 % while BGS trap catches significantly increased by almost fivefold. These important results demonstrate that human-vector contact can indeed be diminished in the presence of a suitable spatial repellent and attractive trapping system. It should also be noted that such an effect was not observed in the presence of picaridin, demonstrating that the new room test procedure also differentiates between spatial- and contact-repellent properties.

However, the perforated tube system had a few drawbacks. The utilization of pressurized air presents problems with an easy conversion to use in the field. In addition, variations between individual spatial repellent trials occurred, e.g. in push-only trials with catnip essential oil, BGS trap catch rates ranged between 10% and 70%. In experiments with the human volunteer a second important observation was made; while a portion of the test mosquitoes were found to be hovering in front of the tent, some still managed to fly through the curtain unaffected. The assumption that the created repellent air curtain contained gaps that allowed easier access was later on confirmed in smoke experiments using potassium chlorate (for a schematic drawing, see Supplemental Information, Fig. S2). Consequently, the system needed to be improved by creating more homogenous conditions and dispensing modules that could easily be implemented in a field situation. This led to the development of the five fan system (FFS) which was the first system that provided a homogenous dispersal of active ingredients over an area of 2 m^2. By using fans instead of pressurized air, the system could also easily be transferred to the field. In addition, a new repellent dispensing sachet was developed that allowed a continuous release of active ingredients from polymer granules over a period of 8 to 10 hours. Through thermal desorption gas chromatography coupled to mass spectrometry (TD-GC-MS) quantification experiments, we were able to demonstrate that the repellent air curtain created by the FFS contained greater nepetalactone quantities compared to the y-tube olfactometer. Nepetalactone quantities were on average 40 % higher (91 µg/m^3 in the FFS curtain compared to 55 µg/m^3 in the olfactometer). Behavioral experiments confirmed the efficacy of the FFS. In push-only trials with BGS trap, catnip essential oil led to a 70 % reduction in trap catch rates, the greatest reduction that was observed in room test set-ups until then. While the perforated tube system yielded an average trap catch reduction of 50 % using

25 mg of catnip oil per push-only trial, the FFS required an average of only 15 mg to achieve a comparable effect. The great potential of the FFS was also confirmed in push-pull trials with a human volunteer, now human landing rates were reduced by 50 % while BGS trap catch rates increased by 3-fold in the presence of catnip oil. As described earlier, the perforated tube system yielded a 40% reduction in human landing rates in push-pull trials using a mix of 50 mg catnip oil and 50 mg homopiperazine[4]. The new FFS provided a comparable protection of the human volunteer by using only a third of the amount of catnip essential oil.

Other studies on effective concentrations of non-pyrethroid spatial repellents are scarce. Hao et al. (2008) investigated the host-seeking and blood-feeding inhibiting potential of different essential oil constituents, however, they overexposed mosquitoes to vapors that contained more than 2500-fold higher quantities of active ingredients compared to our studies, which renders their data incomparable with ours. Field studies on the spatial repellent effect of metofluthrin coils reported a 60 % reduction in *Ae. aegypti* house entry at average indoor metofluthrin concentrations of only 0.06 $\mu g/m^3$. However, control experiments with untreated coils achieved a 50 % reduction of mosquito hut-entry, thus the reduction is most likely correlated with the produced smoke and to a lesser extent with the presence of metofluthrin. Overall, a comparison of pyrethroids and essential oil based spatial repellents is difficult, due to their different mode of actions.

In the last set of experiments, the FFS was evaluated under more rigorous conditions in a semi-field environment. Due to the limited availability of the semi-field study site, the experimental design was restricted to two important trials that had been successful when tested indoors: (1) push-only trials using a BGS trap as attracting stimulus and (2) push-pull trials involving a human volunteer and BGS trap. While catnip oil yielded a 70 % reduction in trap catch rates in push-only trials indoors, such an effect could not be observed in the semi-field setting: BGS trap catch rates in control trials did not differ from trials with catnip. Other groups have reported an inhibiting effect of catnip oil in the field. As described earlier, Menger et al. (2014) reported a 55 % reduction in the attraction of *An. gambiae* to a trapping system in the presence of catnip oil. In the presence of a novel spatial repellent compound, delta-undecalactone, trap catch rates were reduced by more than 80 %. However, the group used 4.0 g of the repellent materials per 10h trapping interval (or 0.4 g/h), which is a far greater amount compared to the 0.05 g/h used in our semi-field tests.

