Olfactory mediation of oviposition in *Anopheles* mosquitoes:
a new strategy for malaria vector control

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Thesis

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Chapter 1

General introduction

1.1 Mosquitoes and malaria transmission

Vector-borne diseases account for about 17% of the infectious diseases in the world; of these, three quarters are mosquito borne (Townson et al. 2005). Malaria, one such mosquito-borne disease, is one of the most serious public health problems facing low and middle-income countries. The disease remains one of the most important causes of morbidity, mortality and economic loss (Breman et al. 2006). Malaria is caused by *Plasmodium* parasites which are transmitted by female anopheline mosquitoes. Malaria transmission of varying degrees occurs in 100 countries inhabited by over 40% of the world population. In 2015, 212 million cases occurred globally, leading to 429,000 deaths, most of which occurred in children under the age of five years in Africa (Shretta et al. 2017). Most malaria cases in 2017 were in the WHO Africa Region (200 million or 92%), followed by the WHO South-East Asia Region with 5% of the cases and the WHO Eastern Mediterranean Region with 2% (WHO 2018). Due to a hugely stepped-up and massive global malaria control programme, between 2000 and 2015 the *Plasmodium falciparum* prevalence in Africa dropped by 50% and clinical disease by 40%, (Bhatt et al. 2015), data for the period 2015–2017 indicate that no significant further progress in reducing malaria cases was made during this timeframe (WHO 2018).

In Tanzania, the burden of malaria is very high: every year, 14–18 million new malaria cases are reported resulting in 120,000 deaths. Of these deaths, 70,000 are very young children. The annual incidence rate is 400–500/1,000 people and this number doubles for children less than five years of age (NBS 2016). The United Republic of Tanzania has an estimated population of 50 million, whereby over 90% of its population lives in areas where malaria transmission is high (WHO 2018). The endemicity and risk of transmission varied as shown by the project “Mapping Malaria Risk in Africa” (Tanser and Le Sueur 2002). Tanzania has the third largest population at risk of stable malaria in Africa, after Nigeria and the Democratic Republic of Congo. The socio-economic impact of malaria is high and contributes continuously to poverty and underdevelopment (Mboera et al. 2007). Malaria has been estimated to cost Tanzania more than US$ 240 million every year in lost gross domestic product, although it can be controlled for a fraction of that sum (Makundi et al. 2007).

Current control programmes for malaria vectors have a number of weaknesses, both actual and potential. First, they tend to rely almost entirely on indoor control measures, such as the use of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRSs) of insecticides. Such measures prevent only biting/transmission events that occur indoors; they have no impact on mosquito vectors that target and feed upon their hosts outdoors. Furthermore, over time, this may lead to the selection for malaria transmission by mosquitoes that bite outdoors, gradually undermining the disease prevention efficacy that has been achieved by indoor control measures (Russell et al. 2011, Govella and Ferguson

2012). Secondly, both ITN and IRS rely on conventional insecticides, typically synthetic pyrethroids, to prevent blood feeding. This presents a great danger of the development of resistance in the target vectors, a recurring challenge in previous efforts to control mosquito vectors. Recent research demonstrated that insecticide resistance is spreading rapidly across malaria-endemic countries (Ranson and Lissenden 2016). These developments underscore the need for a safer and more effective solution to control mosquito vectors.

Due to the devastating and often preventable impacts of malaria, efforts need to be put forth to not only suppress the prevalence and mortality, but to completely eradicate the disease from the endemic regions. In order to accomplish this ambitious goal, it is critical to establish effective, environmentally friendly, and sustainable long-term control of the mosquito vectors responsible for the transmission of malaria. Rather than focusing solely on the use of adult-targeting conventional insecticides, as mosquito control programmes have done in the past, today's vector control efforts must be expanded to include all possible methods of mosquito suppression at all life stages, such as targeting different behaviours that enable mosquitoes to avoid conventional strategies outdoors and indoors (Takken and Knols 2009). These tools include methods to target mosquitoes in their behaviours, which can impact on blood feeding and reproduction.

1.2 The behaviours of mosquitoes that can be exploited for malaria control

There are several behaviours that can be targeted for mosquito control (Takken 1999). Most of the existing control tools have been developed by exploiting such behaviours, as highlighted by (Killeen 2014). These tools target mosquitoes at different stages in their lifecycle (Fig. 1).

Extensive ecological research must precede the development of such tools to understand how malaria mosquitoes search for mates, sugar and blood meals, resting sites and oviposition sites (Ferguson et al. 2010). As chemical cues (infochemicals) are involved in many mosquito resource-finding processes, elucidation of these cues can help to develop alternative strategies of mosquito control. For example, infochemicals can be made into attractive baits for use in push-pull or attract-and-kill techniques, or in the surveillance of vector populations.

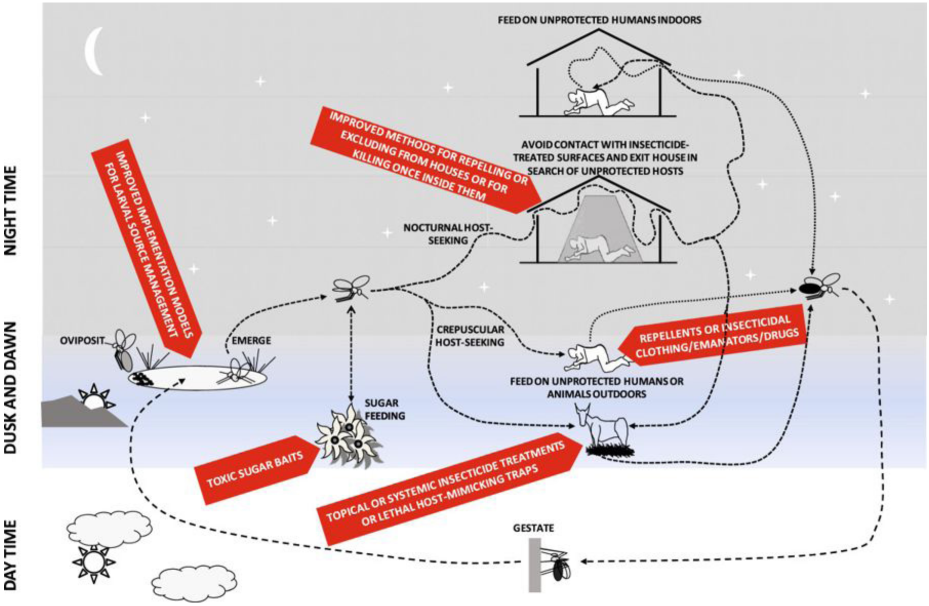


Figure 1 - Lifecycle of malaria vectors showing insecticide avoidance behaviour and novel tools that can target mosquitoes at different stages in their lifecycle (Source: Killeen 2014)

1.3 The biology of aquatic stages of mosquitoes and its role in malaria control

Mosquitoes spend the most crucial period of their life in an aquatic environment. In the early days of their life, human-biting mosquitoes such as African malaria mosquitoes develop in aquatic environments in the vicinity of human dwellings. Eggs are laid in a body of water, the larvae hatch and several larval stages and pupal formation occur in the water. Moreover, adult emergence, which is another crucial moment, occurs in the aquatic environment. In the tropics, mosquitoes spend 10 – 14 days from egg to adult emergence; during these aquatic stages, they are in a restricted environment and therefore can easily be controlled if accessed. Moreover, most adult anopheline mosquitoes do not live more than two weeks under natural conditions. Therefore, as short-living organisms, female mosquitoes lay many eggs to maximize their number of progeny. In a nutrient-enriched site, a single mosquito may lay 50 - 500 eggs at one time (Clements, 1992). The productivity of larval habitats is a key factor which governs the size of mosquito populations and, in the case of anopheline vectors, the transmission of human malaria parasites.

In the field, some water bodies contain high densities of anopheline larvae, whereas many others have none despite high densities of adult mosquitoes in the immediate environment (Minakawa *et al.*, 2002). Therefore, there is a huge variation in adult density, depending on the more or less suitable conditions prevailing in larval habitats. Among adverse conditions, desiccation of habitats, competition for food or space and predation either among themselves or other species are of primary importance. These adverse conditions, which occur during larval development, are the main reason for larval mortality, and may also have profound effects on life-history traits, e.g., growth rate, fecundity and longevity (Telang et al. 2012, Muriu et al. 2013). These are important factors affecting pathogen transmission through changes in general adult fitness and vectorial capacity, which measures the transmission potential of a given infectious agent by a vector population (Bara et al. 2015, Roux et al. 2015, Vantaux et al. 2016).

For these reasons, adverse conditions that compromise the life of aquatic stages of mosquitoes have an influence on the adult population and the probability of *Plasmodium* infections. Moreover, the mobility of aquatic stages of mosquitoes is limited by the boundary of a larval habitat; this reduces the operational costs in the control of these vectors at larval stages by treating only sites that produce high numbers of mosquitoes. Consequently, integrating targeted larval control measures with other strategies that leverage selective oviposition behaviour, such as luring ovipositing females to gravid traps or egg sinks are recommended in order to improve the efficiency of control (Wong et al. 2012).

1.4 Mosquito oviposition behaviour and the distribution of larvae

In mosquitoes, the distribution of larvae is an accurate predictor of the oviposition site(s) selected by gravid females (Pates and Curtis 2005). Consequently, biotopes occupied by the immature stages represent sites selected by their mothers (Clements 1999). The fact that many anopheline larvae are often found in only a few sites (Keating et al. 2004) suggests that females exhibit a selective oviposition behaviour, thus there is a need to investigate the site-selection behaviour of female anophelines. Previous studies on oviposition behaviour of mosquitoes indicate that many species are capable of using tactile, chemo-tactile, olfactory or visual cues to assess such site characteristics as colour, reflectance, texture, moisture, salinity, surrounding vegetation, bacterial growth, fungal infusion, conspecific population density, and the presence of a variety of chemicals (Sivagnaname et al. 2001, Sumba et al. 2004, Rejmankova et al. 2005, Okal et al. 2013). Recent studies (Asmare et al. 2017, Eneh et al. 2019) have revealed that volatile substances released from larval habitats may be potential olfactory cues mediating oviposition. Hence there is a need to study specific larval habitats of *An. gambiae* and *An. funestus*, the most important malaria vectors in Africa in order to identify new putative chemicals that may be used to manipulate gravid mosquitoes.

1.5 Oviposition-site selection by gravid mosquitoes

Oviposition-site selection by mosquitoes is a critical factor for fitness of individual females and is therefore an essential part of the life history of all species (Mboera 1999). Gravid female mosquitoes seek a suitable site for oviposition depending on the physiological requirements of their immature stages. Larvae of *An. minimus* are found in the grassy margins of lightly shaded, flowing streams. These sites are readily recognizable during the daytime on the criteria of shade, and air and water temperatures in the breeding site and outside, but these differences do not apply when gravid *An. minimus* are searching for an oviposition site after midnight on moonless nights. At night, there was no difference in illumination under the light shade of the breeding site and in the open, and differences in air and water temperatures, which had been so marked in the afternoon, were no longer apparent. However, *An. minimus* only deposited eggs in those sites in the field where the larvae could survive. This suggests that species of *Anopheles* mosquitoes are guided by specific cues when searching for a suitable oviposition site, and this confirms the principle by Shannon that the larvae of each mosquito species are more or less restricted to a special type of habitat and consequently, gravid mosquitoes of each species and often each group of species, subgenus or genus has its own preference type of habitat (Hopkins, 1952). The role of infochemicals in the oviposition behaviour of mosquitoes has now been well described, and can be considered a guiding principle in the selection of oviposition sites by gravid mosquitoes (Okal et al. 2013, Brown 2015, Eneh et al. 2016)

1.6 Mosquito breeding habitats

Mosquito larval habitats can be classified as stagnant aquatic and flowing aquatic habitats or natural and man-made habitats. Stagnant aquatic habitats can form either temporary or persistent water biotopes. Habitats of *Anopheles* mosquito species differ amongst each other having species-specific characteristics. Therefore, habitat requirements of *An. gambiae* differ from that of *An. funestus* despite the fact that adult stages of both species exist in the same settings (Paaijmans 2008, Emidi et al. 2017).

Mosquitoes of *An. gambiae* s.l. occur in a great variety of water bodies, and the most common sites consist of shallow, open sun-lit pools. The origin of such pools is diverse, and may range from borrow pits, drains, brick-pits, ruts, car tracks, and foot/hoof-prints around ponds and water holes. Others include the overflow from rivers, pools left by receding rivers, back waters, and rain water collecting in natural depressions (Fillinger et al. 2009, Hamza and El Rayah el 2016). The stamp of human activity is implicit in many of these sites, such as overstocking and subsequent loss of plant cover, erosion and scouring (Moreno et al. 2007, Olson et al. 2010). More fundamental is the partial drainage and exploitation for crops of permanent or seasonal swamps. For example, seasonally waterlogged pans provide numerous breeding sites if cleared for cattle or dug up for crop or vegetables production (Kweka et al. 2016, Paul et al. 2018). *Anopheles funestus* breeds in bodies of clear water that are either large and more or less permanent, e.g., swamps (near the edge if these are deep), weedy sides of streams, rivers, furrows or ditches, protected portions of a lake shore, ponds, especially when weedy, or water such as seepages coming from underground permanent sources (Minakawa et al. 2008). Emergent vegetation is found to be the most important ecological feature of *An. funestus* habitats. This may be in the form of short grasses with very little shading of water surface or of tall reeds or trailing vegetation providing a much greater degree of shade (Gillies 1987, Tchigossou 2018).

1.7 Infochemicals mediating the oviposition behaviour in mosquitoes

Mosquitoes choose breeding sites preferentially by selecting sites that are most suitable for their young, based on indicators of suitability in the candidate sites. They do so using volatile chemical compounds, which in most cases are produced by various organisms in the breeding sites. Chemicals that convey information are widely distributed in nature; they may comprise even toxins and nutrients (Nordlund 1981, Dicke 1988). My study focuses on “infochemicals” in the oviposition behaviour of malaria mosquitoes. Infochemicals are defined as all chemicals that mediate specific responses altering the behaviour and/or physiology of the recipient organism (Dicke and Sabelis 1988).

Mosquitoes have evolved to identify various infochemicals produced in the breeding sites and associate them with the suitability of the site. This assumption was verified by studying the oviposition behaviour of *Culex tarsalis*, which preferred to lay eggs in water with conspecific larvae rather than in distilled water (Osgood 1971). Later, it was found that eggs of *Cx. quinquefasciatus* produce an apical droplet, which contains an infochemical just after the eggs hatch (Bruno and Laurence 1979). This compound is volatile and as it diffuses, it invokes other gravid *Cx. quinquefasciatus* mosquitoes to lay eggs in that breeding site. The compound is produced only after the larvae have hatched, which is 2-3 days after the eggs were deposited. It will not be produced if larvae have not hatched due to unfavourable conditions or otherwise consumed by predators. In other words, the infochemical indicates to the gravid female that a certain site can support immature development, as the larvae have hatched successfully. The active compound was identified as erythro-6-acetoxy-5-hexadecanolide, which became known as oviposition pheromone (Laurence and Pickett 1982). A synthetic oviposition pheromone (SOP) was later produced and successfully tested in the laboratory and in the field (Mboera et al. 2000).

Plants and wood infusions have long been associated with mosquito larvae and were eventually found to attract gravid females (Bentley et al, 1979, Millar et al., 1992). P-cresol from wood infusion and phenol, 4-ethylphenol, 4-methylphenol, indole and 3-methylindole from grass infusion attract *Aedes triseriatus* and *Cx. quinquefasciatus* gravid females to their oviposition sites. Furthermore, scientists evaluated the influence of either plant infusion or a synthetic composition of their chemicals when mixed with a known oviposition pheromone to see whether there is any synergistic effect. For example, an additive effect of a mixture of 3-methylindole and a synthetic oviposition pheromone (SOP) was found (Millar et al. 1994). As well, the synergistic attraction of gravid females of *Cx. quinquefasciatus* to oviposition stimuli of grass infusion mixed with SOP has been established (Mboera et al. 1999). Moreover, it was realized that oviposition by *An. albimanus* is mediated by stimuli from certain plants, which after being extracted and analyzed, yielded guaiacol, phenol, isoeugenol, longifolene, caryophyllene, phenyl ethyl alcohol, and *p*-cresol (Torres-Estrada et al. 2005).

Several fatty acid esters have been found to mediate oviposition behaviour in *Ae. aegypti* and *Ae. albopictus*. Propyl octadecanoate attracts gravid *Ae. aegypti* to an oviposition substrate while tridecyl octanoate, hexadecyl pentanoate and tetradecyl heptanoate have been shown to significantly repel gravid *Ae. aegypti* (Sharma et al. 2008). Similarly, the same authors reported that decyl undecanoate, nonyl dodecanoate, pentyl hexadecanoate and propyl octadecanoate elicit concentration-dependent positive oviposition responses (Sharma et al. 2009). This mechanism is also displayed in *Aedes* mosquitoes, whereby an infochemical that stimulates other gravid *Ae. aegypti* to lay eggs is also produced by larvae. Water from larval habitats was analysed and a chemical compound, heneicosane, was identified as the most abundant compound that acts as an oviposition attractant (Mendki et al. 2000). On the other hand, some researchers extracted from *Ae. aegypti* eggs several acids and esters that elicit oviposition attraction in gravid mosquitoes (Ganesan et al. 2006). These include decanoic and (Z)-9-hexadecanoic acids. Thus, culicine mosquitoes are reported to signal first the successful hatching of eggs and then the survival and development of larvae in the breeding site. This is very important for the fitness of a female mosquito because in nature, not all hatched eggs may survive and develop to larval stages.

Bacterial volatiles play an important role in informing gravid mosquitoes of the presence of suitable breeding sites (Rockett 1987, Huang et al., 2006, Lindh et al., 2008, Ponnusamy et al., 2008, Trexler et al., 2009). Several bacterial species are known to produce infochemicals that mediate oviposition behaviour in mosquitoes. Bacterial culture filtrates were found to attract more *Cx quinquefasciatus* than *p*-cresol, and they were found to consist of *Bacillus thuringiensis* var *israelensis* (*Bti*) and *Bacillus cereus* (Poonam et al. 2002). Moreover, it was confirmed that water containing the bacteria *Psychobacter* *immobilis* and *Sphingobacterium multivorum* elicited significantly more oviposition of *Aedes albopictus* than control water without bacteria (Trexler et al. 2003). Gravid mosquitoes also respond to volatiles produced by microbial metabolic activities in hay or leaf infusions (Lindh et al., 2008, Ponnusamy et al., 2008; 2010). These infusions are often used in ovitraps for monitoring the egg-laying activities of *Aedes* and *Culex* mosquitoes in the field (Ritchie et al., 2009). In addition, Sumba et al. (2004) and Eneh et al. (2016) have shown that the mediation of oviposition-site selection behaviour of *An. gambiae* is mediated by infochemicals of microbial origin.

1.8 The role of oviposition-stimulating infochemicals in mosquito control

Knowledge on insect behaviour can be exploited in order to design sustainable surveillance and control strategies for disease vectors and other pests (Githeko et al. 1996). Such behaviour includes feeding behaviour, host and sugar seeking, reproductive behaviour, mating and oviposition (Takken 1999). For example, attraction to traps in the field has been demonstrated mainly when exploiting host-seeking behaviour, where traps baited with infochemicals of host origin increased trap catches over non-baited traps (Mboera et al. 1997). Recently, oviposition infochemicals have been shown to attract gravid mosquitoes in the laboratory, semi-field and real field conditions (Schorkopf et al. 2016, Mafta-Nero 2018). In field settings, 3-methyl-indole, which is an attractant of gravid *Culex* species, has been shown to attract *Cx. quinquefasciatus, Cx. stigmatosoma* and *Cx. tarsalis,* (Beehler et al. 1994), as well as *Cx. tigripes* and *Cx. cinereus* (Mboera et al. 1999). Furthermore, Mboera et al. (2000) concluded that traps baited with oviposition pheromone and grass infusion can be used effectively to sample gravid *Cx. quinquefasciatus.*

Attractants can be combined with a killing agent, in a lure-and-kill system, whereby an attractant is used to lure gravid mosquitoes to lay eggs in an area where their offspring will be killed (Mboera 2003, Ong and Jaal 2015). In a study conducted in north-east Tanzania (Schorkopf et al. 2016), a mixture of attractants (acetoxy hexadecanolide) and biological larvicides (*Bacillus thuringiensis israelensis* and *Bacillus sphaericus*) was shown to synergize the control of mosquito disease vectors. A similar study was conducted recently in Kenya, where mosquitoes were lured by the oviposition attractant cedrol to bait stations and contaminated by an insect growth regulator pyriproxyfen, after which they transferred it to breeding sites and thereby contaminated larvae (Mbare et al. 2019). All these findings demonstrate the potential of infochemicals to be used in surveillance and control of mosquito vectors. Such understanding of what drives mosquito resource selection can have important implications for control (Mboera 1999). To date, only few studies have examined the role of infochemicals in the oviposition-site selection by *An. gambiae* and, consequently, these compounds have not been exploited for developing vector surveillance and control programmes. Provided such infochemicals can be identified, an ideal control strategy would involve an integrated vector-control approach using attractants and environmentally friendly larvicides to reduce the abundance of mosquitoes. Strategies based on the concept of oviposition-attractants will be effective and novel.

2.0 Problem definition and research goals

Currently, the major malaria vector control techniques employ the use of insecticide-treated bednets (ITNs) or indoor residual spraying of insecticides (IRS) (WHO 2018). However, insecticide resistance and environmental hazards remain the major problems facing most malaria vector control programmes in Africa (Mboera et al. 2015). An additional complicating factor is the ability of mosquito vectors to detect the presence of insecticides or larvicides from treated surfaces and divert their biting or ovipositing activities towards unprotected areas (Gerold 1977, Lines et al. 1987). Therefore, mosquito control techniques that target gravid females and/or larvae may be incorporated into integrated vector management (IVM) programmes, especially as this reduces or prevents the addition of adult mosquitoes in the existing vector population.

As an alternative strategy, I propose to use a novel concept of integrating the manipulation of a behavioural trait, namely oviposition, with an effective but environmentally-friendly biolarvicide to stop the production of future mosquito generations. In my research, olfactory cues that direct gravid *Anopheles* female mosquitoes to lay eggs in selected sites were identified. These infochemicals were used to enhance the trapping of gravid mosquitoes in oviposition traps treated with appropriate biocides (Insect Growth Regulators or *B. thuringiensis israelensis*). Attacking the initial stage of the mosquito life cycle, by manipulation of oviposition behaviour and control of young larvae using biolarvicides, could be an important alternative method of control, by killing the vectors in their cradle. The focus on oviposition behaviour has the advantage that only selected breeding sites would be treated by larvicides, as mosquitoes lay eggs in a limited number of sites. Moreover, mosquitoes do not have the opportunity to escape as they might do with bed nets or a wall surface. The immature stages of mosquitoes are confined to discrete, often small, aquatic habitats from which they are unlikely to escape (Killeen et al. 2002). The proposed strategy of mediation of oviposition behaviour and larval killing is a potentially powerful lure-and-kill system for mosquito control.

In summary the specific objectives of the work presented in this thesis are:

1. To determine and characterize aquatic habitats of *An. gambiae* and *An. funestus* in central-eastern Tanzania;
2. To investigate biotic factors that determine oviposition-site selection by gravid malaria mosquitoes of the *Anopheles gambiae* complex;
3. To investigate and characterize compounds from habitat materials that exhibit oviposition attraction;
4. To investigate the use of oviposition attractants in surveillance and control of human-biting mosquitoes;
5. To evaluate the efficacy of a lure-and-kill strategy with candidate oviposition attractants and larvicides in a natural settings.

Thesis outline and hypotheses

In **Chapter 2,** the chemical ecology of oviposition behaviour in mosquitoes is reviewed. In the literature, the culicine oviposition behaviour has been thoroughly explored, and the possibility of extending this line of research to anophelines is discussed. The potential of exploiting knowledge of the chemical ecology of oviposition behaviour of mosquitoes into development of control and surveillance techniques is emphasized.

Chapter 3 presents a study that aimed to examine if oviposition behaviour of gravid *Anopheles gambiae* was affected by the presence of conspecific instars at different development stages. In order to understand the phenomenon of preferential oviposition behaviour, we hypothesized that gravid females choose breeding sites randomly. Dual choice laboratory experiments were designed with early and late conspecific instars. To explain the nature of cues involved, a double-cup experiment was designed to distinguish between visual and chemical cues. Therefore, the results showed how larval stages of mosquitoes can manipulate their parent's oviposition behaviour and enhance their fitness.

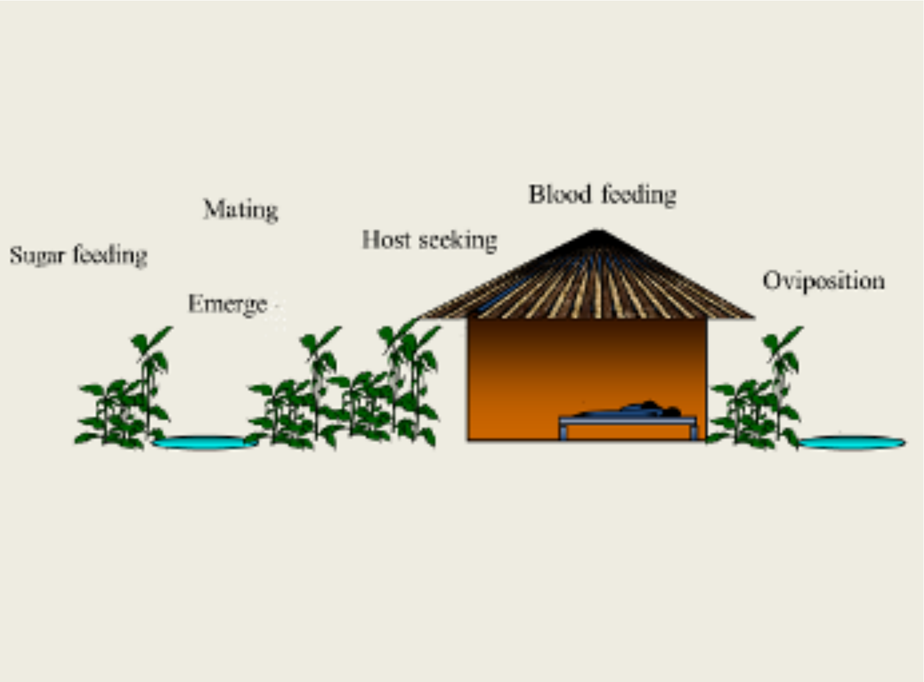
In **Chapter 4**, candidate chemicals mediating oviposition were identified and tested in the laboratory and semi-field environment against laboratory-reared *An. gambiae* mosquitoes. To establish an appropriate dose, a series of dose-response experiments were conducted in the laboratory. The established doses were verified in a dual-choice test in the laboratory and doses that performed well were selected for further testing. The selected doses were tested in a sticky-screen experiment to find out whether the chemicals emitted from first larval instars are attractants or stimulants. Physiological experiments were also designed to understand the probable effects of infochemicals on mosquitoes' fitness and behaviour. Finally, we tested the selected doses in a semi-field environment in Tanzania, which allowed high through-put testing of attractants and repellents with a colony of laboratory-reared mosquitoes in a more realistic environment than that of the laboratory. It was concluded that the identified infochemicals mediate oviposition behaviour in *An. gambiae* in opposite ways. The results obtained in the semi-field were used to design further field experiments with the most potent oviposition attractant.

In **Chapter 5**, the natural breeding sites of *Anopheles* mosquitoes were characterised in relation to land-use patterns in central Tanzania. Land-use patterns are known to influence the oviposition-site selection process, larval development and therefore general mosquito productivity. We hypothesized that practices resulting from such land-use activities influence the behavioural ecology of mosquitoes and especially oviposition strategies. The main land-use patterns in the study area were identified and the breeding sites from each type of land use were studied extensively. The density of adult mosquitoes was strongly correlated to the density of larvae in the breeding sites. The results were discussed in terms of contributions of various practices towards anopheline larval productivity in the study area.

The combined effects of candidate oviposition attractants and soil microbes from a natural breeding site are investigated under natural conditions in **Chapter 6**, in order to determine the synergistic effects of nonane and soil infusion in attracting gravid mosquitoes. Wild populations of *Anopheles gambiae* s.l. and *Culex quinquefasciatus* were exposed to clay bowls filled with watery solutions of the oviposition attractants and the microbials. Production of larvae and pupae was examined daily to establish if there was enhanced oviposition well as synergy between the treatments.

Chapter 7 describes the development of a “lure-and-kill” strategy by combining oviposition attractants (pheromones and kairomones) with biolarvicides; *B. thuringiensis israelensis* (Bti) and Temephos. The oviposition attractant of larval origin identified in Chapter 4 and then tested in Chapter 5, was now introduced to simulated breeding sites either singly or in combination with attractants. Our aim was to (i) determine the attractiveness/stimulatory effects of these compounds to natural population of ovipositing mosquitoes, (ii) determine the effects of direct sunlight/rain on attractants (iii) assess the possible repellent effects of Temephos and Bti on natural populations of ovipositing mosquitoes and (iv) assess the efficacy of Temephos and Bti on growth and development of mosquitoes when incorporated with oviposition attractants. *We concluded that the “attract-and-kill” method has high potential to be implemented as a novel tool for mosquito vector control.*

In **Chapter 8**, I discuss how the findings of this thesis contribute to the general understanding of the chemical ecology of the oviposition behaviour in anopheline mosquitoes with special emphasis on *Anopheles gambiae*. The chapter discusses whether the objectives of the thesis have been achieved, and how the results follow from previous assumptions and hypotheses. Furthermore, the consequences of the findings on vector-borne disease management, i.e., the improvement of control and surveillance techniques for mosquito vectors are discussed. The results of the research described in this thesis were synthesized to generate an overview of the entire project. New concepts that were not captured in the previous chapters are discussed and the results of the entire thesis are placed in broader contexts that provide suggestions for future research.



Oviposition behaviour is an important aspect within the mosquito life cycle.

Chapter 2

Exploiting the chemical ecology of mosquito oviposition behaviour in mosquito surveillance and control: a review

Victor Mwingira,
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To be submitted

Abstract

Vector control is an important component of the interventions aimed at mosquito-borne disease control. Current and future mosquito-control strategies are likely to rely largely on the understanding of the behaviour of the mosquito vector, by exploiting mosquito biology and behaviour, while using cost-effective, carefully timed larvicidal and high-impact, low-volume adulticidal applications. This chapter reviews the knowledge on the ecology of mosquito oviposition behaviour with emphasis on the potential role of infochemicals in surveillance and control of mosquito-borne diseases. A search of PubMed, Embase, Web of Science, Global Health Archive and Google Scholar databases was conducted using key words: mosquito, infochemical, pheromone, kairomone, allomone, synomone, apneumone, attractant, host-seeking, oviposition. Articles in English from 1974 to 2019 were reviewed to gain comprehensive understanding of current knowledge on infochemicals in mosquito resource-searching behaviour. Our findings show that oviposition of many mosquito species is mediated by infochemicals. These comprise pheromones, kairomones, synomones, allomones and apneumones. The novel putative infochemicals that mediate oviposition in the mosquito subfamilies Anophelinae and Culicinae were identified. The role of infochemicals in surveillance and control of these and other mosquito tribes is discussed with respect to origin of the chemical cues and how these affect gravid mosquitoes. In conclusion, oviposition attractants and deterrents can potentially be used for manipulation of mosquito behaviour by making protected resources unsuitable for mosquitoes (push) while luring them towards attractive sources (pull). In this review, strategies of targeting breeding sites with environmentally friendly larvicides with the aim to develop appropriate trap-and-kill techniques are discussed.

KEY WORDS: Culicidae, oviposition, infochemicals, olfactory cues, mosquito behaviour, surveillance, control.

Introduction

Exploring the chemical ecology of oviposition behaviour in mosquitoes may lead to the ecological understanding of the origin, role and significance of natural chemicals mediating various interactions within and between mosquito species and their surroundings. The observation of such interactions in nature and the elucidation of chemical compounds involved may contribute to the development of novel control and surveillance strategies necessary for the management of mosquito and mosquito-borne diseases. Most of the existing mosquito control strategies interfere with mosquito behaviours, especially biting and resting (Pates and Curtis 2005).

The need to develop new vector control methods and improve implementation of existing ones has led to the exploration of interactions in the oviposition phase of mosquitoes in an attempt to understand the mechanism of interactions and elucidate the structures of the chemical compounds regulating oviposition behaviour. A control strategy that deploys chemicals attracting/stimulating or deterring gravid females intending to oviposit is considered to be novel. It would contribute to addressing the global demand for the reduction of toxic chemicals in the environment and therefore could be an essential component of integrated vector management strategies in the future (Cook et al. 2006).

Many mosquito species exhibit oviposition behaviour through specific selection of breeding sites (Osgood 1971, Laurence et al. 1985, Blackwell et al. 1993, Chadee et al. 1993, Zahiri et al. 1997, Allan and Kline 1998). Generally, mosquitoes avoid ovipositing in sites that are occupied by competitors or predators (Kiflawi et al. 2003a, Blaustein et al. 2004). They tend to oviposit in habitats with conspecific larvae, as these indicate the suitability of the habitat for the survival of the next generation (Blaustein and Kotler 1993a, Allan and Kline 1998, Mwingira et al. 2019). Therefore, mosquitoes are discriminating in selecting sites for oviposition, as they occupy a non-random set of aquatic habitats (Heard 1994, Minakawa et al. 2004).

Biotopes occupied by the immature stages are sites selected by their mothers (Clements 2000, Mwingira et al. 2009, Mwingira et al. 2019). Consequently, oviposition-site selection can influence the distribution and dynamics of the next generation (Spencer et al. 2002). Thus, female mosquitoes can increase their contribution to the next generation by selecting oviposition sites without predators and competitors (Kiflawi et al. 2003b, Blaustein et al. 2004) or with abundant food (Blaustein and Kotler 1993b, Sherratt and Church 1994).

Oviposition-site selection by mosquitoes is a critical factor for fitness and is therefore an essential part of the life-history of all species (McCall and Cameron 1995, Mboera 1999). Understanding insect oviposition decisions may provide further insight into the factors affecting population dynamics and the epidemiology of mosquito-borne diseases, and assist in predicting population responses to control measures (Nylin 2001, Pates and Curtis 2005, Vonesh and Blaustein 2010). As only few breeding sites are of epidemiological significance (Keating et al. 2004, Fillinger et al. 2009), investigating the site-selection behavioural process of female mosquitoes in an attempt to develop a control strategy is crucial.

Previous studies on oviposition-site selection of mosquitoes indicate that many species are capable of using tactile, taste, olfactory and visual cues to assess such site characteristics as colour, reflectance, texture, moisture, salinity, surrounding vegetation, bacterial growth, fungal infusion, conspecific population density and the presence of a variety of chemicals (Millar et al. 1992b, Blackwell et al. 1993, Takken and Knols 1999, Sivagnaname et al. 2001a). Several studies have confirmed the mediation of oviposition behaviour in mosquitoes by intra- and inter-specific chemical signals across different species (Afify and Galizia 2015). Most of these findings came from the Culicinae subfamily, which has been the mosquito group most intensively studied. The objective of this review is to explore the role of infochemicals in mediating oviposition behaviour of mosquitoes, and evaluate how these cues may be used for mosquito control.

Physical stimuli

Many studies have explored possible roles of visual, tactile, olfactory and taste stimuli in mediating oviposition behaviour in mosquitoes (Beehler et al. 1993, Reiskind and Zarrabi 2012). The function of the senses of mosquitoes is summarized by Montell and Zwiebel (2016).

Visual cues

The major sense organs of most insects include a pair of compound eyes, three simple eyes and a pair of antennas. Simple eyes are found in many immature and some adult stages. The structure of the eye varies depending on whether the insect is habitually active only by day or by night. Vision is most widely used by diurnal insects, which live in open habitats (Lehane 1991). In many nocturnal and semi-nocturnal insects, the eye is large enough to allow maximum photon capture, and contrast sensitivity is improved at low ambient light levels (Land 1981). Grousp of facets in different parts of the eye may vary for different kinds of vision; for example, separate groups of facets may have specialized sensitivity to colour and light. This is illustrated by the relationship between theoretical optimum eye parameters and various illuminance conditions as observed for *Aedes aegypti* (Muir et al. 1992).

Most mosquitoes have apposition eyes, which do not function well at low light intensities (Clements 1999). However, *Anopheles gambiae* have exceptional, conically-shaped eyes that allow photons to be intercepted by the lens (Land et al. 1997). Their apposition eye is adapted for high sensitivity in dim environments, where high photon capture takes precedence over fine resolution (Kirschfeld 1974). Therefore, *An. gambiae* appears to be more sensitive to light intensity than other mosquito species (Land 1999).

Most mosquito species are capable of discriminating wavelengths of various ranges during oviposition. *Aedes aegypti*, oviposition-site acceptability is negatively correlated with intensity of illumination (Clements 1999). Oviposition water treated with a colour dye has been reported to be more attractive to gravid *Ae. triseriatus* and *Cx. quinquefasciatus* than untreated water (Williams 1962). Black- and red-coloured containers are preferred for oviposition by *Cx. annulirostris* and *Cx. molestus,* respectively (Dhileepan 1997).

Similarly, *Toxorynchites amboinensis* and *T. moctezuma* oviposit preferentially into black containers rather than white, yellow, green or blue (Collins and Blackwell 2000). Black ovitraps are the most preferred target for gravid *Ae. albopictus* seeking artificial oviposition sites (Hoel et al. 2011). Black substrates have been reported to elicit most oviposition by both laboratory and house-collected *An. gambiae* mosquitoes (Huang et al. 2006b, Huang et al. 2007). In general, the majority of mosquito species prefer a black-, followed by blue-coloured oviposition background.

Tactile cues

Despite the fact that antennas are the chief site of the sense of touch in insects, the entire insect body is covered by a variety of tactile bristles, scales, and pits which are used for detecting cues through physical contact. The touch receptors of the antennas are the fine hair-like bristles with which they are covered. Tactile cues are important stimuli in the activation and orientation of many blood-sucking insects (Allan et al. 1987). Taste organs occur not only on the mouth parts but also on the antennas, palpi, feet and egg-laying appendages. Unlike visual stimuli, which are important signals when the insect is still at some distance from an oviposition site, heat and moisture become important when the insect is near an oviposition site (Lehane 1991, Okal et al. 2013b, Spitzen and Takken 2018).

Gravid mosquitoes often touch water in the breeding site using either mouth parts or legs before the onset of oviposition. Taste as a mode of infochemical reception has been observed clearly in *Ae. albopictus* and *Ae. triseriatus,* where oviposition responses were mediated by contact chemostimulants rather than attraction to odorants (Trexler et al. 1998). Therefore, for a better understanding of infochemical perception by mosquitoes during oviposition, it is important to separate physical from odorant reception of cues (Sumba et al. 2004a, Mwingira et al. 2019).

Moisture and temperature

The antennas of most insects also bear receptors sensitive to moisture content in the air and to temperature. Moisture content in the air was identified as one of the most important variables explaining the dynamics of oviposition, given the need for moist landscapes in which mosquitoes may search for oviposition sites (Day et al. 1990, Edman et al. 1998). On the other hand, there is a strong positive correlation between soil moisture content, quantified as surface conductivity and the degree of oviposition activity, which peak at saturation with standing water (Koenraadt et al. 2003, Huang et al. 2006a).

Temperature increase contributes to the rise of water vapour in the vicinity of potential breeding sites, and therefore influences the ability of gravid mosquitoes to locate breeding sites (Okal et al. 2013b). It has been shown that environmental factors including temperature, humidity and air pressure are significantly correlated with oviposition of *Culex pervigilans* (Zuharah and Lester 2010). Water vapour is, therefore, a long-range cue and close to the oviposition site, the insects detect other cues through tactile and taste receptors (Amarakoon et al. 2008).

Chemical stimuli

Infochemicals influencing oviposition behaviour

Insect resource-seeking processes are mediated through chemical senses. Insects use infochemicals from their environment at all stages of development to locate food, oviposition and hibernation sites, to come together with conspecifics and sexual partners, and to avoid dangerous situations or unsuitable habitats and hosts (Agelopoulos et al. 1999). Olfaction and taste are employed in recognizing infochemicals by insects (Lewis 1984). Some of the earliest examples of infochemical-mediated activities reported for mosquitoes are summarized in Table 1.

When searching for potential breeding sites, mosquitoes are guided by long-range infochemicals such as pheromones to identify the presence of breeding sites (Pickett and Woodcock 1996, Okal et al. 2013a). When close to the site, the mosquito uses short-range infochemicals to discriminate between a suitable and unsuitable breeding site for their next generation. At an oviposition site, infochemicals play a crucial role in the gravid female’s assessment of chemical properties of the prospective habitats for the immature stages (Takken and Knols 1999). Mosquitoes use contact stimuli to evaluate water quality and other factors prior to oviposition (Bentley and Day 1989b).

Mechanisms of odour reception and signal transduction

Insects have small cuticular extensions of various forms, called sensilla, which are involved in stimulus perception and can be found on various parts of the body including the head and legs (McIver 1982, Hansson 1999). Due to widespread presence of olfactory cues in their environment, insects have a highly sensitive and specific system of odour discrimination (Carey and Carlson 2011). They can find their resources precisely, despite being exposed to complex odour profiles from various sources (Hansson and Anton 2000, Zwiebel and Takken 2004). Insects detect odours by means of olfactory receptor neurons (ORNs) (Carey et al. 2010) housed in the sensilla, which are odorant-specific as they are expressed by individual odorant receptor genes (Su et al. 2009). A standard insect olfactory sensillum contains one to several bipolar ORNs that send their sensory dendrites into the sensillum lumen.

Mosquitoes have several types of olfactory sensilla. These include sensilla trichodea, grooved peg sensilla, capitate peg sensilla and coeloconic sensilla which they use as infochemical receptors. The genes encoding expression of various classes of these organs are regulated depending on the timing of a specific activity within the gonotrophic cycle. For example, after ingestion of a blood meal, the mosquito stops host seeking, as receptors of oviposition cues become more sensitive to stimuli used in the search for an oviposition site. Host-seeking behaviour is restored again 24 h after oviposition (Klowden and Lea 1979a, b, Takken et al. 2001). Furthermore, it has been reported that blood feeding results in the activation of a new functional class of trichoid sensilla, which is not usually expressed in mosquitoes that have never had a blood meal (Qiu et al. 2006). Apparently, several chemicals activate this sensillum (Table 2), indicating that they are putative infochemicals mediating oviposition behaviour in mosquitoes (Liu et al. 2018). Some of these chemicals have already been shown to mediate oviposition behaviour in mosquitoes through behavioural assays (Eneh et al. 2016b, Gaburro et al. 2018). Such compounds are 3-methylindole, 2-ethylphenol, 4-ethylphenol, 4-methylphenol (Table 3).

Table 1: Principal behaviours in mosquitoes that are mediated by infochemicals

Process	Infochemicals	Signal source(s)	Effect(s) of the signal	Species	Reference
Mating	Pheromones	Conspecifics	to bring both sexes together	*Mansonia* spp.	McIver *et al*., 1980
Plant feeding	Synomones	Fruits, floral nectar & plant spp.	to inform mosquitoes about presence of food	*An. gambiae*	Foster & Hancock, 1994
					Foster & Takken, 2004
					Impoinvil *et al*., 2004
					Nyasembe *et al*., 2012, 2014
				An. sergentii	Junnila *et al*., 2010
				Ae. aegypti	Healy & Jepson, 1988
					Martinez-Ibarra *et al*., 1997
				Cx. nigripalus	Hancock & Foster, 1997
				Cx. pipiens	Jhumur *et al*., 2006, 2007, 2008
					Otienoburu *et al*., 2012
					Bowen *et al*., 1992
Host seeking	Kairomones	Vertebrates	to inform mosquitoes about presence of food	*An. albimanus*	Knols *et al*., 1994
				An. gambiae	Smallegange *et al*., 2005
				Ae. aegypti	Njiru *et al*., 2006
				Ae. albopictus	Mweresa et al, 2016
				Culex spp.	Williams *et al*., 2006,
					Bernier *et al*., 2007
					Xie *et al*., 2019
					Takken *et al*., 1999
					Smallegange *et al*., 2010
					Tian *et al*., 2018
	Allomones	Vertebrates	to mask attractive-ness of hosts to mosquitoes	*An. gambiae*	Brady *et al*., 1997
					Mukabana *et al*., 2004
Oviposition	Allomones	Fungi, predators	to induce mosquitoes to oviposit	*Cx. quinquefasciatus*	Mboera *et al*., 1999
				Aedes spp.	Allan & Kline, 1998
	Apneumones	Hay infusion		*Cx. quinquefasciatus*	Millar *et al* 1992
	Kairomones	Plants		*An. albimanus*	Torres-Estrada *et al*., 2005
				An. arabiensis	Wondwosen *et al*., 2016
				An. arabiensis	Asmare *et al*., 2017
				An. coluzzii	Asmare *et al*., 2017
	Pheromones	Egg rafts, larvae	to induce mosquitoes to oviposit	*Cx. quinquefasciatus*	Mboera *et al* 1999
				Aedes spp.	Allan & Kline, 1998
				An. coluzzii	Mwingira *et al*., 2019
	Synonomes	Microorganism		*An. gambiae*	Sumba *et al*., 2004,
					Lindh *et al*., 2005, 2008
					Eneh *et al*., 2016

Table 2 - Response spectra of ORN innervating Sensilla tricodea, Grooved peg and Capitate pegs of mosquitoes in relation to oviposition stimulus.