A satisfactory protective effect was also not observed in push-pull trials with a human volunteer. Even though BGS catch rates increased by 35 % compared to repellent-free controls, human landing collections were only reduced by 15 %. In contrast to the

[4] Due to its unpleasant odor and safety precautions, homopiperazine was excluded from further indoor tests after the initial trials

laboratory test set-up, several variations occurred in the semi-field setting: (1) the test arena had a space of approx. 800 m^3, which was 20 times greater than the laboratory setting of 40 m^3, thus spatial effects were most likely diluted, (2) the test arena was fully exposed to outdoor conditions, thus individual trials were influenced by air movement, precipitation and temperature changes and (3) the inside of the tent provided a black background (white in laboratory trials), which could have been an additional optical stimulus for the test mosquitoes. The positive outcome of laboratory tests is most likely correlated with the confined space and stagnant air, two parameters that differed completely in the semi-field study. Due to scheduling restrictions, the quantification of nepetalactone in the semi-field was not possible, however, such an analysis would have provided important indications on the dispersal of active ingredients within the outdoor setting. Most likely, greater amounts of active ingredients are required to achieve an effect, as indicated by other field studies involving nepetalactone or catnip essential oil (Menger et al., 2014; Chauhan et al, 2012). In addition it would be of great interest to include other promising candidates in future field tests, such as valerian oil, which showed great spatial repellent effects in olfactometer assays and is more cost-effective compared to catnip oil.

To date, other push-pull field studies involving catnip oil are unavailable. Field trials in western Kenya on the aforementioned novel spatial repellent delta-undecalactone resulted in contradictory conclusions. In a first study, *Anopheles* hut entry rates were reduced by more than 50 % in the presence of delta-undecalactone compared to untreated huts. In these trials, delta-undecalactone was slowly released by diffusion from microcapsules on cotton fabric (hung close to the eaves, which are one of the entry points for *Anopheles* mosquitoes), thus no active dispersal was necessary. Interestingly, outdoor catch rates of attractant and CO_2 -baited traps rarely increased compared to pull-only trials and the authors concluded that the use of push components alone is well suited for a cost-saving short-term intervention (Menger et al., 2015). The use of push-only, however, does not serve to eliminate the vector from the population and thus offers no real control. In a follow up study the same outdoor trapping device was used and eaves were mechanically sealed by wire mesh. Again, delta-undecalactone was used as the push-component in push-pull trials. This time outdoor trap catch rates of *Anopheles* slightly increased but also in control experiments without additional repellent. Thus, the mechanical barrier itself was very effective and it was advised to combine eave screening with an outdoor trap (pull-only) in a long-term study for potential population reduction (Menger et al., 2016). Wagman et al. (2014) investigated a combination of outdoor CDC light traps and indoor transfluthrin dispensers to reduce hut entry of local *Anopheles* species in Belize, Central America. Outdoor trap catches of *An. albimanus* did not increase in push-pull trials, even though hut entry was significantly reduced. The author concluded that outdoor trapping may have been more effective if performed on a larger scale, over longer periods of time and using more sophisticated traps. Identifying the proper combination of spatial repellent and trapping device for the target species remains a complex challenge in developing push-pull systems. The tested push and pull components showed varying efficacy, non-pyrethroid spatial repellents do not seem to be as powerful as pyrethroid-based active ingredients. On the other hand, pyrethroid-based ingredients may impact the success of the pull component by temporarily paralyzing the target species. To further complicate this situation, the chosen pull component may not be effective in attracting and removing the target species from the environment, even if mosquitoes are deterred by the push component.

Unfortunately, individual differences in trap sensitivity and efficacy are quite common (Lühken et al., 2014; Irish et al., 2008; Williams, et al., 2006; Kröckel et al. 2006; Kline, 2006). The BGS trap has been proven to be the superior trapping tool currently available for *Ae. aegypti* (Kröckel et al., 2006; Maciel de Freitas et al., 2006; Williams et al., 2006). In our push-pull trials in both the laboratory and semi-field, we observed a significant increase in trap catch rates in the presence of catnip, even though this effect was less prominent in the field setting. Thus, the BGS trap has a great potential to serve as an alternative host in a push-pull system for *Ae. aegypti*.