Stimulus	**Mosquito species**	**Response**	**Sensillum**	**Reference**
Ammonia and Amines				
Ammonia*	*An. gambiae*	+	ST	Braks et al., 2001, Smallegange et al., 2005
		+	GP	
1-Pentylamine***		+	ST	Qiu et al., 2005
		+	GP	
Carboxylic acids				
3-Methylbutanoic acid*	*An. gambiae*	+	ST	Qiu, 2005
		+/−	GP	
Pentanoic acid*		+	ST	Qiu, 2005
		+/−	GP	
Hexanoic acid*		+	ST	Qiu, 2005,
		+/−	GP	Smallegange et al., 2002
Heptanoic acid**		+		Qiu, 2005
Octanoic acid***		+		
Nonanoic acid**		+		
		-		
7-Octanoic acid*		+		Qiu, 2005,
2-Methyl-2-hexenoic acid*		+		Constantini et al., 2002
Alcohol and heterocyclics				
1-Hexen-3-ol*	*An. gambiae*	+	ST	Qiu et al., 2005
2-Phenoxyethanol***		+	ST	
Phenol*		+	ST	
2-Methylphenol*		+	ST	
4-Methylphenol*		+	ST	
4-Methylphenol	*Ae. aegypti*	+	ST	Bentley et al., 1982
4-Ethylphenol*	*An. gambiae*	+	ST	Qiu et al., 2005
4-methylcyclohexanol	*Ae. aegypti*	+/−	ST$_2$, sb, sp	Bentley et al., 1982
	Cx. tarsalis	+/−		
	Cx. pipiens	+/−	ST$_2$	Bowen et al., 1992
	An. stephensi	+/−		Bentley et al., 1982
Indole**		+	ST	Qiu, 2005
		+/−	CP	Tan Lu et al., 2007
3-Methylindole***		+/−	ST	Qiu et al., 2005

table continues

Stimulus	**Mosquito species**	**Response**	**Sensillum**	**Reference**
		+/−	CP	Tan Lu et al., 2007
Ketones				
Butanone***	*An. gambiae*	+/−	ST	Qiu et al., 2005
6-Methyl-5-hepten-2-one*		+	ST	
2-Nonanone***		-	ST	
Geranyl acetone***		-	ST	
Others				
Heptanal***	*An. gambiae*	+	ST	Qiu et al., 2005
Water vapour	*Ae. aegypti*	+	GP	Kellogg et al., 1970
Oviposition site related	*Ae. aegypti*	+/−	GP	David & Sokolove 1976
Oviposition site related	*Ae. aegypti*	+	ST$_2$	Davis et al., 1976
Oviposition site related	*Ae. aegypti*	+/−	GP & ST$_2$	Davis et al., 1976
Terpenes	*Cx. pipiens*	+	ST$_2$	Bowen et al., 1992
Green plant volatiles		+		
Fatty acid esters		+		

Key: + represent excitation (attraction), − inhibition (deterrence), +/− excitation & inhibition

ST - Sensilla trichodea, GP - Grooved peg, CP – Capitate peg, Sb - short blunt, Sp - short pointed

* represents excitation response that occur before and after blood feeding

** represents excitation response that increase relatively after blood feeding

*** represents excitation response that occur only after blood feeding

Table 3 - Response of gravid mosquitoes to infochemicals mediating oviposition behaviour

Chemical compounds	**Response(s)**	**Mosquito species**	**References**
Alcohol/cyclics			
Phenol	0	*Cx. quinquefasciatus*	Millar et al., 1992
4-ethylphenol	0	*Culex quinquefasciatus*	Millar et al., 1992
	+	*Culex quinquefasciatus*	Zhu et al., 2013
2-methylphenol	+	*Tx. moctezuma, Tx. amboinensis*	Collins and Blackwell, 2002
3-methylphenol	+	*Tx. moctezuma, Tx. amboinensis*	Collins and Blackwell, 2002
4-methylphenol	+	*Aedes triseriatus*	Bentley et al., 1981

table continues

Chemical compounds	Respon-se(s)	Mosquito species	References
	0	*Cx. quinquefasciatus*	Millar et al., 1992
	+	*Culex quinquefasciatus*	Zhu et al., 2013
	-	*Aedes albopictus*	Trexler et al., 2003
	+	*Tx, brevipalpis, Tx. splendens*	Linley et al., 1989
	+	*Tx. moctezuma, Tx. amboinensis*	Collins and Blackwell, 2002
4-methylcyclohexanol	+	*Aedes triseriatus*	Bentley et al., 1981
	+	*Tx. moctezuma, Tx. amboinensis*	Collins and Blackwell, 2002
Indole	0	*Cx. quinquefasciatus*	Millar et al., 1992
	0	*Aedes albopictus*	Trexler et al., 2003
	0	*Aedes albopictus*	Trexler et al., 2003
3-methylindole	+	*Cx. tarsalis, Cx. stigmatosoma*	Beehler et al., 1993, 1994
	+	*Cx. quinquefasciatus*	Blackwell et al., 1993, Seenivasagan et al., 2013
	-	*Ae. albopictus*	Trexler et al., 2003
	+	*Tx. brevipalpis,*	Linley et al., 1989
	+	*Tx. amboinensis, Tx.moctezuma*	Collins and Blackwell, 2002
	0	*Tx. splendens*	Linley et al., 1989
Cedrol	+	*An.gambiae, An. Arabiensis*	Eneh et al., 2016
Carboxylic acids			
(Z)-9-hexedecenoic acid	+	*Ae. aegypti*	Kumaran et al., 2006
Decanoic acid	+	*Ae. aegypti*	Kumaran et al., 2006
Nonanoic acid	-	*Ae. aegypti, Cx. tarsalis,*	Schultz et al., 1982
	-	*Cx. quinquefasciatus*	
Oleic[(Z)-9-octadecenoic]acid	-	*Cx. quinquefasciatus*	Hwang et al., 1983
Butyric acid	-	*Cx. quinquefasciatus*	Hwang et al., 1979
Octanoic acid	-	*Ae. aegypti, Cx. tarsalis,*	Schultz et al., 1982
	-	*Cx. quinquefasciatus*	
Fatty acid esters			
Aryl hydrozono	+	*Ae. albopictus*	Bandyopadhyay, 2011
	+	*Ae. aegypti*	Guha et al., 2012

table continues

Chemical compounds	Respon-se(s)	Mosquito species	References
Decyl undecanoate	+	*An. stephensi*	Sharma et al., 2009
Heptadecyl butanoate	-	*An. stephensi*	Sharma et al., 2009
Hexadecyl pentanoate	-	*Ae. aegypti, Ae. albopictus*	Sharma et al., 2008
	-	*An. stephensi*	Sharma et al., 2009
Nonyl dodecanoate	+	*An. stephensi*	Sharma et al., 2009
Octyl tridecanoate	+	*Cx. quinquefasciatus*	Seenivasagan et al., 2013
Octadecyl propanoate	-	*An. stephensi*	Sharma et al., 2009
Pentyl hexadecanoate	+	*An. stephensi*	Sharma et al., 2009
Propyl octadecanoate	+	*An. stephensi*	Sharma et al., 2009, Seenivasagan et al., 2012
	+	*Ae. aegypti*	Sharma et al., 2008
	+	*Cx. quinquefasciatus*	Seenivasagan et al., 2013
Tetradecyl heptanoate	-	*Ae. aegypti, Ae. albopictus*	Sharma et al., 2008
	-	*An. stephensi*	Sharma et al., 2009
Tridecyl octanoate	-	*Ae. aegypti, Ae. albopictus*	Sharma et al., 2008
Other chemicals			
Dimethyl disulphide	0	*Aedes albopictus*	Trexter et al., 2003
Trimethylamine	0	*Ae. albopictus*	Trexter et al., 2003

Key: + represents attracting, −: deterring, 0: no response

Principal oviposition chemical signals

The chemical signals that convey information in biological systems are referred to as infochemicals. Previously, they were classified according to the origin of the compounds in specific interactions. However, the classification was ambiguous because in reality the producer or emitter may be a different organism from the same or even another trophic level, being associated to at least one of the interactants (Dicke and Sabelis 1988). Consequently, the discussion on classification of mosquito infochemicals is based on cost-benefit analysis criteria. An infochemical is therefore defined as a chemical that conveys information that mediates an interaction between two individuals by evoking physiological and/or behavioural responses that benefit the emitter, receiver or both (Dicke and Sabelis 1992). Several infochemicals have been described and are discussed below.

Allelochemicals

Mosquitoes interact with organisms outside their species mainly through allelochemicals. These are infochemicals that mediate interactions between two individuals of different species. They are grouped into the following classes: kairomones, allomones, and synomones.

Kairomones - A kairomone evokes a behavioural or physiological response that benefits the receiver but not the emitter (Dicke and Sabelis 1988). Owing to their ability to assist insects/predators to locate their hosts, kairomones have been exploited to develop control and surveillance systems for host-seeking insects (Dicke et al. 1990). The development of traps which use infochemicals such as carbon dioxide (CO2), lactic acid, ammonia, carboxylic acids, 1-octen-3-ol etc. to trap host-seeking insects resulted from the exploitation of a kairomonal behavioural response (Kline et al. 1990, Kline et al. 1994, Becker et al. 1995, Kline and Mann 1998, Gibson and Torr 1999, Rueda and Gardner 2003). In the mosquitoes' oviposition phase, kairomones play a big role in assisting gravid females to locate suitable breeding sites. The known kairomones mediating oviposition behaviour are mainly produced by aquatic plants and algae and the receiver (mosquitoe) benefits while the emitters (plants/algae) do not.

There is a positive correlation between mosquito larvae and plants present in breeding waters. Various plants are associated with immature stages of mosquitoes. A strong positive association has been observed between larvae of *An. albimanus* and specific vegetation forms including: *Brachiaria mutica*, *Cynodon dactylon*, *Jouvea straminea*, *Fimbristylis spadicea* and *Ceratophyllum dermersum* (Rodriguez et al. 1993, Hernandez et al. 1997, Torres-Estrada et al. 2005). Also the presence and density of *An. farauti* larvae was positively associated with aquatic emergent plants (Bugoro et al. 2011).

Not only specific plants have been observed to play a role in guiding oviposition site selection of mosquitoes, but also the extracts from certain green plants such as vegetable dye have been found to guide gravid *Ae. triseriatus* (Beehler and DeFoliart 1990). This suggests that a gravid female may be using cues from these plants to select suitable oviposition sites. A mixture of terpenoid and alcohol compounds identified through GC-MS analysis has been found to mediate *Anopheles* oviposition (Rejmankova et al. 2005b, Torres-Estrada et al. 2005).

Grass infusions have been shown to contain oviposition stimuli for culicine mosquitoes, including *Ae. albopictus* (Allan and Kline 1995), *Ae. aegypti* (Chadee 1993a), *Ae. triseriatus* (Holck et al. 1988), *Cx. pipiens*, *Cx. restuans* (Reiter 1986, Jackson et al. 2005), *Cx. tarsalis* (Reisen and Meyer 1990) and *Cx. quinquefasciatus* (Millar et al. 1992a). Consequently, a number of infochemicals, mainly kairomones, have been identified from grass infusions. The attractive compounds include among others, phenol, 4-methylindole, 4-ethylphenol, 3-methylindole, 4-methylphenol and indole (Bentley et al. 1981, Millar et al. 1992a, Du and Millar 1999). Two compounds in particular, 3-methyl-indole (skatole) and 4-methylphenol (*p*-cresol), have been investigated in more detail (Mboera et al. 2000b). Gravid females of *Cx. quinquefasciatus* had a higher electrophysiological sensitivity for skatole (3-methyl-indole) than males (Blackwell et al. 1993).

A positive correlation between the presence of filamentous algae and a high number of mosquito larvae has been well documented (Schaefer 1983, Kramer 1989, Vazquez-Martinez 2002, Torres-Estrada 2006). The presence of certain algal species is the main characteristic in mosquito larval habitats (Savage et al. 1990, Fernandez-Salas et al. 1994, Manguin et al. 1996b). Algae have been found in over 90% of *Anopheles* larval habitats (Manguin et al. 1996b) and larval productivity was low in the absence of algae (Rejmankova et al. 1992, Manguin et al. 1996a). Some of the algal species that are associated with mosquito breeding sites are *Spirogyra* spp., *Chladophora* spp., *Oedogonium* spp., and *Closterium* spp., (Fernandez-Salas et al. 1994). In this association, mosquito larvae benefit by feeding on a diet consisting of algae (Gimnig et al. 2002). Filamentous algae *Spirogyra majuscula* and *Cladophora glomerata* were found to represent 47% of the gut content of *An. pseudopectipennis* (Bond et al. 2005). Similarly, phylogenetic analysis of the gut contents from *An. gambiae* larvae revealed that 50% consisted of green algae of the Chlamydomonales and Chlorococcales families (Garros et al. 2008). Volatile compounds released by algae are likely to be the main short-range attractants for gravid mosquitoes. For example, ethyl acetate, and higher alkanes (docosane, tricosane, tetracosane, pentacosane, hexacosane, heptacosane and octacosane) were identified to be infochemicals originating from *S. majuscula* and mediate the oviposition behaviour of *An. pseudopectipennis* (Torres-Estrada 2006).

Allomones - Allomones are allelochemicals that evoke a behavioural or physiological response that benefits the emitter but not the recipient (Dicke and Sabelis 1988). They are of significant importance in the biological methods of vector control. Allomones of interest in mosquito oviposition behaviour are mainly emitted by natural enemies of mosquitoes such as fungi and aquatic animals.

The association between mosquitoes and fungi has been reviewed in detail with much of the attention being directed to entomopathogenic fungi (Scholte et al. 2004). Several species of entomopathogenic fungi are known to infect mosquitoes successfully, using mosquito bodies for their propagation and dispersal (Scholte et al. 2005). It is not suprising that some fungal infusions have been shown to attract ovipositing mosquitoes. For example, breeding water treated with aqueous *Polyporus* spp. infusions received significantly more eggs of mosquitoes than other substrates (Sivagnaname et al. 2001b). Cedrol is an infochemical emitted by fungi that are living in rhizomes of the grass *Cyperus rotundus* and attracts both *An. gambiae* and *An. arabiensis* (Eneh et al. 2016a).

This may be a stratergy of fungi to emit infochemicals that attract mosquitoes to lay eggs in infected sites for the benefit of the fungi.

Certain aquatic animals have been associated with the mediation of oviposition behaviour in mosquitoes. Crustaceans and some mollusc species have been investigated for candidate oviposition attractants of mosquitoes. The copepod species, *Mesocyclops longisetus*, which previously was used for the biological control of mosquitoes (Calliari et al. 2003), has been found to attract gravid *Ae. aegypti*. These crustaceans release various chemical compounds such as: 3-carene, α-terpinene, α-copaene, α-cedrene and δ-cadinene that mediate oviposition behaviour in mosquitoes (Torres-Estrada et al. 2001). Moreover, water that had been conditioned with carpet shells (*Paphia undulate*) and giant tiger prawns (*Penaeus monodon*) has been found to be highly attractive to gravid *Ae. albopictus* (Thavara et al. 2004). It is plausible for these organisms to attract ovipositing mosquitoes so that they can eat their larvae.

Synomones - Synomones are allelochemicals that evoke a behavioural or physiological response that benefits both the emitter and receiver (Dicke and Sabelis 1988). Synomones affecting oviposition behaviour are produced by bacteria that are present in soil, water, plants and fermenting organic matter and attract mosquitoes to lay eggs. Mosquitoes rely on their gut microbes for rapid growth and development (Coon et al. 2014). Consequently, gravid female mosquitoes use volatiles of bacterial origin to assess nutrient availability and durability of habitats, both of which are vital determinants for the survival of their offspring, and hence their fitness (Sumba et al. 2004a). On the other hand, bacteria benefit from mosquito site-selection behaviour as they feed on food processed by mosquito larvae. Moreover, bacteria have been found to live symbiotically in the gut of mosquito larvae, where they benefit from the availability of nutrients and a suitable growth medium (Guegan et al. 2018).

The role of bacteria and their volatiles in mediating the oviposition responses of a gravid mosquito is well documented in a number of studies (Poonam et al. 2002, Trexler et al. 2003, Lindh et al. 2008, Ponnusamy et al. 2008, Ponnusamy et al. 2010). Bacterial species producing infochemicals that mediate oviposition behaviour in *An. gambiae* have been identified and belong to the genera *Aeromonas, Pasteurella, Pseudomonas, Vibrio* and *Acetinobacter* (Sumba et al. 2004a). Other species such as *Psychrobacter immobilis*, *Sphingobacterium multivorum* and *Bacillus* species have been shown to significantly stimulate oviposition behaviour in *Ae. albopictus* (Trexler et al. 2003).

Infochemicals of bacterial origin have also been shown to mediate oviposition in *Cx. quinquefasciatus* (Beehler et al. 1994) and *An. gambiae* (Lindh et al. 2008) mosquitoes. Culture filtrates of *Bacillus thuringiensis* var *israeliensis* (wild type) and *Bacillus cereus* are known to attract gravid *Cx. quinquefasciatus* better than *p*-cresol (Poonam et al. 2002). Moreover, several species of mosquitoes feed on bacteria during their larval stages (Merritt et al. 1992). Also cyanobacteria have been associated with high anopheline larval productivity in breeding sites, and the most frequently isolated taxa include: *Phormidium* sp, *Oscillatoria* sp, *Aphanocapsa* cf *littoralis*, *Lyngbya lutea*, *P. animalis*, and *Anabaena* cf. *spiroides* (Vazquez-Martinez 2002). It is clear that mosquito breeding sites produce infochemicals of microbial origin that mediate mosquito oviposition behaviour (Lindh et al. 2015). However, the chemical composition and mode of action of chemicals involved are not yet fully explored.

Bacteria are also involved in the decomposition of organic matter, which leads to emission of volatile compounds that attract gravid mosquitoes to oviposit. In most cases volatile chemicals produced by decomposition of organic debris are the principal attractants of culicine mosquitoes (Bentley and Day 1989b, Mboera et al. 1999, Takken and Knols 1999). Log-ponds are particularly attractive oviposition sites for *Cx. tarsalis* and *Cx. quinquefasciatus* and water contaminated with chicken manure or rice straw infusions are very attractive to *Cx. pallens* (Service 1993). Moreover, cow manure infusions have been found to be very attractive to *Cx. quinquefasciatus* and *Cx. nigripalpus* (Allan et al. 2005) as well *Cx. pipiens* and *Cx. restuans* (Jackson et al. 2005). Furthermore, it was reported that water polluted by various materials, including rabbit faeces, have an additive effect when used with an oviposition pheromone (Agelopoulos et al. 1999). Gas chromatography - mass spectrometry (GC-MS) analysis of the volatiles emitted from these materials identified compounds including phenol and indole. Moreover, the identification of olfactory receptor for 3-methylindole enabled laboratory and field assays using the pure compound, which significantly increased oviposition activity (Mboera et al. 2000b, Hughes et al. 2010).

Pheromones

Pheromones are infochemicals that mediate interactions between individuals of the same species in which the benefit is to the origin-related individual, to the receiver or to both (Dicke and Sabelis 1988). They can originate from specialized secretory glands, body orifices, organs involved in digestion and reproduction (e.g. mouth, anus, aedeagus) (Wertheim et al. 2005). Pheromones act as chemical releasers of specific factors that trigger fixed action patterns in mosquitoes, such as aggregation for mating and oviposition. Aggregation pheromones on a breeding substrate are often released by other females (Judd and Borden 1992, Jiang et al. 2002, Wertheim et al. 2002) or by eggs, larvae or pupae (Bentley and Day 1989a, Leonard and Sanai 1993, McCall and Cameron 1995).

In several dipteran species, pheromones released by females are male-derived. Males produce the pheromone and transfer it to females during copulation (Bartelt et al. 1985, Bentley and Day 1989a, Schaner et al. 1989). Examples can be found in the housefly, *Musca domestica* whereby infochemicals from ovaries of gravid females attract gravid females (Jiang et al. 2002), and several

Culex species in which oviposition-mediating pheromone can be extracted from eggs (Laurence and Pickett 1982, 1985)

In mosquitoes, oviposition pheromones are known to originate from eggs and larvae of conspecifics (Chadee 1993b, Zahiri et al. 1997, Blackwell and Johnson 2000). This hypothesis was first made when *Cx. tarsalis* was found to have an oviposition preference for water containing conspecific larvae above distilled water (Osgood 1971). The responsible pheromone was identified as a mixture of estrolide 1,3-diglycerides (Starratt and Osgood 1973). Methanolysis of the mixture yielded methyl esters of mono- and dihydroxy fatty acids, of which *erythro*-5,6-dihydroxyhexadecanoic acid was the major component among the dihydroxy compounds. A similar mechanism was observed with *Cx. pipiens*, in which the oviposition response was due to droplets present on the eggs' apex (Bruno and Laurence 1979). The responsible chemical compound was *erythro*-6-acetoxy-5-hexadecanolide, henceforth called mosquito oviposition pheromone (Laurence and Pickett 1982, 1985, Otieno et al. 1988).

Furthermore, several compounds have been extracted from the eggs of *Ae. aegypti* and identified as 6-hexanolactone, methyl dodecanoate, dodecanoic acid, methyl tetradecanoate, tetradecanoic acid, methyl (Z)-9-hexadecenoate, octadecanoic acid, methyl hexadecanoate (Z)-9-hexadecenoic acid, hexadecanoic acid, methyl (Z)-9-octadecenoate and methyl octadecanoate (Z)-9-octadecenoic acid (Table 4). All identified chemical compounds deterred gravid *Ae. aegypti*, except dodecanoic and (Z)-9-hexadecenoic acid which showed significant positive oviposition response at different concentrations (Ganesan et al. 2006).

Evidence for pheromones of larval origin has been based on chemical analysis of water from larval breeding sites following the observation that certain mosquito species prefer to lay eggs on water containing conspecific larvae but not eggs. This hypothesis was supported by the fact that preference for water containing conspecific larvae of *Cx. annulirostris* was density dependent (Dhileepan 1997). In a similar way, water from *Ae. aegypti* breeding sites was analysed and the following compounds were identified: docosane, heneicosane, isopropyl myristate and nonacosane (Table 4). Heneicosane had the strongest effect on ovipositing *Ae. aegypti* (Mendki et al. 2000).

Table 4 - Effects of oviposition cues from living organisms on the response of gravid mosquitoes

Source of oviposition cue	Species/type emitting	Chemical composition of emitted cues	Effects of the semiochemicals	References
Mosquito				
Mosquito eggs	*Cx. quinquefasciatus*	6-acetoxy-5-decanolide	Attracts *Cx. quinquefasciatus*	Laurence & Picket, 1982, 1985
			Cx. tarsalis and	Trexler et al., 2003 Braks et al., 2007
			Cx. cinereus	Mboera et al., 1999
	Ae. aegypti	Dodecanoic acid (Z)-9-hexadecenoic acid Methyldodecanoate 6-hexanolactone Methylhexadecanoate Hexadecanoic acid Methyl(Z)-9-octadecanoate Methyloctadecanoate Octadecanoic acid (Z)-9-octadecanoic acid Methyltetradecanoate Tetradecanoic acid Methyl(Z)-9-hexadecenoate	*Ae. aegypti* & *Cx. quinquefasciatus* are attracted by Dodecanoic & (Z)-9-hexadecenoic acid but deterred by the rest of chemicals	Ganesan et al., 2006 Sivakumar et al., 2011
Mosquito larvae	*Ae. aegypti*	Heneicosane Octadene, Isopropyl myristrate, Docosane and Nonacosane	Heneicosane was the most attractant to *Ae. aegypti*	Mendki et al., 2000
	An. gambiae	2,4 Pentanedione, Nonane,	Attracts *An. gambiae* Attracts *An. gambiae* and *Cx. quinquefasciatus*	Schoelitzs et al.2009 Mwingira et al 2013
		Dimethyldisulphate, Dimethyltrisulphate	Deters *An. gambiae*	Schoelitzs et al 2009

table continues

Source of oviposition cue	Species/type emitting	Chemical composition of emitted cues	Effects of the semiochemicals	References
Microorganisms				
Bacteria	*P. aeruginosa*	7,11-dimethyl-octadecane	Attracts *Ae. aegypti*	Ikeshoji et al., 1979
	Psychrobacter immobilis *Sphingobacterium multivorum* *Bacillus* spp	Unknown	Attracts *Ae. albopictus*	Trexler et al., 2003
	Pseudomonas *Stenotrophomonas* *Enterobacter* *Pantoea,* *Klebsiella,* *Acinetobacter* *Aeromonas* *Bacillus* *S. matrophilia*	Chemical compounds produced by these bacteria are not known	Except of *S. matrophilia* which deterred *An. gambiae,* odour from all other bacteria attracted *An. gambiae*	Huang et al., 2006
	Vibrio metchnikovii	2-Methyl-3-decanol	Attracted *An. gambiae*	Lindh et al., 2008
	Proteus spp	3-Methyl-1-butanol, Indole, 2-phenyl ethanol		
	Micrococcus spp	3-Methyl-1-butanol		
	Exiguobacterium spp	3-Methyl-1-butanol, 3-Methylbutanoic acid		
	Bacillus spp	Alkyl-pyrazines		
	Comamonas spp	Phenylmethanol, 2-phenylethanol		
	Unknown species	Carboxylic acid and Methyl esters	Attracts *Ae. aegypti*	Ponnusamy *et al.*, 2008, 2010
	Aeromonas	Unknown	Deters *An. sinensis*	Mei & Lin-Hua, 2010

table continues

Source of oviposition cue	Species/type emitting	Chemical composition of emitted cues	Effects of the semiochemicals	References
	Leptolyngbya	Unknown	Volatiles from cynobacterial mats attract An. albimanus and An. vestitipennis when at low concentrations.	Rejmankova *et al*., 2005
Fungi	*Fusarium falciforme*	Cedrol	Attracts *An. arabienis, An. gambiae*	Eneh *et al*., 2016
	Polyporous spp	Unknown	Attracts *An. subpictus* Attracts *Ae. aegypti* Deters *Cx. quinquefasciatus*	Sivagnaname *et al*, 2001
	Tricoderma viride	Unknown	Attracts *Cx. quinquefasciatus*	Geetha et al., 2003
Protist	*Ascogregarina taiwanensis*	Unknown	Attracts *Ae. aegypti*	Reeves 2003
Yeast	*Candida* near *pseudoglaebosa*	Unknown	Attracts *Ae. aegypti*	Reeves 2003
Trematode	*P. elegans*	Unknown	Deters *Ae. atropalpus*	Zahiri et al., 1997
Predators				
Dragonfly	*Anax imperator*	Unknown	Deters *C. longiareolata*	Stav et al., 1999
Diving beetle	*Eretes griseus*	Unknown	Deters *Cx. tritaeniorhynchus*	Shin-ya Ohba, 2012
Backswimmer	*Notonecta maculata*	Unknown	Deters *C. longiareolata* and *An. gambiae*	Spencer et al., 2002, Blausten et al, 2004 Munga et al., 2006 Silberbush et al., 2011
Tadpole	*L. peronei*	Unknown	Deters *O. australis*	Mokany & Shine 2003
	Bufo viridis	Unknown	Deters *C. longiareolata*	Blaustein & Kotler 1993
Copepods	*Mesocyclops longisetus*	3-carene, α-terpinene, α-copaene, α-longipinene, α-cedrene, δ-cadinene	Attracts *Ae. aegypti*	Torres-Estrada, 2001

table continues

Source of oviposition cue	Species/type emitting	Chemical composition of emitted cues	Effects of the semiochemicals	References
Molluscs: Carpet shell Giant tiger prawn	*Paphia undulata* *Penaeus monodon*	Unknown	Attracts *Ae. albopictus*	Thavara et al., 2004
Fishes	*Gambusia affinis*	Unknown	Deters *Cx. pipiens* & *Cx. quinquefasciatus* Deters *Cx. tarsalis*	Angelon et al., 2002 Van Dam & Walton, 2008
	Betta splendens	Unknown	Deters *Ae. aegypti*	Luciano et al., 2009 Pamplona et al., 2009
Plants				
Bamboo grass	*Arundinaria gigantea*	Unknown	Attracts *Cx. stigmatosoma*	Beehler et al., 1994
Bermuda grass	*Cynodon dactylon*	3-methylindole	Attracts *Cx. tarsalis* & *Cx. quinquefasciatus*	Beehler et al., 1994 Mboera et al., 2000
		4-methylphenol 4-ethylphenol	The phenols were neither attractive nor deterrent	Millar et al., 1992 Blackwell et al., 1993
Other grasses	*Brachiaria mutica* *Jouvea staminea* *Fimbristylis spadicea* *Ceratophyllum-demersum*	Guaiacol, Phenol, Isoeugenol, Longifolene, Caryophyllene, Phenyl ethyl alcohol, P-cresol	Higher concentrations deterred while lower concentrations attracted *An. albimanus*	Torres-Estrada et al., 2005
Cattails	*Typha domingensis*	Unknown	Volatiles from *Typha domingensis* and cyanobacterial mats attract *An. albimanus* and *An. vestitipennis* when at low concentrations. At higher concentrations egg laying is reduced	Rejmankova et al., 2005
	Typha latifolia	Unknown	Attracts *Coquillettidia pertubans*	Serandour et al., 2013

table continues

Source of oviposition cue	Species/type emitting	Chemical composition of emitted cues	Effects of the semiochemicals	References
Algae	*S. majuscula* *C. glomerata*	Unknown	Attracts *An. pseudopuctipennis*	Bond et al., 2005 Rejmankova et al., 2005
White oak leaves	*Quercus alba*	Unknown	*Ae. aegypti was* attracted to infusions made over short time fermentation period while *Ae. albopictus* was attracted to infusions made over long fermentation period.	Ponnusamy et al., 2012
			Ae. triseriatus deposited largest number of eggs in older age infusion	Trexler et al., 1998
Hard orange	*Poncirus trifoliata*	Poncirin Rhoifolin Naringin Marmesin	The flavonoids exhibited oviposition deterrence against *Ae. aegypti*, and are ovicidal at higher concentrations	Rajkumar & Jebanesan, 2008
Water oak	*Quercus nigra*	Unknown	Attracts *Ae. albopictus*	Obenauer et al., 2012
Longleaf pine	*Pinus palustris*	Unknown	Deters *Ae. albopictus*	
Industrial fertilizers	NPK	Aqueous solution of Nitrogen, Phosphorus and Potassium	Moderate concentration of aqueous solution attracted *Ae. aegypti.* Low or high concentrations did not induce significant attraction	Darriet & Corbel, 2008 Darriet et al., 2010

Mosquito responses to oviposition infochemicals

Site location and selection

Before and during oviposition, many mosquito species exhibit several behavioural and physiological traits. Although for most species pheromones are yet to be fully characterised, efforts to identify their roles have implicated long-range attractive and short-range arresting constituents. For example, in several laboratory studies, it was suggested that at a long range, acetoxyhexadecanolide stimulates upwind flight of *Cx. quinquefasciatus* towards the pheromone source and at close range, this compound produces an orthokinetic reduction in flight speed (Pile et al. 1991, 1993). Moreover, at an oviposition site, the pheromone induces a higher proportion of visiting females to oviposit. This suggests that the same infochemical can elicit different behavioural responses when the gravid mosquito is near or far from a prospective breeding site. However, the role that the infochemical plays in space, and the distance over which infochemicals are effective, requires similar investigations with other species of mosquitoes.

Additive and synergistic effects

The oviposition response of mosquitoes to a mixture of attractants has been of great interest recently. In a natural setting, various cues are available to gravid mosquitoes and therefore what has been observed is likely to be a response to multiple stimuli. Most likely there are interactions between pheromones and other infochemicals in guiding gravid mosquitoes to suitable oviposition sites (McCall and Cameron 1995). For example, when the synthetic oviposition pheromone (SOP) (6-acetoxy-5-hexadecanolide) was mixed with grass infusion, or soakage pit water, more egg rafts of *Cx. quinquefasciatus* were laid in the mixture compared to the response of individual attractants alone (Mboera et al. 1999). This means that SOP has a synergistic effect with grass infusion or soakage pit water in attracting female *Cx. quinquefasciatus*.

The response of *Cx. quinquefasciatus* to blends of a fixed amount of SOP with variable doses of 3-methylindole has additive rather than synergistic effects (Millar et al. 1994). The oviposition response of *Cx quinquefasciatus* to the blend increased gradually to a threshold of 0.1 mg. At higher doses, oviposition deterrence was observed. When tested separately at the same dose, which as a blend was repellent, the infochemicals attracted mosquitoes. Additive effects of SOP and 3-methylindole have been observed with *Cx. quinquefasciatus* in different geographical areas (Mboera et al. 2000b, Olagbemiro et al. 2004). Several products originating from soil microbes (Herrera-Varela et al. 2014), plant microbes and conspecific larvae (Mwingira et al. 2019) have been shown to attract ovipositing gravid anopheline mosquitoes. Of particular interest is cedrol, which originates from fungi living in rhizomes of the grass *Cyperus rotundus* and attracts both *An. gambiae* and *An. arabiensis* (Eneh et al. 2016a). As oviposition in nature is mediated by several cues, such individual compounds can be combined to realize their additive or synergistic effects.

Activation of odorant receptors

The *An. gambiae* protein AgOr1, a female-specific member of a family of putative odorant receptors (Fox et al. 2001, Hill and et al. 2002), has been found to respond to certain chemicals found in mosquito larval habitats (Xia et al. 2008). This suggests that chemical oviposition cues activate receptors of this type and may attract the mosquito to suitable breeding sites. Most of the sensory physiology studies undertaken to explore olfactory receptor neural responses in mosquitoes suggest that various infochemicals induce physiological responses (Costantini et al. 2001). Single sensillum electrophysiology (SSR) has revealed that 4-methylphenol, a known oviposition infochemical, confers a strong response in the AgOr1 protein (Hallem et al. 2004). Consequently, chemical cues that were shown to mediate oviposition can be used to identify neurons responsible for the observed attraction to oviposition sites, and determine the sensitivity of identified neurons to other putative behavioural compounds. Increasingly, interactions between behavioural and sensory physiology studies are used for the rapid identification of relevant infochemicals (Zwiebel and Takken 2004, Ray 2015, Lombardo et al. 2017). Thus, knowledge of changes in olfactory sensitivity to kairomones can be applied to increase trap catches of malaria mosquitoes that have taken a blood meal and need to locate an oviposition site (Qiu et al. 2013).

Group oviposition

In dipterans, pheromones are often used in the context of aggregated oviposition within species (Bentley and Day 1989a, Jiang 2002). Although the benefits of group oviposition in mosquitoes have not been studied in detail, in most insects' aggregation is suggested to help conspecifics to find a food source. However, for an aggregation trait to evolve, both the gravid mosquito and the immature or their associated microbes must benefit to some degree. Generally, the benefits of pheromone-induced aggregation in insects have been categorized as increasing the efficiency of resource use, finding mates, protection from natural enemies and protection from environmental conditions (Wertheim et al. 2005). Also, pheromone-induced aggregation results in competition among and between species inhabiting the same niche. Such competition may favour one species over the other and alter the distribution or abundance of competing species (Shragai et al. 2019).

Moreover, the increase in numbers of eggs and larvae may provide physical protection of the group or increased availability of food source in case the emitter of the cue is a microbe. For *Culex* species, a large number of larvae may prevent the formation of scum on top of an organically rich oviposition site (McCall and Cameron 1995). Thus, by forming groups, mosquitoes may change the existing environment to their advantage. Aggregation behaviour ensures high egg density at the oviposition site and resultant spatial cohesiveness of the progeny, which means communal aggregation. As a result, oviposition in mosquitoes constitutes a principal means of transmitting genetic traits to the progeny and facilitating accumulation of these traits across generations (Wong et al. 2012)

Attracting natural enemies

Aggregated oviposition means an increase in the number of mosquito larvae in a limited area, which often results in resource competition among larvae. Mosquito larval competition can hae large effects on emerging adults in terms of longevity, adult size, mating success, etc. Studies with *Cx. pipiens* suggest that larval resource availability and competition influence mosquito population growth correlates and have lasting effects on traits that relate to a mosquito's ability to vector pathogens (Alto et al. 2012).

Studies of *An. gambiae* indicate that competition within the larval environment may indirectly regulate their population by reducing adult body size, which in turn reduces adult survivorship and fecundity (Gimnig et al. 2001, Takken et al. 2013).

On the other hand, aggregation pheromones may also enhance the chances that the site is located by competitors and/or natural enemies (Wertheim et al. 2005). Competitors and predators are able to use the same infochemicals as mosquitoes use to their benefit. In this way, a pheromone for the mosquito is a kairomone for their competitors and natural enemies. For example, the compound (5R, 6S)-6-acetoxy-5-hexadecanolide which is the oviposition pheromone of *Cx. quinquefasciatus,* also attracts *Cx. tarsalis, Cx. cinereus* and *Cx. tigripes* to lay eggs at the same oviposition sites (Mboera et al. 1999). The latter three species are potential competitors of *Cx. quinquefasciatus* and interestingly, *Cx. tigripes* larvae are efficient predators of *Cx. quinquefasciatus* larvae (Mboera 1999).

Apart from these observations, overcrowding and resource competition reduces overall adult fitness (Yoshioka et al. 2012). This may render the resulting mosquito population susceptible to infection by pathogenic microbes (Alto et al. 2008). Despite the risks of aggregation behaviour to safety and individual fitness, mosquitoes continue to respond to aggregation pheromone that indicates the presence of a safe site. The majority of mosquito species suffer serious mortality due to the presence of predators in breeding sites, and have developed a chemical sense to detect and avoid these enemies (Table 1). Gravid mosquitoes of several species can detect predators in prospective breeding sites and divert egg laying activity. These include *Culex* mosquitoes (Blaustein et al. 2005), *C. longiareolata* and *An. gambiae* (Munga et al. 2006, Warburg et al. 2011). Therefore, in theory the benefits of laying eggs into a safe site outweigh the costs of larval competition.

Multiple functions

Infochemicals that mediate mosquito oviposition behaviour have multiple functions depending on their emission rate. In most cases, mosquitoes are attracted to a chemical when presented at low concentration and deterred at high concentration (Hoffmann and Miller 2002). When the emission of chemicals from the source is low, mosquitoes are stimulated to lay eggs, while when the emission is high, they are deterred. For example, in *An. gambiae,* oviposition is stimulated by low larval density and inhibited by high larval density (Sumba et al. 2008). Similarly, *An. albimanus* and *An. pestitipennis* are attracted to low concentrations of organic extracts, while deterred from high concentrations of organic extracts (Rejmankova et al. 2005a, Torres-Estrada et al. 2005).

Potential application of oviposition infochemicals

In nature, infochemicals affect insect behaviour. Humans exploit this knowledge by introducing artificial chemicals for the manipulation of this behaviour. Infochemicals that mediate oviposition in mosquitoes may play an important role in disease and vector control strategies in the future. Some of these potential applications are discussed below.

Vector surveillance

Mosquito sampling is a prerequisite to most vector population studies (Githeko et al. 1994, WHO 2017), and therefore various sampling techniques as well as entomological indicators have been used to monitor and evaluate the impact of vector control interventions (Mboera 2005). Ovitraps are often used for mosquito population monitoring and surveillance (McHugh and Hanny 1990). These traps can also be used to estimate vector population size and structure. The use of oviposition attractants or stimulants as baits, to increase the sensitivity of these traps as well as gravid female traps, has considerable potential (Freier and Francy 1991). Such traps have been effectively used to sample gravid mosquitoes (Mboera et al. 2000a) or attract them to breeding sites treated with a biocide (Mboera 2003b, Kampen et al. 2015, Suter et al. 2016).

Most *Anopheles* mosquitoes tend to rest in a sheltered place after a blood meal, and they remain resting until eggs are fully developed, after which they fly out in search of a suitable oviposition site (Takken et al. 2001). However, some do not rest in human-made shelters; they fly out and rest in natural shelters. Sampling gravid females by using oviposition attractants could be very rewarding, as gravid females will be attracted from various resting places, even those of species that are not sensitive to other methods of trapping. Therefore, traps baited with an oviposition pheromone are likely to be more efficient and thus are excellent tools for detecting the presence of mosquitoes, monitoring their flight range as well population levels.

Gravid female traps have been used not only for surveillance of vector populations, but also for the surveillance of vector-borne diseases (Allan and Kline 2004). The entomological parameter being studied and the behaviour of the mosquito species being sampled determine the choice of a sampling method (Davis et al. 1995). Unfortunately, most of the available mosquito sampling methods may not allow for such rational choices to be made, as there are major limitations associated with their use (Service 1977). Consequently, new tools for sampling mosquito populations are required to determine various entomological parameters of epidemiological importance.

Oviposition pheromones that specifically attract gravid mosquitoes exposed to infection during their previous blood meals, can be used in traps to aid the determination of population infection rates more effectively than traps that attract host-seeking mosquitoes (McCall and Cameron 1995). Traps baited with chemical oviposition stimuli can aid effectively in the estimation of the entomological inoculation rate (EIR), which is the standard parameter for estimating transmission, representing the number of infectious bites any person receives per year (Kilama et al. 2014). As the likelihood of getting sick is influenced by EIR, its estimation is important for disease monitoring. It follows that the use of infochemicals affecting oviposition in vector surveillance can increase the chance of sampling mosquitoes that are infected with parasites. Therefore, when released from mosquito traps, oviposition infochemicals will provide an objective monitoring tool for parasite transmission.

Control of adult mosquitoes

In addition to increasing the sensitivity for monitoring population and surveillance, the use of oviposition infochemicals in traps has a potential role in the control of adult gravid mosquitoes when traps are inoculated with a suitable pesticide (Mboera 2003a). Lethal ovitraps with an oviposition strip treated with deltamethrin insecticide, have been found to affect natural populations of *Ae. aegypti* (Perich et al. 2003, Sithiprasasna et al. 2003). Traps baited with a synthetic oviposition pheromone, authentic plant volatiles or a blend of these chemicals are expected to become excellent tools for the control of gravid mosquitoes particularly when combined with an environmentally-friendly pesticide such as Insect Growth Regulators (IGRs). Some IGRs such as pyriproxyfen (Chism and Apperson 2003, Mbare et al. 2019) and methoprene (Nishiura et al. 2003, Braga et al. 2005b, Braga et al. 2005a) have been shown to be highly active against mosquitoes; consequently, when used in combination with oviposition infochemicals they may provide a novel approach to mosquito control. When combined with insect-growth regulators in Kenya, the pheromone directed egg laying and inhibited adult emergence of *Culex* (Otieno et al. 1988). This is a highly selective method of vector control with the advantage that it immediately reduces the density of targeted species, with relatively no or little harm to the environment. In other insect species, however, infochemicals are already widely used for population regulation (Gitau et al. 2013, Kelly 2014).

Control of the immature stages

Oviposition infochemicals have considerable potential for delivery of pathogens or pesticides to larval populations (Otieno et al. 1988, Schlein and Pener 1990, Itoh et al. 1994). It has been demonstrated with *Cx. quinquefasciatus* that oviposition attractants might be employed to lure mosquitoes to sites already treated with ovicidal compounds (Mboera 2003b). With more knowledge of mosquito oviposition behaviour source-reduction programmes can more effectively focus on the most productive breeding sites, which resulted from pheromone-induced group oviposition behaviour (Kaur et al. 2003). Consequently, larval control through environmental management or by using biolarvicides will become efficient and cost-effective when the targeted habitats are clustered or when the target area size is limited (Utzinger et al. 2001, Killeen et al. 2002, Fillinger et al. 2003). Attempts to lure gravid mosquitoes to sites already treated with pesticides have been made in Malaysia (Ong and Jaal 2015), Tanzania (Schorkopf et al. 2016), Peru and Thailand (Paz-Soldan et al. 2016).

Relatively few studies using the natural biocides *Bacillus thuringiensis israelensis/B. sphaericus* have been carried out against African malaria vectors (Seyoum and Abate 1997, Skovmand and Sanogo 1999, Fillinger et al. 2003) and they were restricted to experimental and operational research (Barbazan et al. 1998, Fillinger et al. 2009, Magesa 2009). Studies conducted in Tanzania and Kenya indicated that biolarviciding is a cost-effective intervention even in rural settings (Rahman et al. 2016, Derua et al. 2019b). The efficacy of *B. thuringiensis israelensis (Bti)* use for malaria control was for the first time reviewed in a major study comparing all studies known to-date (Choi et al. 2019). Recent studies have underlined the potential of larval control using microbial insecticides and deserve broader application and dissemination (Derua et al. 2019a).

The use of biolarvicides has a great advantage over all other larvicides, as they have a low probability of causing environmental pollution or developing resistance (Charles and Nielsen-LeRoux 2000). Focusing research on the integration of pesticides with oviposition infochemicals will provide the basis for successful use of infochemicals for vector control as an alternative to exclusive use of broad spectrum pesticides. Consequently, there is a great potential of using oviposition infochemicals to manipulate Afro-tropical malaria vectors by leading them to traps or sites treated with biolarvicides.

Disease prevention

Knowledge of mosquito dispersal is critical for vector-borne disease control and prevention strategies and for understanding population structure and pathogen dissemination (Harrington et al. 2005). An increase in adult mosquito dispersal could be a result of gravid females searching for suitable breeding sites, and so a shortage of suitable oviposition sites may accelerate the dispersal of adult females. Larval habitat reduction may encourage the dispersal of these vectors in their search for oviposition sites and thereby contribute to the spread of diseases (Edman et al. 1998). Dispersal is influenced by prevailing wind, longevity of the species and by the presence of a suitable breeding site. The suitability of a breeding site is generally assessed by gravid mosquitoes through the presence of chemical oviposition cues. Therefore, the deployment of oviposition attractants in artificial oviposition sites would restrict gravid female mosquitoes to disperse in search of oviposition sites, and enhance the efficacy of mosquito control for the prevention of vector-borne disease (Mafra-Neto and Dekker 2019).

Conclusions and future prospects

Our analysis reveals that ecological conditions under which infochemicals mediate oviposition behaviour are similar among a wide range of mosquito species from different taxonomic tribes. This enables us to draw general conclusions on the possibility of exploiting mosquito oviposition behaviour and propose a novel strategy for mosquito surveillance and control.

Although some of the earliest studies of the role of infochemicals in the biology of mosquitoes were promising, exploitation of knowledge on chemical ecology for the development of potential control strategies has received limited attention. Further elucidation of the role of infochemicals in mosquito oviposition may lead to a viable and effective tool for vector control. Studies on culicine mosquitoes have demonstrated that we have only just started to unravel the rich and complex chemical communication systems of these important insects.

In this review we have summarised the roles that infochemicals play in the behavioural pattern and responses of the oviposition phase of mosquitoes. In the last decade, notably, much progress has been made in the discovery of infochemicals mediating oviposition behaviour of anophelines. We conclude that there is a great potential of utilizing infochemicals mediating oviposition behaviour in mosquitoes for the development of vector surveillance and control strategies. Studies on culicine mosquitoes and the recent findings obtained from research on anopheline spp. provide a useful model for more studies on these and other mosquito genera.

Aknowledgements

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Laboratory assays were used to understand mosquito oviposition behaviour.

Chapter 3

The influence of larval stage and density on oviposition site-selection behavior of the Afro-tropical malaria mosquito *Anopheles coluzzii* (Diptera: Culicidae)

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Abstract

In the selection of oviposition sites female mosquitoes use various cues to assess site quality to optimize survival of progeny. The presence of conspecific larvae influences this process. Interactive effects of oviposition-site selection were studied in the malaria mosquito *Anopheles coluzzii* Coetzee & Wilkerson in dual and no choice assays, by exposing single gravid mosquitoes to oviposition cups containing (i) larvae of different developmental stages, (ii) larvae-conditioned water and (iii) cups where visual cues of conspecific larvae were absent. Early-stage conspecific larvae had a positive effect on the oviposition response. By contrast, late stages of conspecific larvae had a negative effect. Oviposition choice was dependent on larval density. Moreover, in oviposition cups where larvae were hidden from view, late stage larvae had a significant negative effect on oviposition suggesting the involvement of olfactory cues. Larvae-conditioned water had no effect on oviposition response, indicating involvement of chemicals produced by larvae *in-vivo*.

It is concluded that the presence of larvae in a breeding site affects the oviposition response depending on the development stage of the larvae. These responses appear to be mediated by olfactory cues emitted by the larval habitat containing live larvae, resulting in the enhanced reproductive fitness of the females.

KEYWORDS: *Anopheles coluzzii*, olfactory cues, larval pheromones, oviposition, aggregation

Introduction

Physical and chemical cues allow mosquitoes to assess the suitability of potential larval habitats and hence influence the acceptance of oviposition sites (Blackwell and Johnson 2000, McCall 2002, Abrell et al. 2005, Herrera-Varela et al. 2014). Physical cues originate from vegetation, moisture, optical density, color/contrast, temperature and texture of the substrate (McCrae 1984, Savage et al. 1990, Clements 1999, Koenraadt et al. 2003, Huang et al. 2006b, Huang et al. 2007, Reiskind and Zarrabi 2012). Chemical cues are produced by microorganisms (Trexler et al. 2003, Huang et al. 2006a, Lindh et al. 2008), conspecific eggs (Laurence and Pickett 1982, 1985, Ganesan et al. 2006), conspecific larvae (Mendki et al. 2000, Mokany and Shine 2002, Seenivasagan et al. 2009), water conditioned by eggs and/or larvae (Zahiri et al. 1997, Allan and Kline 1998, Zahiri and Rau 1998), plant infusions (Olagbemiro et al. 2004, Burkett-Cadena and Mullen 2007, Ponnusamy et al. 2010a, Ponnusamy et al. 2010b, Tennyson et al. 2012), odors from pollen (Wondwosen et al. 2017) and predators (Mokany and Shine 2002, 2003, Silberbush et al. 2015, Why et al. 2016).