In the absence of repellent (in laboratory pull-only trials) the BGS trap did not significantly reduce human landing collections, however, a small proportion (9 % to 16 %) of the test mosquitoes was attracted to the BGS trap instead of the human volunteer. Considering the strict conditions of the laboratory set-up and the close proximity of the human host, a trap catch rate of up to 16% however shows great promise for the application of the BGS trap in a more realistic setting. When installed on a domestic level, the trap would not have to compete with human hosts constantly, thus there is a better chance for population reduction. In fact, recent studies have shown that traps can be employed as vector control tools. In Brazil, BGS traps were installed and operated in different clusters within the city of Manaus over a period of 1 year. In treatment areas, each household received one trap whereas control areas received no traps. During the rainy season, when *Ae. aegypti* abundance reaches its peak, the population in the treatment clusters was significantly reduced compared to the control clusters and dengue infections were also less common (Degener et al., 2014). A field study in Italy showed that the BGS trap is capable of reducing *Ae. albopictus* human landing collections. When used at densities of 1 trap per 150 m^2 up to 1 trap per 350 m^2 in urbanized intervention sites, *Ae. albopictus* landing rates were reduced by up to 87 % compared to control sites (Englbrecht et al., 2015). In New Jersey, continuous use of a homeowner version of the BGS trap in treatment clusters resulted in a significant reduction in *Ae. albopictus* biting pressure as compared to control clusters (Isik Unlu, 2015, pers. comm.) A different approach used autocidal gravid ovitraps (AGO) to control natural *Ae. aegypti* populations in Puerto Rico. The use of three to four traps per household led to a significant reduction of the vector population. Over the course of the 1 year study, *Ae. aegypti* capture rates were reduced by 50 % to 70% (Barrera et al., 2014). All these studies provide a strong indication, that traps can achieve a reduction in the vector population when used constantly over longer time periods. This aspect was not investigated within the chosen semi-field set-up but it would have been very interesting to evaluate our push-pull approach over longer periods in the absence and presence of a human host. Such an experiment could also help to clarify the impact of the push-component: would it be greater if mosquitoes were exposed over longer periods before the human host is present or does the push component indeed not contribute to an increase in trap catches, i.e. would the pull component be sufficient to reduce human vector contacts? The success of our push-pull system may also have been greater if the trap had been equipped with CO_2. Carbon dioxide was excluded from our trials as (1) the BGS trap and BG lure alone are very efficient in attracting *Ae. aegypti* and (2) the frequent use of CO_2 requires logistics and creates additional costs (Kröckel et al., 2006). Adding CO_2 as a supplemental attractant is another interesting aspect for the improvement of our push-pull system and should be included in future trials.

In the end, the most important question remains: is push-pull a suitable approach for mosquito vector control? Although the idea of push-pull is decades old, to date only very few successful examples are known, for instance, the control of the cotton bollworm *Helicoverpa armigera* Hübner (Pyke et al., 1987; Duraimurugan & Regupathy, 2005), the mountain pine beetle *Dendroctonus ponderosae* Hopkins (Lindgren & Borden, 1993) and the cherry fruit fly *Rhagoletis cerasi* L. (Aluja & Boller, 1992). Extraordinary success was only reported from managing stem borers (*Chilo partellus* Swinhoe*, Eldana saccharina* Walker*, Busseola fusca* Fuller and *Sesamia calamistis* Hampson) in maize in sub-Saharan Africa (Khan et al., 1997 & 2000). By intercropping non-host plants that emit repellent volatiles, the abundance of stem borers in maize plantations is reduced. At the same time, stem borers are lured to alternative host plants located outside of the maize field. However, these alternative plants do not allow larval development. This example is the gold standard of push-pull pest management, as it is non-toxic, ecologically sound, sustainable and highly effective. It remains a great challenge to replicate this success in other systems, mainly because most push-pull research focuses on long-range stimuli deterrence rather than looking into the full range of cues, thereby missing important information on short distance and contact effects (Eigenbrode et al., 2016). In mosquitoes, long- and short range attraction is well studied, but so far, effective long-range repellents have not been identified – and they might not exist. Thus, at this point in time it should be concluded that contact with the vector might be inevitable, but the current state of the art provides evidence that human-vector contact can be reduced through the combined use of effective trapping systems and spatial repellents.

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Acknowledgments

It is about time to thank those who made this work possible and provided their continuous support. First, I would like to express my sincere gratitude to my supervisor Prof. Dr. Joachim Ruther. Thank you for accepting me as an external PhD student and providing me with the freedom to pursue my projects without objections; at the same time I knew that your door was always open and I greatly appreciate your scientific advice and our constructive and insightful discussions about my work. I would also like to thank you for allowing me to use the equipment and materials needed to run the TD-GC-MS analyses.