Gravid mosquitoes may be attracted to or deterred by habitats containing conspecific larvae, because the presence of such larvae may indicate the suitability of habitats, an important factor in maximizing the fitness of their offspring (Blaustein and Kotler 1993, Allan and Kline 1998). Studies on the influence of conspecific eggs/larvae on site selection suggest that culicines are generally attracted to conspecifics (Laurence and Pickett 1982, 1985, Mendki et al. 2000). So far, studies on anophelines have given conflicting results regarding the influence of conspecifics on oviposition behavior. Some authors have reported oviposition deterrence in the presence of conspecific larvae (Bentley and Day 1989, Munga et al. 2006) whereas others have reported that several females may oviposit at the same site (Chen et al. 2006, Chen et al. 2008), which suggests attraction. Olfactory mediation of oviposition behavior in *Anopheles gambiae* Giles *sensu stricto* by conspecific larva was reported, and a density-dependent factor has been suggested to regulate this behavior (Sumba et al. 2008). In these studies, no difference was made between the effects of larval stages which may determine the decision taken by the ovipositing female.

The co-existence of various developmental stages of mosquito larvae (instars) in a breeding site may lead to resource competition and cannibalism (Koenraadt and Takken 2003). First instars of the malaria vector *An. gambiae s.s.* have been reported to be consumed by fourth instars of the same species. It is therefore plausible for a gravid mosquito to avoid oviposition in sites where late instars are present. Therefore, mosquitoes must make a careful assessment of breeding sites before selecting one for their offspring. It has been shown, however, that mosquitoes tend to avoid oviposition in habitats with predators and competitors (Kiflawi et al. 2003, Mokany and Shine 2003). Therefore, mosquitoes are faced with the challenge of choosing a suitable habitat while minimizing the costs of intra and/or inter-specific competition (Blaustein et al. 2004). However, it is not clear whether gravid females can express an optimal oviposition strategy by using only olfactory cues or in combination with other cues emitted from the larval habitat.

The ability of a female mosquito to assess breeding site suitability relies on her ability to detect and estimate the presence and density of conspecifics, in a single breeding site as well as within and among surrounding potential sites. The contribution of breeding sites with high or low larval density to the adult population and to the epidemiology of vector-borne diseases is vital as density-dependent effects may mediate the fitness of mosquitoes emerging from the site. The mechanism, with which a gravid female recognizes various densities of larvae in the breeding site, is not well understood. Only few studies have investigated oviposition site-selection behavior, despite its large consequences for individual fitness, population dynamics and community structure (Blaustein et al. 2004).

In insects, infochemicals can originate from specialized secretory glands, body orifices, organs involved in digestion and reproduction (e.g., mouth, anus, aedeagus) (Wertheim et al. 2005). Also infochemicals can be emitted by microorganisms living symbiotically with larvae, and attract or repel conspecific adults. Odors from bacteria are known to mediate oviposition behavior in mosquitoes (Ponnusamy et al. 2008, Ponnusamy et al. 2010a).

Studies with the mosquito *Culiseta longiareolata* Macquart, (Silberbush et al. 2010) and *An. gambiae* (Warburg et al. 2011) reveal that gravid females avoid larval habitats containing the cues of the predatory backswimmer, *Notonecta maculata* Fabricus (Hemiptera: Notonectidae), as a result of detecting the hydrocarbons (n-heneicosane and n-tricosane) produced by *N. maculate*. On the other hand, an oviposition pheromone n-heneicosane (C21), was identified and characterized from the larval cuticle of *Aedes aegypti* (L.) (Mendki et al. 2000). Furthermore, it was confirmed that low doses of n-heinecosane, attract gravid *Ae. aegypti* whereas at higher doses they are repelled by the pheromone (Seenivasagan et al. 2012). It is not surprising that this same compound repels *Culiseta*. Recent studies based on electro-antennographic responses (GC-EAD) and additional oviposition assays confirmed n-heneicosane to be an oviposition pheromone in *Ae. aegypti* and a behavior modifier of *Ae. albopictus* (Skuse) in larval habitats (Gonzalez et al. 2014).

Understanding mosquito oviposition behavior can provide a tool for behavioral manipulation of mosquitoes in the field and enable the development of an effective vector surveillance and control strategy. The objective of the present study was to investigate the influence of larval stages and density of conspecifics on the oviposition strategy of *An. coluzzii* Coetzee & Wilkerson and whether this strategy was chemically mediated. Specifically, the study aimed to explore the age at which larvae attract or deter conspecific gravid females to oviposit, and identify respective intraspecific cues involved in oviposition-site selection.

Materials and Methods

Mosquitoes: Experiments were performed using gravid *An. coluzzii* (Suakoko line) mosquitoes reared at the Laboratory of Entomology of Wageningen University, The Netherlands. Mosquitoes have been kept in the Laboratory of Entomology, Wageningen University since 1988 and maintained on human blood. *Anopheles coluzzii* were reared in a climate-controlled room at 28ºC and 80% relative humidity, with 12:12 LD photoperiod. Adults were kept in a 30cm cubic cage, with constant access to 6% glucose solution. Larvae were reared in 2.5 L plastic trays filled with tap water and fed Tetramin® (Tetra Werke, Melle, Germany) fish-food. Pupae were collected daily and placed in small trays inside the adult cage for emergence.

Bioassay conditions: All bioassays were conducted in a standard 30cm x 30cm x 30cm (length, width, height) netting cage, placed inside a climate-controlled room at 28 ºC and 80% relative humidity, with 12:12h LD photoperiod. Thirty 5-6 d old mosquitoes were placed in one cage, and offered a blood meal for 10 min. Unfed mosquitoes were removed from the cage and blood-fed mosquitoes were kept for two days while provided with 6% glucose solution on filter paper until they became fully gravid. Individual mosquitoes were randomly selected among these gravid females and used in the experiments. Selected females were placed singly in a cage with 6% glucose *ad libitum* and provided with two plastic oviposition cups (5cm diameter x 4cm height) in a two-choice bioassay. Each oviposition cup was placed diagonally in the corner of the cage at the farthest possible distance (~35cm) from the other cup. Oviposition was checked at 24 h and 48 h after the start of the experiment.

First and fourth instars (aged two and seven days' post oviposition) were taken from the colony trays, using a plastic pipette. Water drops containing the larvae were placed on the bottom of an empty 2.5 L-rearing pan and the larvae counted. Rearing water was removed as much as possible by rinsing larvae through a sieve and rinsing them once with tap water. Subsequently, larvae were transferred to the oviposition cups and tap water of 28ºC was added to each cup to a volume of 30ml. Each cup contained either first instars, fourth instars or both, depending on the experimental set up. Each experimental cage was assigned a pair of oviposition cups containing 30 ml of tap water (control) or 30 ml of tap water with larvae.

Oviposition response in the absence of larvae: An oviposition response experiment in the absence of larvae was designed to investigate mosquito oviposition preference when given a dual choice of cups filled with tap water only (no treatment, negative control).

Oviposition in response to first instars: The influence of the density of conspecific first instar larvae on oviposition-site selection of gravid *An. coluzzii* was investigated. Four cages were used and each was assigned two oviposition cups containing water with or without larvae. The numbers and densities (larvae/ml of water) of conspecific larvae tested against controls were 10 (0.3), 30 (1), 50 (1.7) and 100 (3.3). On each experimental day, the four treatments were tested simultaneously.

Oviposition in response to fourth instars: The potential effect of fourth instars on oviposition of gravid *An. coluzzii* was investigated at two larval densities, each tested against a control of tap water. One cup contained 10 fourth instars and the other contained 50 fourth instars (0.3 and 1.7 larvae/ml of water, respectively). Larvae were obtained from the rearing stock and they were not fed during the experiment. On each experimental day, the two densities were tested simultaneously.

Oviposition in response to first and fourth instars: Dual choice tests were designed to investigate whether gravid *An. coluzzii* make a trade-off between first-stage and fourth-stage conspecific larvae of low and high densities during oviposition site selection. Two cages were used; one with 10 first instars and 10 fourth instars in in cups within one cage. A second cage had 50 first and 50 fourth instars.

Oviposition response to larvae-conditioned water: This experiment investigated whether oviposition attractant/deterrent cues are present in larvae-conditioned water (LCW). Fifty first or fourth instar larvae were kept in oviposition cups for 24 h, and provided with Tetramin® baby-fish food (Tetra Werke, Melle, Germany) at 0.01 g per cup. A control was prepared by adding the same amount of Tetramin to a cup of tap water and let age for 24 h. The water from these cups was filtered through Whatman filter paper no. 2 into clean cups after 24 h and used in the experiment. Two treatments tested water from first or fourth instar larvae against a negative control of water conditioned with Tetramin and against each other.

Oviposition response to Tetramin-conditioned water: To investigate if the oviposition response observed with larvae of different developmental stages was caused by larval cues or by nutritional cues, gravid females were exposed to oviposition cups containing Tetramin-conditioned water (tcw) and tcw + first or fourth instars in a dual-choice assay. Tetramin (0.02 g) was added to 30 ml of tap water, shaken vigorously and left for 12 hours. Then the water was filtered by using Whatman filter paper no. 2 into other cups and used in this experiment. Fifty first and fourth instars, respectively, were placed in oviposition cups and provided with 30ml of Tetramin-conditioned water. In a dual-choice set up, in the standard 30x30x30 cm bioassay cage, single gravid mosquitoes were exposed to i) tcw vs tcw+first instars, ii) tcw vs tcw + fourth instars, and iii) tcw + first instars vs tcw + fourth instars. Cups were inspected for presence of eggs after 24 and 48 h.

Oviposition response to cups covered with filter paper: This experiment separated the influence of physical and chemical cues on oviposition-site selection by gravid mosquitoes. Larger plastic cups (7.5cm diameter) that contained 100 first or fourth stage larvae and water were prepared. A small cup (5cm diameter) with distilled water was immersed on the large cup, serving as an oviposition cup and then, a filter paper was inserted to touch the water in the small cup but not the larval water in the large cup (Fig. 1). The entire set up was covered by a filter paper. The small cup prevented contamination of the filter paper with larval water while the wet filter paper prevented gravid females from seeing larvae in the cup, and at the same time served as an oviposition substrate. In this set up, dark color or shade due to the presence of larvae was controlled as a larva that darkens the oviposition substrate was hidden from view.

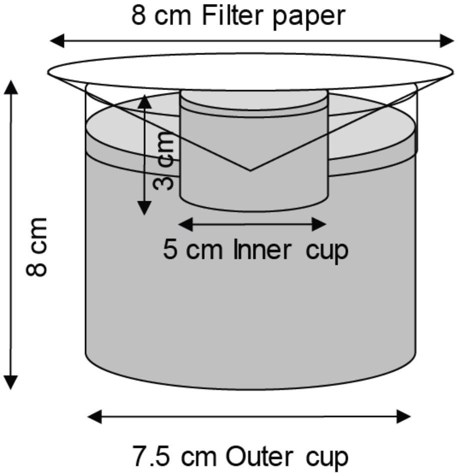

Figure 1 - Diagram of the set-up of the double cup experiment in which visual cues from larvae are hidden from the mosquito view by filter paper.

No-choice experiment: The aim of this experiment was to assess the response of ovipositing females to larvae of different instars (L1 and L4) in the absence of alternative oviposition sites. Individual gravid mosquitoes were exposed to a cup containing either 100 first instars or 50 fourth instars for 72 h. Oviposition was checked at 12, 24, 48 and 72 h. Any eggs remaining in the ovaries and not oviposited were counted following dissection.

A flow diagram of the experimental procedures is presented in Figure S1.

Statistical Analysis: The nature of a dual-choice assay with a single gravid female per cage yields discrete data as a female may lay all eggs in one oviposition cup only. Therefore, we used non-parametric statistical procedures to determine the difference in number of eggs laid on the paired oviposition substrates. Wilcoxon signed rank tests for paired samples were conducted using IBM SPSS statistics 20 for Windows®. The response variable reported here is the total number of eggs collected after 48 h and each experimental set up was replicated at least eight times on different days. Data from the no-choice experiment were analyzed by a Kruskal-Wallis test.

Results

Oviposition response in the absence of larvae: There was no significant difference in the mean number of eggs that mosquitoes deposited in either cup. In 20 replicates, females laid eggs randomly in one of the two cups only, with no preference for any cup (mean number of eggs per cup: 23.7 ± 5.7 and 22.5 ± 5.6, N=20, $p = 0.779$).

Oviposition in response to first instars: Gravid *An. coluzzii* deposited significantly more eggs in cups containing 50 (N=17, $p = 0.49$) or 100 first instars (N=17, $p < 0.001$) than in negative control cups. Mosquitoes laid four times as many eggs in cups with 50 first instars and 30 times as many eggs in cups containing 100 first instars compared to control cups. The difference between eggs laid in cups with 10 or 30 first instars and their controls was not significant (N=17, $p = 0.124$) and (N=17, $p = 0.088$), respectively (Fig. 2A).

Oviposition in response to fourth instars: Gravid *An. coluzzii* deposited significantly more eggs in control cups than in the cups containing 10 fourth instars (N=17, $P = 0.009$ or in cups with 50 fourth instars (N=17, $p < 0.001$) (Fig. 2B).

Oviposition in response to larvae in two different development stages: Gravid mosquitoes did not discriminate between cups with 10 first instars and cups with 10 fourth instars (N=17, $p = 0.586$). However, when cups with 50 first instars were tested against cups with 50 fourth instars, no eggs were deposited in the cups containing fourth instars (N=17, $p < 0.0001$). The cup containing 50 first instars received on average 45 ± 9.8 eggs (Fig. 2C).

Oviposition response to larvae conditioned water: There was no significant difference in the number of eggs that females deposited in the control cup and the cup with water conditioned by first instars (N=17, $p = 0.981$) or by fourth instars (N=17, $p = 0.906$) (Fig. 3). Also, in a dual choice test, there was no significant difference between eggs laid on water conditioned by first or fourth instars (N=17, $p = 0.705$) (Fig. 3).

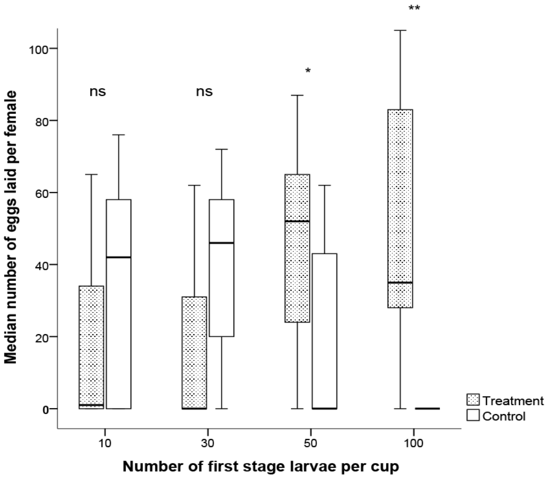

Figure 2A - Number of eggs laid by single female *An. coluzzii* per cup in a dual-choice essay, using cups with conspecific 1st instars in ascending densities and cups containing water only (controls). Median and quartiles are given; asterisks indicate statistical differences between treatment and control for a given density (* *P* <0.05, ** *P* <0.01, Wilcoxon signed rank tests). The number of replicates was 17.

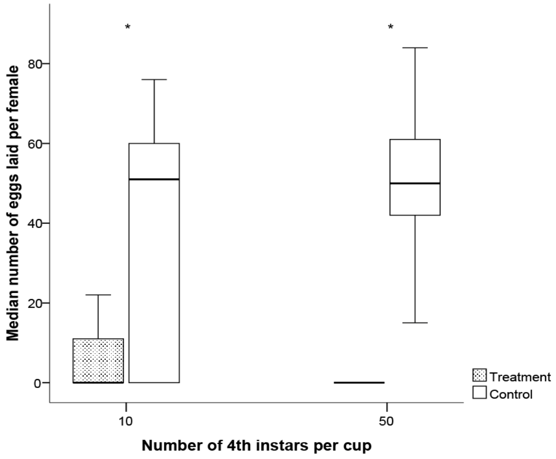

Figure 2B - Number of eggs laid by single female *An. coluzzii* per cup in a dual-choice essay, using cups with conspecific 4th instars in densities of 10 and 50 per cup, respectively, against cups containing water only (controls). Median and quartiles are given; asterisks indicate statistical differences between treatment and control for a given density (* *P* <0.05, ** *p* <0.01, Wilcoxon signed rank tests). The number of replicates was 17.

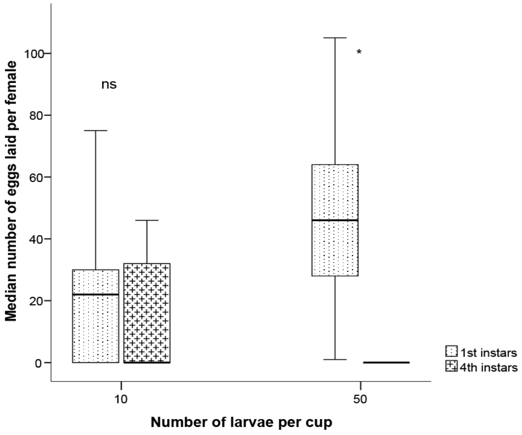

Figure 2C - The number of eggs laid by *Anopheles coluzzii* in a dual choice test between cups treated with low and high densities of conspecific first and fourth instars. Median and quartiles are given; asterisks indicate statistical differences between the larval stages at a given density (** *P* <0.01, Wilcoxon signed rank tests). The number of replicates was 17.

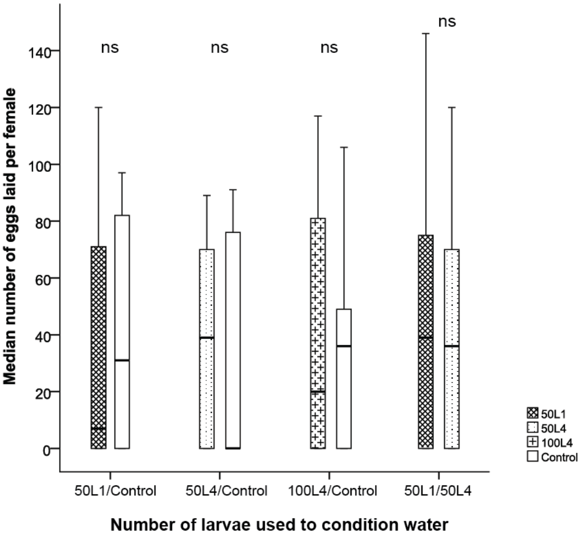

Figure 3 - *Anopheles coluzzii* oviposition (measured as the number of eggs laid per female) in cups treated with larvae-conditioned water (LCW) and respective controls. Set 1 consists of LCW from 50 first instars and a control, set 2 consists of LCW from 50 fourth instars and a control, set 3 consists of LCW from 100 fourth instars and a control, and set 4 consists of LCW from 50 first instar larvae and LCW from 50 fourth instar larvae. Median and quartiles are given; there were no significant differences in any of the 4 treatments (Wilcoxon signed rank tests). The number of replicates was 20.

Oviposition response to Tetramin-conditioned water: In dual choice tests, there were no differences in the mean number of eggs laid per female in cups with tcw than in first instars + tcw or fourth instars + tcw (Table 1). In a choice between tcw and larvae + tcw, however, females more often selected the tcw-only cups. When first instars + tcw were tested against fourth instars + tcw, females selected more often the cups with first instars, but there was no difference in the mean number of eggs laid between treatments.

Table 1 - Oviposition response of *Anopheles coluzzii* in Tetramin conditioned water (tcw) and larval instars to which tcw has been added. Number of replicates per treatment: n = 13

	Treatments					
Dual choice set up	**L1+tcw**	**tcw**	**L4+tcw**	**tcw**	**L1+tcw**	**L4+tcw**
No of females laid eggs in either cup	4	9	4	9	9	5
Total eggs	342	781	368	736	924	401
Mean no. eggs per female	85.5	86.8	92.0	81.8	102.7	80.2
S.E.	12.53	14.77	13.91	15.63	18.28	11.66

Oviposition response to cups covered with filter papers: When larvae were hidden from view by covering the oviposition cups with filter paper using the double cup technique, there was no significant difference between eggs laid in cups with 100 first instars and the control cups (N=17, *p* = 0.758), but in the test with fourth instars, significantly more eggs were laid in the control cups than in cups with 100 fourth instars (N=17, *p* = 0.26) (Fig. 4). When cups with 100 first instars were tested against cups with 100 fourth instars, *An. coluzzii* laid significantly more eggs in cups with first instars than in those with fourth instars (N=17, *p* = 0.001) (Fig. 4).

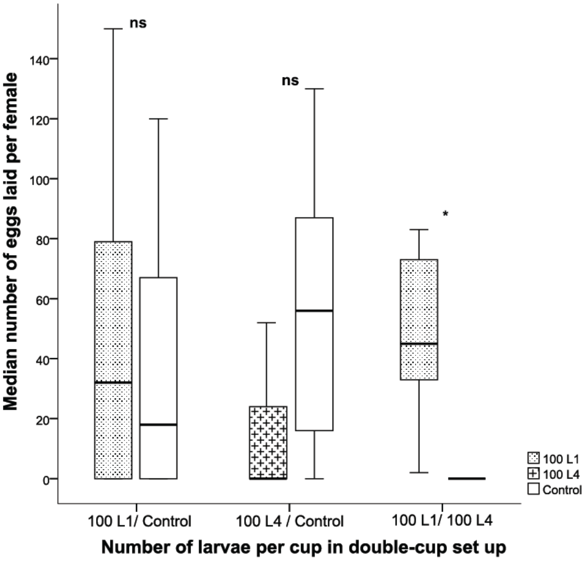

Figure 4 - *Anopheles coluzzii* oviposition, (measured as the number of eggs laid per female) test between cups treated with (i) 100 first instars and a control, (ii) 100 fourth instars and a control and (iii) between 100 first instars and 100 fourth instars using a double cup set up. Median and quartiles are given (*: *P* <0.05, Wilcoxon signed rank tests). The number of replicates was 17.

Oviposition response in single cup (no-choice experiment): In 15 replicates, 100% of females exposed to cups with first instars laid eggs. By contrast, of the females exposed to fourth instars, only 73.3% laid eggs. Cups with first instars received significantly more eggs 103.4 ± 10.7 (Kruskal-Wallis test, *p* < 0.001) than cups with fourth instars, which received an average number of 7.6 ± 3.5 eggs. Moreover, all eggs in cups containing first instars were laid within the first 12 h. after exposing gravid mosquitoes to the developing larvae, while females exposed to fourth instars spread their eggs between 12 and 48 h. After 72 h. all mosquitoes were removed and dissected. The mean number of eggs retained in mosquitoes exposed to fourth instars (77.5 ± 12.3) was significantly greater (*p* < 0.001) than the mean number of eggs retained in mosquitoes exposed to first instars (0.5 ± 0.35) (Fig. 5)

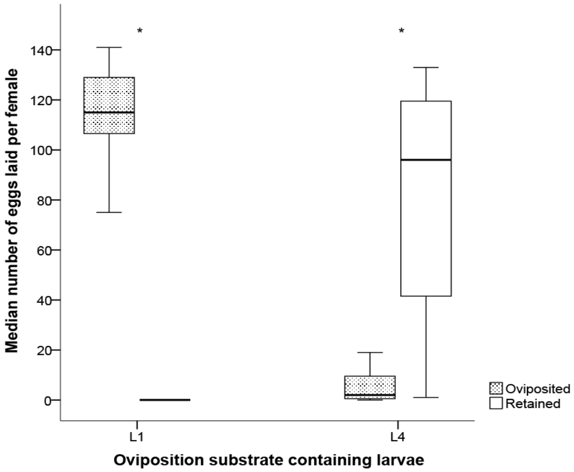

Figure 5 - Egg laying responses of *Anopheles coluzzii* when exposed to oviposition substrates containing either first instar larvae (L1) or fourth instar larvae (L4) under no-choice conditions. The number of replicates was 15.

Discussion

Our study indicated that gravid females of *An. coluzzii* were affected in their oviposition behavior by volatiles produced by conspecific larvae, in which first instars produced attractive chemicals whereas fourth instars produced chemicals that deterred females. This is likely the reason why females select sites with a high density of first instars, while avoiding sites with low densities of such larvae. Studies on *An. gambiae* oviposition behavior showed that members of this group of anophelines tend to lay eggs in groups and several females may oviposit in the same site (Chen et al. 2006, Chen et al. 2008). Therefore, the choice for laying eggs in sites with high densities of conspecifics can be beneficial, unless these sites confer unfavorable traits such as competition, cannibalism or predation. When these conspecifics, however, are in an advanced age of development, they may be dangerous for young siblings, and females avoid such sites to reduce the risk of cannibalism (Koenraadt and Takken 2003). Therefore, the avoidance of oviposition in sites where late stage larvae are present is of great advantage to mosquitoes and reveals how mosquitoes might achieve an optimal oviposition strategy by exploiting sites that confer the best chance for offspring development and survival. In nature, the presence of immature stages could indicate the suitability of that site in terms of food, site persistence, lack of predation and appropriate abiotic conditions (Wong et al. 2011).