Heartfelt thanks to my first mentor Dr. Martin Geier and to Dr. Andreas Rose who enabled me to do this doctoral thesis and funded my research. It has been a long journey and it has not always been an easy one but you confided in me and endowed me with the personal freedom that helped me to get through even the challenging phases of my dissertation. Whenever needed, you were willing to lend an open ear and I greatly appreciate your scientific advice and our constructive discussions.

Sincere thanks to Dr. Ulrich Bernier, my second mentor, for his input and scientific advice and his contributions to chapters 3 and 6. I am especially grateful for having had the chance to do my semi-field work at the facilities of the USDA-ARS-CMAVE in Gainesville, Florida. In this context I would also like to thank Dr. Daniel L. Kline for providing access to the outdoor test cages and Joyce Urban for her help with the test mosquito rearing and preparation.

Special thanks to the members of the Chemical Ecology working group who quickly affiliated me even though I was not around on a day-to-day basis and were always interested to hear how the mosquitoes were doing. I enjoyed the joint seminars, social breakfasts and summerly "beer hikes" which provided the opportunity to exchange ideas in an informal atmosphere. Sincere thanks to Ingmar Weiss and Johannes Stökl for their support with the TD-GC-MS analyses.

I have been working with Biogents for more than 13 years now and I am very grateful to my colleagues. The friendly and familiar work environment were a great support during stressful times. Many thanks to Astrid Schuhbauer and Silke Göttler for their assistance in the early experimental stages.

My friend and colleague Eva Veronesi earns to be mentioned as she was the one who encouraged me to start a PhD and always followed my research with great enthusiasm. Whenever she could, she pushed me forward in my career and she always lend me an open ear when things got complicated. Thank you, dear Eva, for your inspiration and moral support, you truly are one of a kind.

I cannot thank Scott Gordon enough for his ongoing support, motivation, patience and for proof-reading this dissertation and every submitted publication included in this work. Thank you for helping me to stay sane (enough).

Last but not least I would like to thank my parents for their trust, encouragement and moral support. You gave me the liberty to pick my goals in life, always confiding that I would not fail. I consider myself very lucky having the both of you always on my side.

Supplemental Information for Chapter 1

Short History of Mosquito Control

More than 130 years ago, mosquitoes were linked to human diseases for the first time. During the Spanish-American war, between April and August 1898, around 1.500 cases of yellow fever were detected among the American troops, of which more than 200 were fatal. More soldiers died of infectious diseases than in combat. Nineteen years earlier, Carlos Finlay, a Cuban physician, had presented his mosquito-vector theory which was based on the hypothesis that in order "to inoculate yellow fever it would be necessary to pick out the inoculate material from within the blood vessels of a yellow fever patient and to carry it likewise into the interior of a blood vessel of a person who was to be inoculated. All of which conditions the mosquito satisfied most admirably through its bite" (Chaves-Carballo, 2005). Finlay also had a strong suspicion on the responsible vector species, he believed it to be *Culex fasciatus* which was found close to inhabited areas and bit in the early morning and afternoon. *Culex fasciatus* was later on classified as *Stegomyia fasciatus* and eventually as *Aedes aegypti*. He started inoculation experiments on human volunteers using contaminated mosquitoes, hat had been feeding on yellow fever patients. Nine days after the experiments, the first subject developed typical syndromes, like fever, jaundice and albuminuria (Chaves-Carballo, 2005).

In 1900, the United States of America sent a newly formed yellow fever commission headed by Walter Reed to Cuba. The commission scanned through Finlay´s experimental notes and publications, however some members of the commission remained skeptical about his mosquito-vector theory. During their stay in Havana, James Carroll and Jesse Lazear were bitten by infected mosquitoes (Carroll voluntarily, Lazaer by a stray individual): four days after the bite Carroll developed severe symptoms, Lazaer died after 12 days (Chaves-Carballo, 2005).

In 1898, William Gorgas became chief sanitary officer in Havana with the task to eradicate yellow fever and malaria. Influenced by Finlay´s theory which had been confirmed by Walter Reed and the yellow fever commission, Gorgas started his program of eliminating or reducing mosquito infestations in 1901. His team cleared out ditches, burned insecticides and oiled the streets with kerosene to kill mosquito larvae. Within only 7 months, yellow fever was eradicated from Cuba (Reeves, 1980; Patterson, 1989). As a consequence, Gorgas was called to take the same measures against yellow fever and malaria in Panama, when the U.S. had decided to continue the constructions of the canal. First constructions had been undertaken in 1881 by the French Panama Canal Company, at a time when the role of mosquitoes in disease transmission was yet unknown. Yellow fever and malaria killed one third of the company´s workers every year, by 1889 an estimated 22.000 lives were lost and almost \$ 300.000.000 US had been spent (Cadbury, 2003). Gorgas started his *Ae. aegypti* elimination program in 1904, first by improving the living conditions in Panama city. A central water supply and sewage system was installed, along with indoor toilets and proper garbage disposal. The entire city was fumigated. By 1906 yellow fever was eradicated from Panama. Fighting malaria was a greater challenge, however, Gorgas and his team of 1.200 workers succeeded again by draining swamps, burning insecticides and even killing mosquitoes by hand. Within 10 years, malaria infections in workers of the canal construction site dropped from 80 % to 4 % (Patterson, 1998).

It became clear that disease control can be achieved through rigorous reduction of the vector population. In 1932, the Rockefeller foundation started their mosquito eradication program in collaboration with the Brazilian Government. Led by Fred Soper, a team of 40.000 workers eliminated mosquitoes from vast areas of the country which led to one of Soper´s greatest achievements: the eradication of *Anopheles gambiae* from northeastern Brazil within a period of only 22 months (Reeves, 1982). The great success of former eradications program was predicated on resolute administration and management: clear lines of command, careful planning and recording, explicit task delineation and rigorous and disciplined execution.

Fred Soper has been described as a physically imposing man, very cold and very formal. "Fred Soper was the General Patton of entomology" (Gladwell, 2001). His eradication plan was based on a highly disciplined and rigorous protocol: "he would map an area to be cleansed of mosquitoes, give each house a number, and then assign each number to a sector. A sector in turn would be assigned to an inspector, (…) the inspector´s schedule for each day was planned to the minute, in advance, and his work double-checked by a supervisor" (Gladwell, 2001). Rumor has it Soper sent condolences to the family of one of his inspectors after he had heard about an explosion in an ammunition dump in one of the work sectors (thanks to his elaborate schedule he easily identified the responsible inspector). When the inspector showed up at work the other day, Soper fired him right away.

Encouraged by the examples of Gorgas and Soper, the Pan American Health Organization (PAHO) developed a hemispheric campaign in 1947 to eradicate *Ae. aegypti* from the American continent. It was the advent of dichlorodiphenyltrichloroethane (DDT) and mosquitoes showed great susceptibility to this new insecticide. The elimination program started in northeastern Brazil and continued in most parts of Latin America by applying the so-called "perifocal" method: a 5% suspension of DDT was used to treat any open water container that could serve as a mosquito breeding site (Camargo, 1967). By 1962, 18 countries and some Caribbean islands had achieved eradication (PAHO Report, 1997). The great success of the campaign was accredited to the well-trained personnel, programs were executed with a military-type organization, clear lines of command, strict supervision and high levels of discipline. Unfortunately after 1962, eradication programs slowly deteriorated. Political importance of *Ae. aegypti* control was lost in countries that had achieved eradication. The development of insecticide resistances, rapid urbanization and increased international and domestic travel further contributed to mosquito re-infestations and expanded their geographic distribution (Brathwaite Dick et al., 2012). Today, *Ae. aegypti* is endemic in more than 100 countries in Africa, the Americas, South-East Asia and the Western Pacific (WHO, 2015).

Supplemental Information for Chapter 6

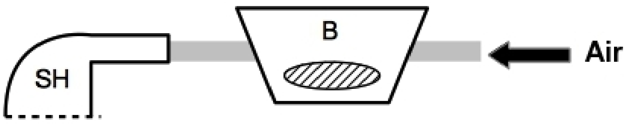

Fig. S1: Schematic drawing of the SHS (one unit)

Table S1: Wind speeds [m/s] within the FFS air curtain. Wind speeds were measured with an anemometer at 137 cm above ground (position 1), 107 cm (position 2), 77 cm (position 3) and 44 cm (position 4). Each position shows the minimum and maximum speed recorded in three individual measurements.