The findings of our experiment suggest that in the absence of conspecific larvae, mosquitoes oviposit in water bodies selected at random. The mean number of eggs laid in cups with water only was less than the mean number of eggs laid in cups containing larvae with densities starting from 1.7 larvae/ml of water. This indicates that females retain some of their eggs when there is no clear evidence of site suitability. Moreover, in the presence of first instars, oviposition responses increased with increasing larval density starting from 1.7 larvae/ml of water. Likewise, the strength of deterrence caused by fourth instars increased with increasing larval density. We can therefore conclude that the oviposition response in the presence of conspecific larvae is density dependent. This was also found by Ognufabor and Sumba (2008), who reported similar olfaction-guided oviposition behavior of *An. gambiae* with density dependence. In Ognufabor and Sumba's study, however, no distinction was made between young and older instars. In fact, their study reported deterrence of oviposition by all larval densities in distilled water. As we learned from our study, this is possibly caused by volatiles produced by fourth instars, which under all circumstances, at densities of 0.3 to 3.3 larvae per ml, caused deterrence. Our findings also supported an earlier hypothesis that breeding sites with low larval density are perceived as unsuitable by gravid mosquitoes (Munga et al. 2006). Higher larval densities are common, especially in small breeding sites such as hoof/foot prints, tire prints, and road banks. In a nutrient enriched site, a single mosquito may lay 50 - 500 eggs at one time (Clements 1992). As conspecific sharing of breeding sites is common among mosquitoes (Chen et al. 2008) and egg-hatching rate is high (Phasomkusolsil et al. 2013), small sites are often harboring high larval densities. Therefore, the existence of breeding sites with low larval density suggests unfavorable conditions. *Anopheles gambiae* s.l. tends to breed in such small, shallow and temporary sites due to absence of predation (Service 1993, Mala and Irungu 2011, Muriu et al. 2013).

In natural conditions some habitats contain high densities of anopheline larvae, whereas many others have none despite high densities of adult mosquitoes in the immediate environment (Minakawa et al. 2002). These observations are in line with the findings from our study and observations by other authors that some aquatic habitats are more attractive or suitable for oviposition and larval development than others (Minakawa et al. 1999), and as a result, mosquitoes perform selective oviposition behavior (Davis et al. 2015). Randomly amplified polymorphic DNA used to estimate the number of full sibling family size revealed that the average family size of *Ae. aegypti* mosquito larvae in a container is 11 and the family size distribution among containers is skewed toward containers with one or two families (Apostol et al. 1994). Studies on oviposition behavior of *An. gambiae* using a pair-wise genetic relatedness method substantiate that average genetic relatedness tends to be low for breeding sites with high larval populations (Chen et al. 2006). This means that *An. gambiae* prefers to oviposit where others have oviposited. Our study suggests that the presence of an optimum density of first instar stage larvae in selected breeding sites may induce other conspecific mosquitoes to oviposit in the same breeding sites.

Results from the no-choice experiments suggest that substrates containing first instars attract egg-laying behavior of conspecific gravid females, whereas substrates containing fourth instars deter females intending to oviposit and induce egg-retention behavior. Egg retention is common in skip oviposition where mosquitoes do not lay all eggs in one site but retain some (Williams et al. 2008) and lay in multiple sites (Colton et al. 2003, Reiter 2007, Snell et al. 2010).

In our study we found that egg retention is common when fourth instars are present in oviposition cups, and oviposition in more than one container occurred when nutrient-poor substrates were used in both oviposition choices. This suggests that skip oviposition occurs when there is no clear indication of site suitability, as mosquitoes do not display a clear preference. Moreover, our results suggest that in natural settings, where the only available water body contains predators (e.g. conspecific fourth instars), *An. coluzzii* will lay few eggs in that risky environment on the first day. On the second day, *An. coluzzii* will lay even fewer eggs as compared to the first day while retaining the rest of the eggs. These observations are akin to skip oviposition behavior, which is displayed by other mosquitoes. For example, in *Ae aegypti,* significantly higher oviposition occurred in one site and residual eggs were distributed in groups of 1-30 eggs (Oliva et al. 2014).

Our data from the double cup experiment, where visual stimuli from water and/or larvae were excluded, indicate that olfactory compounds are involved in oviposition behavior. Chemical communication has been demonstrated to operate in mosquito oviposition behavior in several mosquito species (Bentley and Day 1989, McCall and Cameron 1995) including *An. gambiae s.s.* (Blackwell and Johnson 2000), a sibling of *An. coluzzii*. However, our results demonstrate that only alive, conspecific larvae produce these cues, as larvae-conditioned water did not cause a behavioral response. These effects are clearly dose-dependent, as the magnitude of the influence increases with increasing larval density and at low larval densities the influence was not observed. Ognufabor and Sumba (2008) also reported the presence of oviposition-mediating olfactory cues derived from immature larvae of *An. gambiae*. In their study, however, larval water from different instars was mixed, and hence the chemical cues from one instar may have overridden the deterrent effects from fourth instars.

The effects of the larval cues appeared to be overruled by cues produced by Tetramin. Nutritional substrates are well known to affect *An. gambiae* oviposition behavior (Lindh et al. 2008) and an oviposition attractant cedrol was identified and associated with the presence of microbes in the breeding habitat (Lindh et al. 2015). Therefore, it is likely that chemical cues produced from nutritional constituents such as Tetramin impact mosquito oviposition behavior, masking the effects of the larval cues. Significant differences in cues from first and fourth instars, however, are evident as gravid females laid more eggs in larval substrates with first instars than with fourth instars.

When female *An. coluzzii* were offered a choice between oviposition cups with fourth instars and a control, they deposited more eggs in the control cups. When subjected to a choice between same high densities of early and late stage larvae, female mosquitoes opted for the former exclusively. These results indicate the presence of different types of larval pheromones involved in the oviposition strategy of *An. coluzzii*. Pheromones produced by early instars that signal to the gravid female suitability of the site and pheromones produced by late instars that signal unsuitability. The pheromone might be of the same nature, but produced at a different concentration by early and late stage larvae. On the other hand, the difference between eggs laid in cups with low densities of first and fourth larval instars was not significant, and confirms our earlier findings that a low density of first instars does not signal the quality of a site. It is possible that the emission of chemical cues that are likely to mediate oviposition behavior is too low when larval density is low.

Under laboratory conditions, gravid *An. gambiae* usually touch the oviposition substrate briefly or hover 5-10 cm above the site before depositing her eggs (Huang et al. 2006b). Such an assessment procedure may involve visual, tactile or olfactory cues, alone or in combination. Experiments with larvae-conditioned water did not show a significant difference between conditioned water and controls. The disappearance of attractiveness or deterrence after the removal of larvae may suggest two things: first, vision is also involved in the previously displayed behavior. Secondly, the presence of live larvae is necessary to evoke the observed behavior. Chemicals released by mosquito larvae are highly volatile and present in low concentrations (Allan and Kline 1998). Therefore, it is possible that the removal of larvae and volatilization of chemicals have contributed to absence of attractiveness or deterrence response in the larvae-conditioned water experiments.

Studies by Huang *et al.* (2018) emphasized that cannibalization of newly hatched *An. gambiae* larvae by fourth-instar larvae was a result of egg reduction in egg counts and not olfactory deterrent cues. However, results from the double cup experiments, in which the larvae are hidden from view by a filter paper (Fig. 1), suggest that chemical cues are involved in the oviposition behavior of *An. coluzzii*. Our study further suggests that oviposition-deterrent chemicals are responsible for counteracting resource competition and cannibalism among larvae of *An. coluzzii* in field settings. We therefore confirm recent findings that ovipositing *An. coluzzii* females are less inclined to lay eggs in pools that have late instars compared to those with early instars (Sumba et al. 2008). These observations can partly explain why an initial population peak of *Anopheles* larvae observed at a fixed point in a rice field was not immediately followed by a sustained high population peak of early instars (Mutero et al. 2004). Our study was conducted in the laboratory, using mosquitoes that originated from Liberia. When the study was repeated in Tanzania using mosquitoes that originated from Tanzania, we obtained similar results (V.M., unpublished data). However, further studies need to be conducted in various places with different climatic and geographic conditions to clarify the existence of a region-specific cue in *An. coluzzii* (Ogbunugafor and Sumba 2008).

Larval-based cues can be used for manipulation of mosquito behavior in a push-pull system by making protected resources unsuitable to mosquitoes (by repellents) while luring them towards attractive sources (by attractants) where they can be eliminated by insecticides. It appears evident that larval hydrocarbons can be detected by conspecific (Seenivasagan et al. 2009) and heterospecific mosquitoes (Gonzalez et al. 2014). Moreover, depending on the concentration, female mosquitoes were attracted to a low dose, while higher doses enforced repellency (Seenivasagan et al. 2009). We suspect that the observed pattern in our study might be explained by a similar mechanism whereby early instars emit chemical cues at low concentrations that attract, while late instars emit high concentrations which repel female mosquitoes.

Conclusion

Conspecific larvae, as demonstrated by this study, mediate the oviposition of *An. coluzzii* to enhance successful development of their offspring. Low densities of first instars did not attract gravid mosquitoes, while higher densities attracted gravid mosquitoes significantly. In contrast, fourth instars caused an oviposition-deterring response. These effects occurred only *in vivo,* in the presence of live conspecific larvae, as larvae-conditioned water neither stimulated nor deterred oviposition. The study further suggests that the oviposition attraction and deterring effects of larvae are masked by chemical cues from larval nutriments such as Tetramin, and therefore volatiles from other organisms present in natural breeding sites may interact with the instar- associated cues as found in the present study. The attractive and deterrent effects on ovipositing females were caused, at least in part, by non-visual cues emitted by live larvae and suggests that the oviposition behavior of *An. coluzzii* is chemically mediated.

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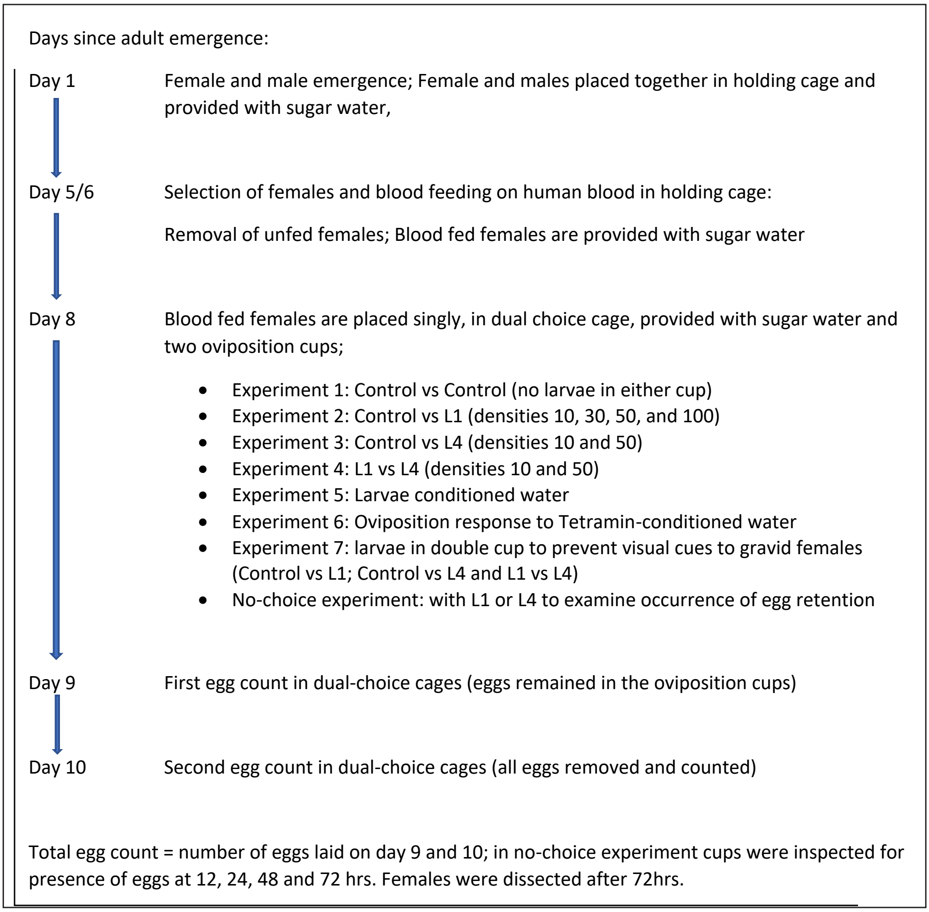

Days since adult emergence:

Day 1 — Female and male emergence; Female and males placed together in holding cage and provided with sugar water,

Day 5/6 — Selection of females and blood feeding on human blood in holding cage:

Removal of unfed females; Blood fed females are provided with sugar water

Day 8 — Blood fed females are placed singly, in dual choice cage, provided with sugar water and two oviposition cups;

- Experiment 1: Control vs Control (no larvae in either cup)
- Experiment 2: Control vs L1 (densities 10, 30, 50, and 100)
- Experiment 3: Control vs L4 (densities 10 and 50)
- Experiment 4: L1 vs L4 (densities 10 and 50)
- Experiment 5: Larvae conditioned water
- Experiment 6: Oviposition response to Tetramin-conditioned water
- Experiment 7: larvae in double cup to prevent visual cues to gravid females (Control vs L1; Control vs L4 and L1 vs L4)
- No-choice experiment: with L1 or L4 to examine occurrence of egg retention

Day 9 — First egg count in dual-choice cages (eggs remained in the oviposition cups)

Day 10 — Second egg count in dual-choice cages (all eggs removed and counted)

Total egg count = number of eggs laid on day 9 and 10; in no-choice experiment cups were inspected for presence of eggs at 12, 24, 48 and 72 hrs. Females were dissected after 72hrs.

Figure S1 – Flow diagram of experimental procedures of dual-choice oviposition experiment with *Anopheles coluzzii*

Mosquito spheres were used to understand short range impacts of infochemicals

Chapter 4

Chemical mediation of oviposition by *Anopheles* mosquitoes: a push-pull system driven by volatiles associated with larval stages.

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Abstract

The oviposition behavior of mosquitoes is mediated by chemical cues. In the malaria mosquito *Anopheles gambiae*, conspecific larvae produce infochemicals that affect this behavior. Emanations from first instar larvae proved strongly attractive to gravid females, while those from fourth instars caused oviposition deterrence, suggesting that larval developmental stage affected the oviposition choice of the female mosquito.

We examined the nature of these chemicals by headspace collection of emanations of water in which larvae of different stages were developing. Four chemicals with putative effects on oviposition behavior were identified: dimethyldisulfide (DMDS) and dimethyltrisulfide (DMTS) were identified in emanations from water containing fourth instars; nonane and 2,4-pentanedione (2,4-PD) were identified in emanations from water containing both first and fourth instars. Dual-choice oviposition studies with these compounds were done in the laboratory and in semi-field experiments in Tanzania.

In the laboratory, DMDS and DMTS were associated with oviposition-deterrent effects, while results with nonane and 2,4-PD were inconclusive. In further studies DMDS and DMTS evoked egg retention, while with nonane and 2,4 PD 88% and 100% of female mosquitoes, respectively, laid eggs. In dual-choice semi-field trials DMDS and DMTS caused oviposition deterrence, while nonane and 2,4-PD evoked attraction, inducing females to lay more eggs in bowls containing these compounds compared to the controls. We conclude that oviposition of *An. gambiae* is mediated by these four infochemicals associated with conspecific larvae, eliciting either attraction or deterrence. High levels of egg retention occurred when females were exposed to chemicals associated with fourth instar larvae.

KEY WORDS - *Anopheles coluzzii, An. gambiae s.s.*, malaria, mosquito, oviposition, dimethyldisulfide, dimethyltrisulfide, nonane, 2,4-pentanedione, behavior

Introduction

Assessment and selection of suitable oviposition habitats is important for the life history of mosquitoes (Bentley and Day 1989). Several studies have shown that the selection of oviposition sites by mosquitoes is influenced by chemicals. *Culex quinquefasciatus* Say, for instance, is known to be attracted to a variety of volatiles from breeding sites, including oviposition pheromones produced by conspecific eggs (Otieno et al. 1988) and compounds originating from organic material such as grass infusions and the compound skatole (Mboera et al. 2000a). It has since been shown that odour blends can be used to manipulate egg-laying females of *Cx. quinquefasciatus* and are therefore suitable for monitoring and control of this species (Mboera et al. 2000b). Several *Aedes* species also use chemical cues originating from microbes to identify oviposition sites (Allan and Kline 1995, Santana et al. 2006, Lindh et al. 2008).

The African malaria mosquito *Anopheles gambiae* Giles *sensu stricto* (hence referred to as *An. gambiae*) is known to be affected by volatiles from micro-organisms in soil and water of breeding sites (Sumba et al. 2004, Huang et al. 2006) and is attracted by water from natural oviposition sites (Sumba et al. 2008, Okal et al. 2013, Herrera et al. 2014). In addition, female mosquitoes of this species show an olfactory-based preference for oviposition sites in which larvae of the same regional population of *An. gambiae* have developed (Ogbunugafor and Sumba 2008). Furthermore, gravid females are repelled by emanations from breeding sites in which third and fourth instars were developing (Suh et al 2016). The repellence caused by larvae on egg-laying behavior of gravid females has also been observed to be affected by the density of larvae (Munga et al. 2006). A low density of young larvae had a positive effect on oviposition, whereas high densities of older larvae negatively affected oviposition (Sumba et al. 2008). More recently, the effects of larval stage and density have been studied in greater detail (Mwingira et al., 2019), suggesting a production of chemical compounds that affect oviposition behavior of conspecific gravid *An. gambiae* females, causing a positive response to cues from first instars and a negative response to cues from fourth instars.

A number of compounds have been shown to attract *Anopheles* mosquitoes to oviposition sites. Recently, Lindh et al. (2015) identified the sesquiterpene alcohol cedrol as an oviposition attractant of *An. gambiae* s.s. The volatiles of grass species *Echinochloa pyramidalis* and *E. stagnina* were attractive to gravid females of *An. coluzzii* Wilkerson & Coetzee and *An. arabiensis* Patton (Asmare et al. 2017); gravid females of *An. arabiensis* were furthermore attracted to volatiles from maize pollen, including *alpha*-pinene*,* limonene*,* *p*-cymene, nonanal and benzaldehyde (Wondwosen et al. 2017). Paradoxically, the compounds dimethyl disulphide (DMDS) and trimethyl disulphide (DMTS), products of decaying plant material, were identified as oviposition repellents for *An. coluzzii* (Suh et al. 2016). Additionally, Bermuda grass hay infusions contained olfactory compounds that repelled *An. gambiae* (Eneh et al. 2016a). These studies show that the oviposition behavior of *An. gambiae* s.l. females are affected by olfactory cues, which may be attractive or repellent, but the nature of these compounds is still poorly understood, especially concerning the interactions between water-associated cues and conspecific cues. While these studies have identified several compounds originating from natural breeding sites affecting oviposition, compounds associated with larvae have to-date not been described.

The identification of infochemicals influencing oviposition behavior is important for a better understanding of the chemical ecology of oviposition, manipulation of mosquito oviposition behavior and application in monitoring and control methods (Sumba et al. 2004, Munga et al. 2006, Sumba et al. 2008). The identification of oviposition attractant chemicals is expected to complement the current methods of monitoring and controlling mosquito populations (Perich et al. 2003, Ponnusamy et al. 2015, Dugassa et al. 2016). The present study was carried out to determine the effect of larval stage on attraction and repellence of gravid females of *An. gambiae* in laboratory and semi-field settings and to identify volatile chemicals produced by larvae of this species that mediate this behavior.

Materials and Methods

Laboratory experiments were conducted at the Laboratory of Entomology of Wageningen University & Research in The Netherlands and at the Amani Research Centre of the National Institute for Medical Research, Muheza, Tanzania. The semi-field study was conducted at the Amani Research Centre in Tanzania.

Insects and rearing procedures - We used *Anopheles coluzzii* originating from Suakoko, Liberia, previously known as *An. gambiae s.s.* M form (Coetzee 2013) that was reared at the Laboratory of Entomology, Wageningen University & Research, The Netherlands. Larvae were raised under standardized conditions (water surface > 2 cm^2 per larva), in a climate-controlled chamber at 28ºC and 80% relative humidity, with a 12:12h LD photoperiod. Larvae were reared in 2.5 l plastic trays filled with acclimatized tap water and were fed 0.003g/larva Tetramin® fish food (Tetra Werke, Germany). Pupae were collected daily and placed in small cups inside a 30 x 30 x 30 cm Bugdorm® cage (https://shop.bugdorm.com) for emergence. Adults (males and females) were kept in a Bugdorm® cage with *ad libitum* access to a 6% glucose solution. When 3-5 days old, females were fed blood by offering a human arm. Gravid mosquitoes from this group were used to study response to volatiles produced by larvae in the laboratory. Ethical approval for blood feeding was not requested as this method of blood feeding is not subject to the Dutch Act of Medical Research involving Human Subjects (WMO). In our anopheline mosquito cultures, no experimental infections took place and mosquitoes were free of any parasite.

At the Amani Research Centre, adult *An. gambiae s.s.* (originating from Ifakara, southern-central Tanzania) were kept in a 30 x 30 x 30 cm metal framed cage covered with netting. Larvae were reared in round aluminium pans with a diameter of 27 cm, filled with filtered tap water to a depth of 2 cm. Larvae were fed on Tetramin® fish food (Tetra Werke, Germany) and were kept in a 12:12h LD light regime. The temperature in the insectarium was 29 ^{o}C. Pupae were removed from the trays daily and were placed in the mosquito cages for emergence. Male and female mosquitoes were kept in the same cages. For blood feeding, 3-5 days old females were offered a human arm. An approval involving human subjects in blood feeding mosquitoes was obtained from the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania. The same volunteer donated blood to all batches of mosquitoes throughout the study, and mosquitoes were fed blood only once during their life time. Gravid mosquitoes from this group were used to study the response to infochemicals in the laboratory and semi-field experiments.

Oviposition in response to larvae - Experiments concerning the oviposition behavior in response to the presence of first or fourth instars were performed at the Laboratory of Entomology in Wageningen. The aim of this experiment was to investigate if the concept that larval habitats of *An. coluzzii* emit chemical cues that mediate oviposition behavior in conspecific adults. Nine-day old female *An. coluzzii* were fed blood on a human arm for ten minutes, two days before the start of the experiment and were kept as described above.

Early-stage larvae (L1) were collected two days after oviposition using a glass pipette. Water drops with larvae were placed on the bottom of a white, dry rearing tray and larvae were counted. Late-stage larval instars (L3/L4) were collected with a plastic pipette (5 ml) and were counted in a similar way as L1 larvae. A total of 100 larvae of the same developmental stage were placed in a plastic oviposition cup (5.25 cm diameter x 3 cm height). The volume of rearing water was removed to a minimum before the transfer of larvae and after the transfer of the larvae cups were filled with tap water to a volume of 30 ml.

Wet filter paper, 125 mm in diameter, Whatman® (Whatman International Ltd, Maidstone, England) was placed over the cup, serving as an oviposition site for the mosquitoes thus preventing stimulation by visual stimuli. To prevent drying out of the oviposition paper, a cylinder made of filter paper was placed in the cups (Fig. 1, left panel). This cylinder ensured that when the water level in the cup decreased, the oviposition paper remained wet. Moreover, because of the cylinder, the oviposition paper did not have to be in contact with the liquid, which would decrease the area of the water surface for the larvae to breathe. Larvae were placed within and outside of the cylinder. As a control the cups were filled with 30 ml tap water.

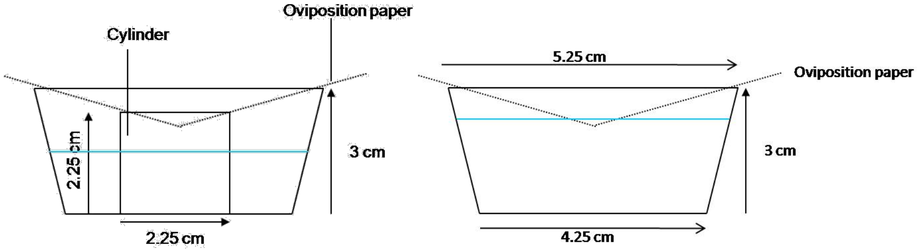

Figure 1 - Schematic representation of oviposition cups used in oviposition experiments; showing the set up with larvae (left) and chemicals (right).

Gravid mosquitoes were held solitary in a 30 x 30 x 30 cm Bugdorm® cage for 48 h – two oviposition periods (Fritz et al. 2008), under the circumstances described above. Each mosquito was given a choice between ovipositing in a treated cup, with either 100 early-stage larvae (L1) or 100 late-stage larvae (L4), and a control cup. Cups were placed diagonally in corners as far from each other as possible, at a distance of approximately 30 cm. Eggs were counted after 24 and 48 h and the total number of eggs after 48 h was taken as the response of the mosquitoes. Each treatment was repeated 17 times.

Collection and identification of chemicals - Three procedures were conducted at the Laboratory of Entomology, Wageningen University & Research: (i) a proof of concept for emission of volatile chemicals from larval habitats, (ii) collection of volatile chemicals from larval habitats by headspace techniques and (iii) identification of entrapped chemicals by GC-MS.

Volatile compounds released by water containing either no larvae, early stage or late stage larvae of *An. coluzzii* were collected from cups filled with 30 ml of tap water placed in separate air-tight cuvettes.

Volatiles were collected using the "purge and trap" approach on an adsorbing polymer: Tenax-TA 20/35 (Alltech, USA). To reduce background volatiles, air was sucked into the cuvette through a carbon filter and a cartridge containing 100 mg Tenax-TA. Headspace volatiles were trapped at a flow rate of 100 ml/min for 24 h on a second cartridge containing 100 mg Tenax TA connected to the outlet of the cuvette. Samples were released from the adsorbent using a thermodesorption unit (Ultra 50:50 TD, Markes, Llantrisant, UK) while re-collected in an electrically cooled cold trap (Unity, Markes) and followed by gas chromatography (Trace GC Ultra) and mass spectrometry (Trace DSQ quadrupole mass spectrometer), both from Thermo (Thermo Fisher Scientific, Waltham, USA).

The program for thermal desorption consisted of dry purging for 3 min and pre-purging for 1 min using helium (residual oxygen removal) at 30 °C. This was followed by tube desorption at 250 °C for 3 min and the volatiles were focused on a cold trap at 0 °C. Injection onto the analytical column was achieved by heating of the cold trap at the maximum heating acceleration (> 60°C per second) to 250 °C in a split mode at a split ratio of 1:6. The transfer line between the cold trap and the GC was kept at 160 °C throughout the analysis.

A 30 m x 0.25 mm ID x 1.0 µm F.T. capillary GC column (Rtx-5 MS, Restek, USA) with helium (5.0 grade) as carrier gas at a flow rate of 1.0 mL/min was used for separation of volatile compounds. The GC temperature was programmed as follows: 45 °C for 3 min, followed by a ramp of 8°C/min to 280 °C and was held at 280°C for 2 min. The transfer line between the GC and MS was set to 275 °C. MS spectra were recorded by ionization of the column effluent by electron impact (EI) ionization at 70 eV, scanning in positive mode from 35-300 *m/z* with a speed of 5 scans per second. The ion source temperature was set to 250 °C and the filament was switched off from 13.6-13.8 min because of a high background peak. Peak identification was performed by comparing the obtained spectra with those in the NIST library (version 2.0 d), experimentally calculated retention indices and using the retention times of authentic synthetic reference compounds.

Chemicals - The synthetic chemicals dimethyl disulfide (DMDS, ≥ 99.0%), dimethyl trisulfide (DMTS, ≥ 98.0%), nonane (≥ 99.0%) and 2,4-pentanedione (2,4-PD), which is also known as acetylacetone (ReagentPlus®, ≥ 99.0%), all from Sigma Aldrich (Sigma Aldrich, Chemie BV, Zwijndrecht, The Netherlands), were used for testing the oviposition response. Since all of these chemicals were insoluble in water, they were dissolved in methanol and Tween20, in the following ratios: 55g (test chemical) + 40ml Methanol + 5ml Tween20. Hereafter, the chemicals were dissolved and diluted in distilled water to make 1 l of diluted chemicals and dilution process continued until the required concentrations for bioassay was reached. The final concentrations of all chemicals ranged from 10^{-7} to 10^{-12} M.

Oviposition bioassays - Identified chemicals were tested for effects on oviposition behavior at the Amani Research Centre, Muheza, Tanzania, using *An. gambiae s.s* mosquitoes (Ifakara strain). Two experiments were conducted: laboratory experiments were performed under the same conditions and with the same materials as was done in Wageningen, with the aim to select and confirm effective doses for each chemical. Semi-field experiments were designed to verify potential attractive/repellent effects of these compounds under natural ambient conditions.

Dose response effects on oviposition - DMDS, DMTS, 2,4-PD and nonane were each tested at six different doses in a four cups choice set up against controls. Gravid *An. gambiae s.s* (48 h post blood feeding) were placed in a 30 x 30 x 30 cm cage. In each cage cups containing 30 ml of a solution of the chemical in concentrations of 10^{-7} M, 10^{-8} M, 10^{-9} M and control, or in concentrations of 10^{-10} M, 10^{-11} M, 10^{-12} M and control were placed. Each of the four oviposition cups was placed in a corner of the cage. Mosquitoes were given a 6% glucose solution as an additional food source. The determination of the most effective concentration was based on the total number and percentage of eggs found in both control and treated cups after 36 h (two nights).

Dual choice experiments with selected doses - Based on the results from the dose-response test, dual choice experiments were performed with single compounds. The following concentrations of single compounds were tested against respective controls:

(a) DMDS: 10^{-7} M and 10^{-9} M
(b) DMTS: 10^{-9} M and 10^{-11} M
(c) 2,4-PD: 10^{-10} M
(d) nonane: 10^{-11} M

Determination of oviposition activity and egg retention - To ascertain the effect of emitted infochemicals as either attractive or repellent, an oviposition activity index (OAI) was calculated using the formula OAI = (Nt-Nc)/(Nt+Nc) (Kramer and Mulla 1979), with Nt = number of eggs laid in the egg cup with larvae or test compound, and Nc = number of eggs oviposited in the cup with control materials. Individual gravid *Anopheles coluzzii* females were exposed to emanations of either 100 first or 100 fourth instars; individual gravid females of *An. gambiae s.s.* were exposed to nonane (10^{-11}M), 2,4-DP (10^{-10}M), DMDS (10^{-7}M) or DMTS (10^{-9}M), respectively. Each treatment was replicated 17 times. At the end of the dual-choice experiments in the laboratory, females were killed and the status of their ovaries was examined for egg retention by dissection of the ovaries. The abdomen of the female was placed on a glass slide, opened with fine surgical forceps and the ovaries were gently pulled out and placed in a drop of physiological saline. The ovaries were examined at 400x magnification under a dissecting microscope. The number of mature eggs present per female were counted (Takken et al. 2013).

Semi-field oviposition experiments - The effects of DMTS at a concentration of 10^{-11} M, DMDS at 10^{-7} M, nonane at 10^{-11} M and 2,4-PD at 10^{-10} M on oviposition response were investigated against their controls (distilled water+methanol+Tween20) in a dual choice assay in a semi-field situation (mosquito spheres) at Muheza in Tanzania, under natural ambient conditions (Knols et al. 2002). The objective was to scale up the exploration into a field situation and compare laboratory with semi-field results. Three mosquito spheres (11.4 x 7.1 x 5.0 m) were used in this study (Fig. 2). During the experimental period, the average temperatures in the spheres ranged from a minimum of 16°C during the night to a maximum of 32°C during the day. The average relative humidity (RH) ranged from a minimum of 40% to a maximum of 100%.

Figure 2 - Mosquito sphere at Muheza in which semi-field oviposition studies took place. The sphere had a small house, banana plants, ground vegetation and 2 oviposition bowls in front of the house.

Two symmetrical holes were dug in the ground at the centre of each sphere, and were located 3 m apart. A green plastic bowl (diameter 26 cm, height 10 cm) was placed in each hole as an artificial breeding site. The bowls were placed in such a way that the rim of the bowls was at ground level. The bowls had a capacity of 5 l and were filled with 3 l of the test solutions of the concentrations mentioned above or with distilled water.

A total of 240 mosquitoes (*An. gambiae s.s.*) were given an opportunity to blood feed twice, on day 3 and day 4 after emergence, and were released on day 5, when eggs had matured (Takken et al. 1998). Mosquitoes were released one hour before dusk (at about 18:00 h), from the centre of the sphere between the bowls. Eggs were counted on the first and second morning after releasing mosquitoes and the solutions were replaced after every experiment. The total number of eggs after two nights was taken as the oviposition response. Each pair in this experiment was replicated 17 times.

Data analysis - Differences in oviposition preferences of mosquitoes were analysed using the Wilcoxon matched-pairs signed rank test and Mann-Whitney test for matched-pairs. This non-parametric test was used because the data were not normally distributed. To compare more than two paired groups, like with the dose response test, the Friedman test was used.

Analysis of OAI data was done by comparing the response value with zero. When the OAI values differed significantly from zero with positive or negative values, the treatment was considered to have significant attractant or repellent effect, respectively, on oviposition behavior of gravid females. Oviposition preference of gravid females was determined by OAI values using the Wilcoxon signed rank test ($\alpha = 0.05$, two-sided). The OAI was also used to compare behavioral assays involving larvae and chemical assays involving identified infochemicals.

The amount of volatiles quantified in headspace collections was analysed using the Kruskal-Wallis test. Differences in egg retention were analysed using the Mann-Whitney U-test.

All tests were performed in SPSS, version 20 (IBM, Armonk, NY, USA).

Ethical clearance - The study was conducted according to Standard Operating Procedures approved by the Medical Research Coordinating Committee (MRCC) of the National Institute for Medical Research (NIMR), Tanzania. It received a research permit from MRCC with reference number NIMR/HQ/R.8a/Vol. IX/573 and a permit from the Tanzania Commission for Science and Technology with reference number CST/RCA 138/225/2008. Ethical approval for blood feeding was not requested as this method of blood feeding is not subject to the Dutch Act of Medical Research involving Human Subjects (WMO).

Results

Oviposition in response to larvae of different development stages - Significantly more eggs were deposited in cups containing L1 larvae than in control cups (p < 0.0001, Wilcoxon signed rank test), with a median (± SE) of 31.0 ± 2.2 over 0 in control, respectively (Fig. 3). When given a choice between 100 L4 larvae and a control, mosquitoes deposited more eggs in the control cups (p < 0.0001, Wilcoxon signed rank test), *i.e.* median of 0 in the cups with L4 larvae compared to 32.0 ± 3.0 in the control cups (Fig. 3). When two cups with water were tested, there was no difference in the median number of eggs per cup (17.0 ± 3.6 and 19.0 ± 3.8 eggs, respectively).

Collection and identification of infochemicals - Analysis of the headspace extract from water containing larvae by GC-MS showed that four out of sixteen volatile compounds that showed a difference between control, early stage larvae and late stage larvae were found (Fig. 4). The compounds were identified by matches with database spectra, and by matching their retention times and mass spectra to standards of synthetic compounds. After analysis, only two of these compounds were significantly different in abundance between the treatments. Dimethyl disulfide (DMDS; p = 0.021, Kruskal Wallis, n = 5) and dimethyl trisulfide (DMTS; p = 0.006, Kruskal Wallis, n = 5) were collected in higher amounts from cups containing late-stage larvae than from the control cups or cups containing early-stage larvae (Fig. 5a, b). Nonane (p = 0.275, Kruskal Wallis, n = 5) and 2,4-PD (p = 0.081, Kruskal Wallis, n = 5) were equally abundant in the headspace from early-stage and late-stage larvae, but differed from the control (p < 0.05). There was no significant difference in abundance of nonane and 2,4-PD between the cups containing early-stage and late-stage larvae (Fig. 5c, d). These four compounds were selected as putative chemicals influencing oviposition because of their marked greater abundance compared to control.

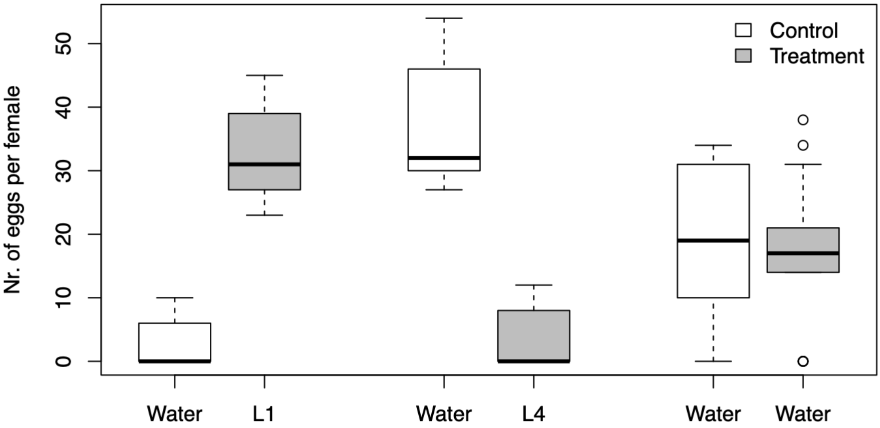

Figure 3 - Number of eggs laid by *An. gambiae s.s.* in a dual-choice test with first and fourth instars. i) a cup containing 100 first instars against a control cup, ii) a cup containing100 fourth instars against a control cup, iii) two cups containing distilled water. Asterisks indicates significance between treatment and control (***, P< 0.001, Wilcoxon signed rank test).

Dose response effects - No significant differences were found in the number of eggs between the different concentrations and control (p > 0.05, Friedman test, $N_{\text{[all chemicals tested]}}$ = 12). The concentration of DMDS that received fewest eggs was 5.5 x 10^{-7} M with an Oviposition Activity Index (OAI) = -0.78, average percentage ± SE of 6.5 ± 6.46% of the total number of eggs oviposited and was selected as concentration used in the follow-up experiments. In the second set of concentrations of DMTS (ranging from 5.5 x 10^{-10} – 5.5 x 10^{-12} M) mosquitoes in two of the three cages did not oviposit at all. At the concentration 5.5 x 10^{-11} M, oviposition bowls received fewest eggs with an OAI equal to -0.17, and this concentration was used for further experiments (Fig. S1).

Differences in oviposition response between the tested concentrations of nonane were small for both ranges and the concentration of. 5.5 x 10^{-11} M with an average percentage of 38.9, mean 72.7 ± 40.3, OAI = +0.21 was selected for further study. When testing 2,4-PD, the concentration of 5.5 x 10^{-10} M received most eggs, with an average percentage of 47.4, mean 52.3 ± 28.3 and OAI of +0.42. These concentrations were selected for further experiments.

Dual choice experiments in the laboratory with selected chemicals - In the laboratory in Muheza, differences in number of eggs laid between treated and control cups were not significant for any treatment (Table 1, Wilcoxon signed rank test). Only 47% and 44% of the mosquitoes exposed to DMDS and DMTS, respectively, developed eggs and oviposited compared to 100% in the control.

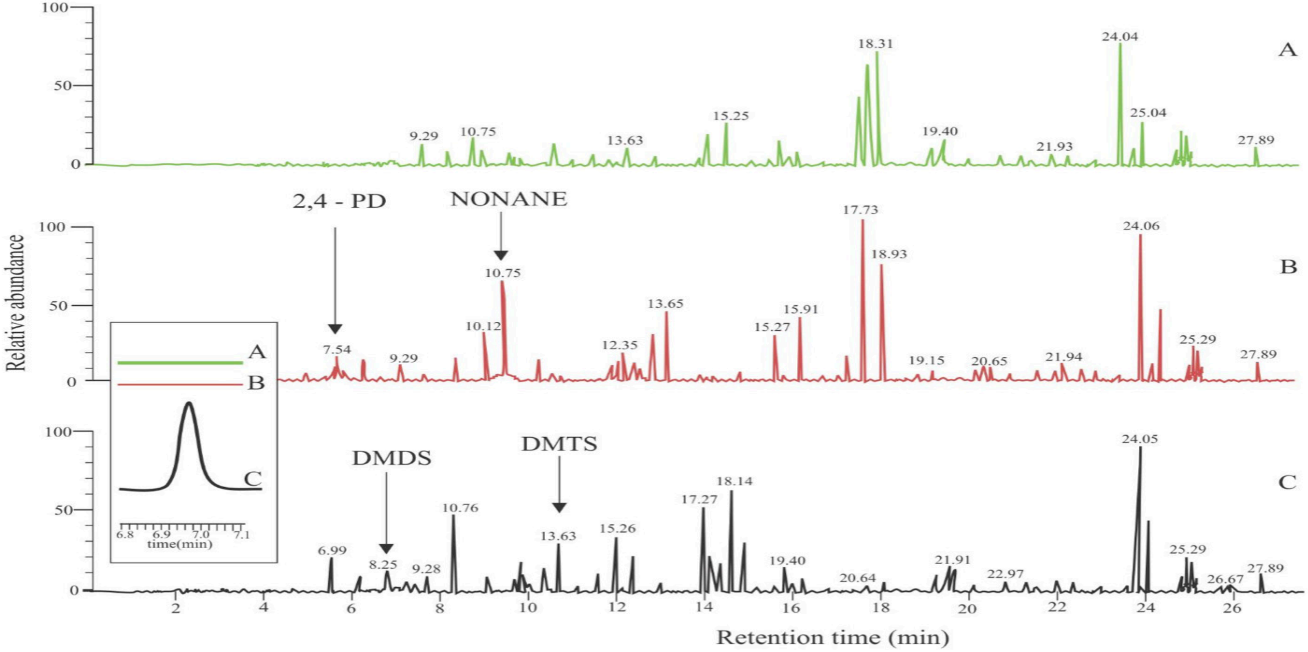

Figure 4 - Partial chromatograms for headspace from water containing larvae and control. Volatile samples were entrapped from control water (A) and water with either of two different larval stages, i.e. early stage (L1) (B) and late stage (L4) (C). A mass range zoom (m/z=94) representing DMDS is added. Y-axis represents equal relative abundances of the different analyses, normalized for the most abundant TIC signal. Peaks for 2,4-pentanedione, nonane, dimethyl disulfide and dimethyl trisulfide are labeled

Table 1 - Results of oviposition response and examination for egg retention of female *An. gambiae s.s.* exposed to selected concentrations of DMDS, DMTS, nonane and 2,4-pentanedione in a dual-choice set up against distilled water

Treatment	Dose	N	Mean no. ± SE of eggs per female			% females that oviposited	Mean no. ± SE retained eggs per female**	
			Treatment	Control	*P**			
DMDS	$5.5*10^{-7}$	17	13.41 ± 5.66	18.12 ± 7.51	0.647	47	52.06 ± 14.43	b
DMTS	$5.5*10^{-11}$	16	4.69 ± 2.48	8.88 ± 5.45	0.799	44	37.13 ± 9.02	b
Nonane	$5.5*10^{-11}$	17	24.00 ± 10.27	47.29 ± 11.29	0.147	88	15.90 ± 10.95	a
2,4-Pentanedione	$5.5*10^{-10}$	14	32.86 ± 10.32	36.43 ± 9.47	0.861	100	6.92 ± 6.92	a
Water (=control)		10	48.30 ± 15.59	19.90 ± 9.62	0.241	100	2.20 ± 2.20	a

N = number of replicates with one female per dual-choice test.

* Wilcoxon signed rank test

** Differences in letters behind each value indicate a significant difference between the mean number of reatined eggs per female (*P*= 0.007 for DMTS, Mann-Whitney U-test)

The remaining mosquitoes of the DMDS and DMTS treatments had fully developed eggs, but did not oviposit. As a result, the number of eggs that were retained by mosquitoes exposed to the methylsulfides were significantly higher than of mosquitoes in cages with water only ($p = 0.018$ for DMDS and $p = 0.007$ for DMTS, Mann-Whitney U). For nonane, 2,4-PD and the control experiments, the percentages of mosquitoes that oviposited were 100%, 88% and 100%, respectively. The number of eggs retained by mosquitoes exposed to nonane and 2,4-PD were not different from the control.

Semi-field experiment - There were marked differences in the oviposition effects of DMTS and DMDS on the one hand, and nonane and 2,4-PD on the other hand (Fig. 6). Bowls treated with DMDS or DMTS received significantly fewer eggs than the controls (for DMDS: $p < 0.0001$, n = 17, Median$_{[DMDS]}$ = 154; Median$_{[control]}$ = 341; for DMTS: $p = 0.049$, n = 17, Median$_{[DMTS]}$ = 35; Median$_{[control]}$ = 353. The bowl treated with nonane received significantly more eggs than the respective controls ($p < 0.0001$, n = 17, Median$_{[nonane]}$ = 958; Median$_{[control]}$ = 384. Likewise, the bowl treated with 2,4-PD received significantly more eggs than the respective controls ($p < 0.001$, n = 17, Median$_{[2,\ 4\text{-}PD]}$ = 726; Median$_{[control]}$ = 406. The oviposition response to control treatments was similar between the different experiments, indicating consistence in results between different experiments (Fig. 6). The OAIs for nonane and 2,4-PD were positive, indicating stimulation of oviposition activity whereas those for DMDS and DMTS were negative, indicating inhibition of oviposition activity in the presence of these infochemicals.

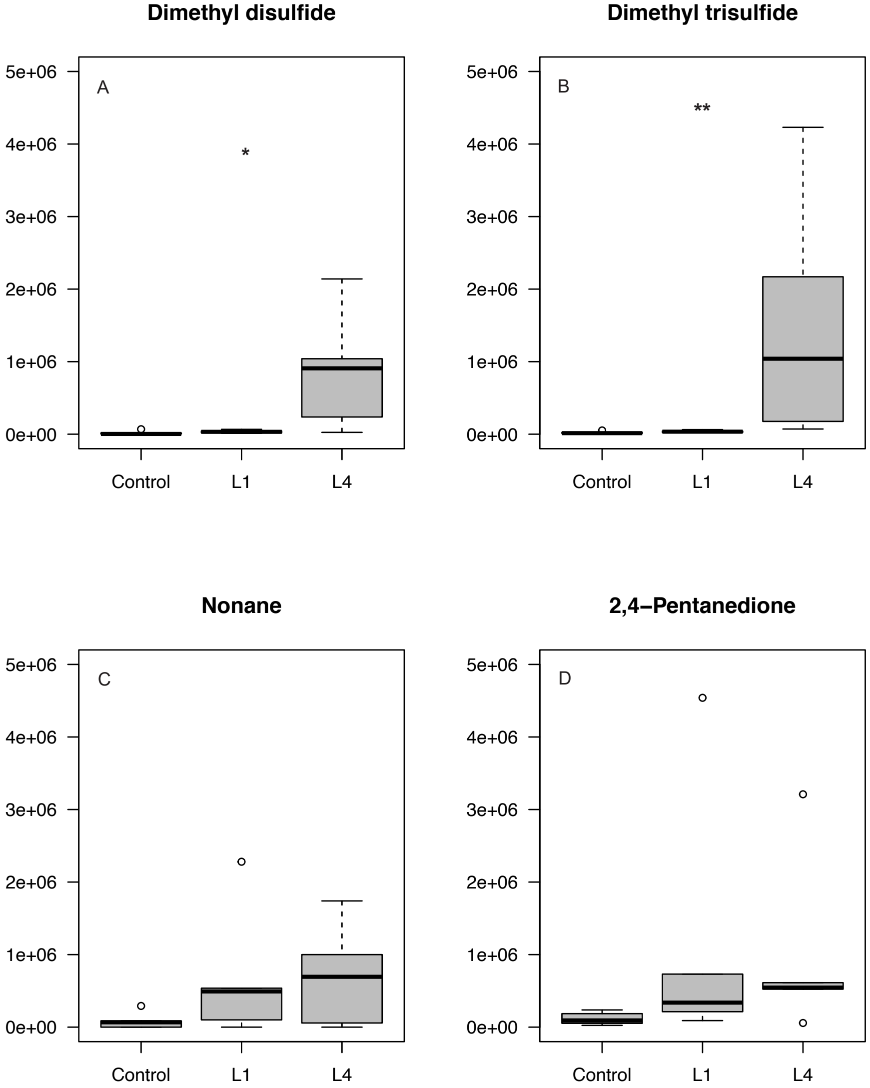

Figure 5 - Relative abundance of the selected chemicals in control cups, cups with early stage (L1) and cups with late stage (L4) larvae. Graphs show mean abundances of DMDS (a), DMTS (b), nonane (c) and 2,4-PD. Asterisks indicates significant RA value different from control (*, P<0.05, **, P< 0.001, Kruskal Wallis, N=5).