Position	3 V	4.5 V	6 V	7.5 V	9 V	12 V
1	0.7 – 1.0	0.8 – 1.5	0.8 – 1.4	0.9 – 2.0	0.9 – 2.0	1.0 – 2.4
2	0.4 – 0.6	0.6 – 0.8	0.6 – 1.0	0.9 – 1.4	0.9 – 1.4	0.8 – 2.0
3	0.3 – 0.5	0.5 – 0.8	0.6 – 0.8	0.6 – 0.7	0.7 – 0.8	0.8 – 1.0
4	0 – 0.1	0.1 – 0.2	0 – 0.2	0.1 – 0.2	0.1 – 0.4	0.2 – 0.4

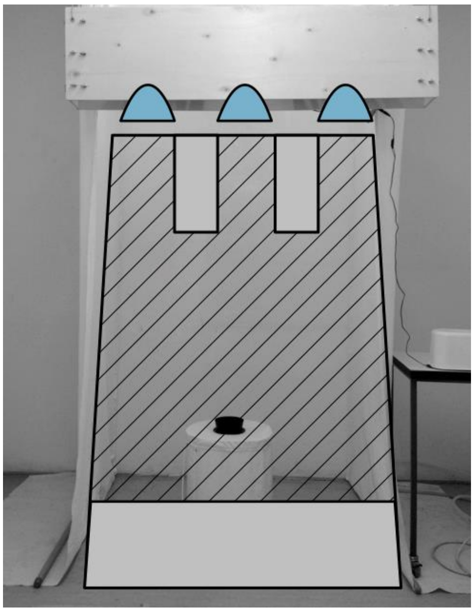

Fig. S2: Sketch of the air curtain generated by the shower head system (SHS). Areas of lower density are indicated in grey. Air volume was estimated at 0.18 m^3 (1.65 m × 1.2 m × 0.09 m).

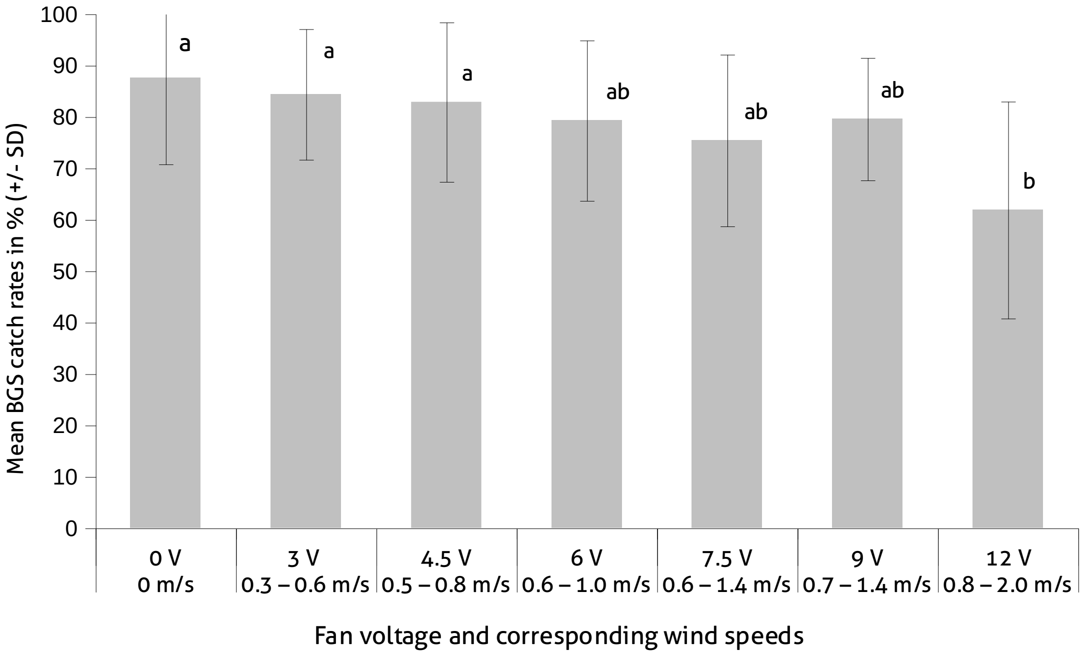

Fig. S3: BGS recapture rates (means ± standard deviation, SD) of *Ae. aegypti* in control trials of the five fan system (FFS). The x-axis gives the different operating voltages with corresponding wind speeds, which were generated in the center of the tent opening (between positions 2 and 3 or at 77 to 107 cm above ground). Different letters indicate significant differences at $P < 0.05$ (Mann-Whitney-*U*-test, n=10).

Fig. S4: Sketch of the air curtain generated by the five fan system (FFS). Air volume was estimated at 0.24 m^3 (1.7 m × 1.2 m × 0.12 m).