Oviposition activity with live larvae and selected chemicals - The oviposition activity in the presence of early and late stage larvae was compared to oviposition activity in the presence of infochemicals entrapped from early and late stage larvae. The oviposition activities of mosquitoes followed a similar trend in response to L1, nonane and 2,4 PD with a positive value that indicates stimulation of oviposition activities (Fig. 7). The median oviposition activity was highest with L1 (OAI = 1.00 ± 0.06, n = 17, $p < 0.0001$) followed by nonane (OAI = 0.36 ± 0.09, n = 17, $p < 0.0001$) and then 2,4-PD (OAI = 0.28 ± 0.07, n = 17, $p < 0.001$). Also, the oviposition activities in response to L4, DMDS and DMTS followed a similar trend, with a negative value suggesting inhibition of oviposition activities (Fig. 7). Fourth instars had the lowest OAI values, (median OAI = -1.00 ± 0.06, n = 17, $p < 0.0001$) followed by DMTS (median OAI = -0.85 ± 0.19, n = 17, $p = 0.0001$) and DMDS (median OAI = -0.4 ± 0.03, n = 17, $p < 0.0001$).

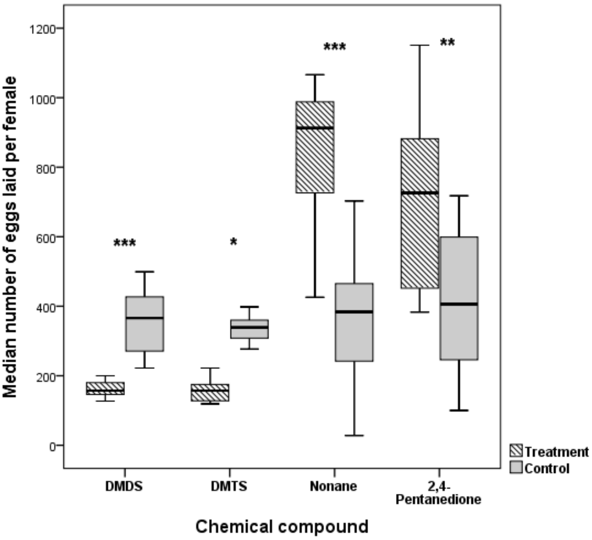

Figure 6 - Oviposition response of *Anopheles gambiae,* expressed as the median number of eggs laid per female, when tested in a dual-choice essay with dimethyl disulfate (DMDS), dimethyl trisulfate (DMTS), nonane or 2,4-pentanedione against distilled water. Asterisks indicate significant differences in number of eggs in treatment versus control (*, P<0.05, **, P< 0.001, ***, P<0.0001; Wilcoxon signed rank test).

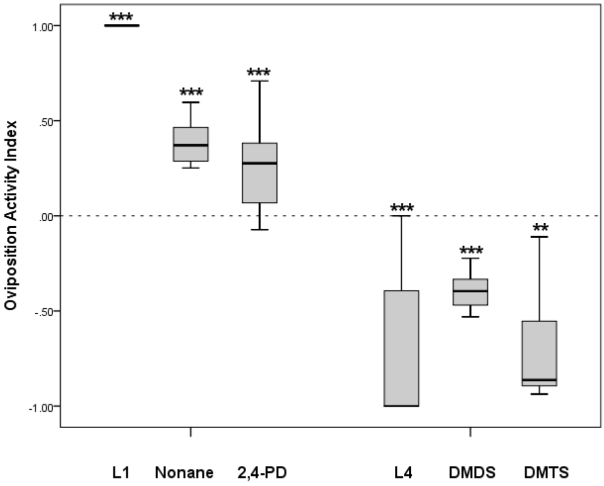

Figure 7 - Oviposition activity index (OAI) of gravid *Anopheles coluzzii* exposed to first and fourth instars,
A) and gravid *Anopheles gambiae s.s.* exposed to nonane, 2,4-pentanedione, dimethyl disulfate or dimethyl trisulfate tested in a dual-choice assay. Asterisks indicates OAI value significantly different from zero (**, P< 0.001, ***, P<0.0001; Wilcoxon signed rank test).

Discussion

Oviposition behavior of *An. gambiae* females is affected by volatile chemicals associated with conspecific larvae, where first instars affect oviposition positively, and fourth instars cause deterrence and even inhibition of oviposition. Nonane and 2,4-PD are putative attractants, and in a semi-field setting water bodies containing nonane or 2,4-PD received significantly more eggs than untreated water. DMDS and DMTS acted as oviposition repellents and caused egg retention. The data suggest that the oviposition response of these anophelines is mediated by infochemicals associated with conspecifics and that instar stage has a strong impact on this behavior.

Several studies have shown the role of breeding-site specific chemical volatiles affecting oviposition behavior of different members of the *An. gambiae* complex, apart from the role of water vapour as a general cue for all mosquito species (Okal et al. 2013). Indole has been identified to act as an attractant for *An. gambiae*, originating from larval water (Blackwell and Johnson 2000). Lindh et al (2008) identified 13 putative oviposition attractants derived from bacteria in breeding water. More recently, cedrol was identified as an oviposition attractant for *An. gambiae s.s*. (Lindh et al. 2015). The compound was associated with water derived from a natural breeding site and shown to be the product of rhizomes of the grass *Cyperus rotundus* (Eneh et al. 2016b). This finding is in line with other studies which also showed a strong association of breeding site water, including infusions of plants growing in the water, and oviposition attraction of various members of the *An. gambiae* complex (Herrera-Varela et al. 2014, Asmare et al. 2017). Other identified chemical cues mediating oviposition behavior in *An. coluzzii* mosquitoes include 2-propylphenol and 4-methylcyclohexanol (Rinker et al. 2013). Recently, DMDS, DMTS and 6-methyl-5-hepten-2-one (sulcatone) were identified from headspace analyses derived from habitats that repelled *An. coluzzii* (Suh et al. 2016), and were considered as putative oviposition repellents. This is corroborated by the findings of our study, where DMDS and DMTS caused a significant reduction in oviposition response.

Insects have evolved a wide range of hydrocarbons to protect against dehydration, which had the potential to become signalling molecules involved in communication. Therefore, most insects produce straight chain n-alkanes (Gibbs 1998) which may serve as water-proofing agent, communication and signalling compound (Hölldobler and Wilson 2009). For a hydrocarbon to act as an infochemical from a distance, it must be volatile (Drijfhout 2009), and nonane (C_9H_{20}) fulfils this criterion. Across the range of environmental temperatures at which mosquitoes are active, nonane is a hydrocarbon which volatilizes easily, hence apt to convey information on suitability of a breeding site to gravid mosquitoes.

Behavioral effects of nonane and 2,4-PD on insects have not previously been reported to our knowledge. Recently, the attraction of gravid female *An. arabiensis* to sugarcane pollen was described and the mosquitoes expressed a positive response to headspace extracts of pollen. Among many headspace volatiles of sugarcane pollen, nonane was identified, but the compound did not elicit an EAG response in *An. arabiensis* (Wondwosen 2016). Nonane has been described to show a slightly increased emission by glass beads contaminated with odors of a person less attractive to the yellow-fever mosquito *Aedes aegypti* (L.) than by a more attractive person. However, hydrocarbons generally contribute little to the overall attraction of host-seeking females of this species (Bernier et al. 2002) and a bioactive role of nonane in mosquito host seeking remained unconfirmed.

The OAI of the diketone 2,4-pentanedione is similar to the results obtained with various ketones studied earlier in different mosquito species (Knight and Corbet 1991). The ketones generally cause a positive ovipositional response (Ganesan et al. 2006). Moreover, the diketone 2,4-pentanedione, is liable to keto-enol tautomerism, which is a process of migration of an atom within the same organic molecule, leading to a change in its structural skeleton, electron density distribution and chemical properties. 2,4-PD undergoes prototropic tautomerism and exists in equilibrium with its enol tautomer and differs just in the location of a double bond and a hydrogen atom (proton) which often migrates. Tautomers are the chameleons of chemistry, capable of changing by a simple change of phase from an apparent established structure to another and then back again when the original conditions are restored (Antonov 2013). Tautomers are interesting because their optical properties make them suitable as signalling molecules in sensors as they can rapidly switch between states. Many biologically important molecules have several tautomers, among which attractants which are used for luring insects (Pickett 1990). Our finding that in a semi-field setup nonane and 2,4-PD elicited high oviposition activity suggests that both compounds may be used for mosquito surveillance and/or control, as odor baits in oviposition traps (Mboera et al. 2000b, Dugassa et al. 2016, Li et al. 2016).

In our study, both DMDS and DMTS had a negative effect on oviposition of *An. gambiae s.s.* and were collected only from the headspace of fourth larval instars, whereas nonane and 2,4-DP were found in the headspace of both first and fourth instars. Most mosquitoes did not oviposit when DMDS or DMTS were present in the cages, neither in the treated cup nor in the control cup. However, the possibility of saturation of air by these chemicals should not be ignored – as the size of the cage may have had an effect on this outcome. DMDS and DMTS are emitted by a broad range of natural sources; both are produced by bacteria (Khoga et al. 2002). DMDS can be found in human feces (Moore et al. 1987) and both compounds are known to be emitted by plants (Du and Millar 1999, Stensmyr et al. 2002, Soler et al. 2007). Insecticidal and repelling properties of both DMDS and DMTS have been previously described. DMDS has been shown to be an effective insecticide against termites (Dugravot et al. 2002) and cockroach species (Dugravot et al. 2003). Our observation that DMDS and DMTS both in the laboratory and semi-field caused strongly reduced oviposition, confirms work by others who reported oviposition deterrence in the presence of these compounds (Suh et al. 2016). Like in our study, Suh et al. (2016) studied the effect of suboptimal larval habitats of *An. coluzzii* in a laboratory bioassay, and identified DMDS and DMTS in the headspace of water that had been pre-conditioned with late-stage larvae. However, here we show that these compounds also induce deterrence in the semi-field, suggesting the important role of these compounds in natural ecosystems. Sulcatone was also identified in the headspace collections by Suh et al. (2016) and shown to cause oviposition deterrence. Unlike DMDS and DMTS, in the present study sulcatone was not identified to be significantly associated with the presence of mosquito larvae. The difference in results between Suh et al. (2016) and our study is likely to be due to different rearing conditions. It is interesting that capitate peg sensilla of *An. coluzzii* were activated when exposed to DMDS, DMTS and sulcatone, providing physiological indications that the oviposition deterrence is mediated by the olfactory system (Suh et al. 2016).

With the exception of 2,4-PD, optimal dose ranges for an effect on oviposition were lower than 10^{-10}M. For nonane, a dose of 10^{-7}M produced a lower oviposition response, while that of 5.5×10^{-9}M was highly attractive. The dose ranges of DMDS and DMTS with most effect on oviposition were between 10^{-7} M and 10^{-9}M, which was in the same range as reported by Suh et al. (2016). The dose-response results demonstrate that testing doses over a wide range is crucial for assessing the potential impact on behavior.

The results of the dual-choice tests with the individual chemicals in the laboratory did not match those from the semi field, where nonane and 2,4-DP elicited high oviposition, and DMDS and DMTS suppressed oviposition. As the results with the controls in the laboratory study were highly skewed, and the experimental sets produced outcomes with high standard errors, it is possible that positioning of the experimental cages may have caused a bias in the results. As most females exposed to nonane and 2,4-DP laid eggs, similar to the controls, and females exposed to DMDS and DMTS expressed high egg retention, we conclude that the tested chemicals affected the oviposition behavior in the same way as observed in the semi field study.

The presence of the oviposition attractants nonane and 2,4-DP in the headspace of water bodies containing both larval stages, and the repellents DMDS and DMTS in those containing older larvae only, suggests that the positive effect of compounds emitted by first instars is masked by DMDS and DMTS in fourth instars. The masking effect of chemical compounds has been suggested in host-seeking *Ae. aegypti* females by Logan et al. (2008), and for *An. gambiae* (including both siblings *An. coluzzii* and *An. gambiae s.s.*) serves as a mechanism to prevent oviposition where late-stage larvae are present. Given the close genetic relationship between the members of the *An. gambiae* complex, the data suggest that these oviposition-mediating chemicals are present in the entire complex.

Our results show that oviposition by *An. gambiae* is influenced by chemical compounds associated with conspecific larvae and that the oviposition response is dependent on the stage of the larvae present in the oviposition site. The behavior of mosquitoes in response to larvae present in oviposition sites is consistent with the behavior described in our earlier study (Mwingira et al., 2019). Early-stage larvae attract gravid mosquitoes that oviposit, whereas late-stage larvae repel them, and both behaviors are mediated by infochemicals, nonane and 2,4-DP as oviposition stimulants, and DMDS and DMTS as repellents. This phenomenon may affect larval site selection strategies within mosquito populations, and could have an important biological effect on mosquito populations such as competition between species (Koenraadt and Takken 2003). It has been found that larvae of different females of *An. gambiae* were sharing the same habitats, suggesting aggregation by different parent mosquitoes (Chen et al. 2006, Chen et al. 2008). Oviposition sites contain a spectrum of factors influencing oviposition behavior like water type (Sumba et al. 2008), food quality and quantity (Munga et al. 2006). The role of conspecific larvae and other biotic or abiotic factors in oviposition site selection needs to be further explored. Evasion of habitats with L4 larvae by gravid females in the field has not been reported to date.

In conclusion, our results indicate that the attractive effects of chemicals associated with early-stage larvae are cancelled out by the chemicals that are associated with late-stage larvae, presumably DMDS and DMTS. Nonane and 2,4-DP, identified in the headspace of anopheline larvae, elicited a strong oviposition response under semi-field conditions. To our knowledge, this is the first report of identified oviposition attractants associated with anopheline larvae *in-vivo*. Our observation that conspecific larvae and chemicals associated with them mediate oviposition behavior warrants further studies, especially under field situations. Compounds with a positive effect on breeding-site selection are interesting as potential candidates for applications in ovitraps (Paz-Soldan et al. 2016). The combined use of nonane and/or 2,4-PD and DMDS and/or DMTS in traps can provide a push-pull system, in which mosquitoes are repelled by DMDS and/or DMTS volatilized from dispensers placed in the vicinity of houses and attracted by nonane and/or 2,4-PD, applied in traps positioned just outside of villages.

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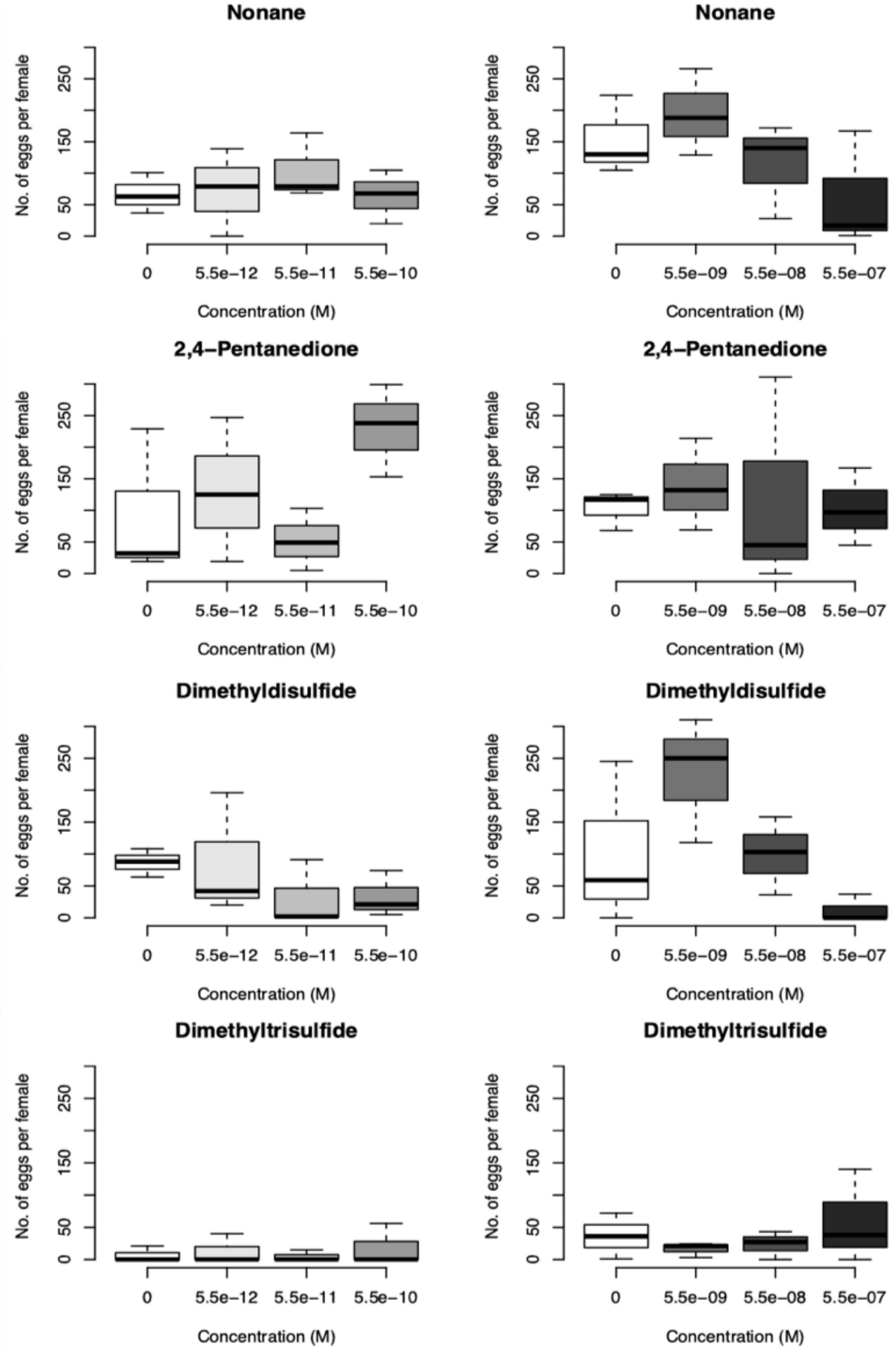

Figure S1 - Number of eggs oviposited by females of *Anopheles gambiae s.s.* in cups containing different concentrations of nonane, 2,4-pentanedione, dimethyldisulfide and trimethyldisulfide. Each treatment was replicated 3 times.

Natural mosquito breeding sites at Mvomero district

Chapter 5

Characterisation of larval habitats for malaria mosquitoes in relation to land use patterns in Mvomero district, Tanzania.

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Leonard E.G. Mboera,
Willem Takken.

To be submitted

Abstract

The development of agricultural water resources influences human settlement in farming areas and alters the environment by making it more suitable for mosquito production. Malaria mosquitoes exploit these habitats and reproduce rapidly due to availability of blood meals from humans and their livestock, which in turn affects human health.

An entomological survey was carried out in various villages of Mvomero district, Tanzania. Larval sampling was done in all available water bodies in selected transects of the study area by a dipping technique. Adult mosquitoes were sampled monthly using Centre for Disease Control (CDC) light traps in ten houses per village.

The presence of anopheline larvae coincided with the presence of anopheline adults caught in the houses. The dynamics of breeding sites and larval density over time was influenced by rainfall. The number of breeding sites and the occurrence of mosquito vectors were larger in the rice producing villages than in areas with different crop systems. Additionally, the presence of brick pits caused a high production of malaria vectors. Exposure to sunlight, shallow water, submerged grassy vegetation and co-habitation with culicine larvae were positively associated with the presence of anopheline larvae, while shade, water pollution, predators and running water were negatively associated with anopheline larvae. The results demonstrate that human-activities and land use have a strong influence on the presence and dynamics of mosquito populations.

KEYWORDS: Brick pits, rice field, anophelines, culicines, larvae, breeding sites, oviposition

Introduction

Malaria and other vector-borne diseases are major contributors to the global burden of disease and a significant impediment to socio-economic development in poor countries (WHO 2005, 2017). It is estimated that 200 to 250 million clinical cases of malaria per year occur globally which results in at least 435,000 deaths (WHO 2018). Over 80% of these deaths occur in Africa where malaria is transmitted primarily by *Anopheles gambiae* s.l. and *Anopheles funestus*. These mosquitoes can easily adapt to man-made habitats that result from human activities.

In most African village settings, man-made mosquito habitats are in close proximity to human dwellings; this enhances stable malaria transmission throughout the year (Mboera et al. 2010a). Human activities which are conducive to the formation of mosquito habitats include: traditional rice irrigation (Service 1989) and mud-brick making for house constructions. Such activities result in the creation of new mosquito breeding sites, and are usually coupled with demographic changes and thus altered human-vector parasite contact patterns (Keiser et al. 2005). In principle, proximity to an irrigation scheme implies proximity to water bodies that can serve as mosquito larval development sites (Klinkenberg et al. 2003a).

Malaria is endemic in most areas under irrigation because climate and environment together present conditions suitable for malaria vectors and parasite proliferation (Okech et al. 2004). Out of all crops under irrigation, rice is considered to pose the greatest danger to health as it is grown in flooded condition, which provides ideal breeding sites for malaria mosquitoes (Ijumba and Lindsay 2001a, Diakite et al. 2015). Mvomero district is one of the rice producing areas in Tanzania, and is characterized by having several broad valleys and rivers that flow from the mountains throughout the year. This area is highly suitable for mosquito production (Mboera 2007, 2010) due to the presence of high temperature and humidity, availability of breeding water in irrigated fields, as well a nearby blood source from humans and domesticated animals.

The level of water in the fields and the period during which water is allowed on the paddies has an impact on the chemistry and stability of the paddies in supporting development of mosquito larvae (Klinkenberg et al. 2003a). The use of industrial (Mutero et al. 2004) or organic fertilizers (Allan et al. 2005) makes the rice fields more attractive to ovipositing females' mosquitoes. Moreover, the practice of processing the harvest in the rice fields leaving out rice straws which eventually decompose is common in Mvomero district. Rice straw infusions are known to produces volatile chemicals that attract gravid *Culex* mosquitoes to lay eggs in a similar way to other plant infusion (Jackson et al. 2005).

It is generally thought that the abundance of sunlit and shallow bodies of water makes rural populations especially vulnerable to increasing contact with mosquitoes (Keating et al. 2005) as larvae of *An. gambiae* frequently occur in small and open aquatic habitats (Service 1993, Minakawa et al. 1999). Just like flooded rice, brick making activities provide ideal breeding sites for members of the *An. gambiae* complex (Carlson et al. 2004, Mboera 2013). Brick making results into numerous small and medium sized habitats that contain brown coloured water and are preferred by ovipositing gravid females of *Anopheles gambiae* s.l. in order to avoid predators (Tuno et al. 2004). Mosquitoes are able to estimate competition (Silberbush et al. 2014), and predation risks among prospective breeding sites (Silberbush and Blaustein 2008). Small and medium sized habitats such as brick pits do not contain a full range of predators and competitors; as a result, they are preferred and colonized easily by ovipositing *An. gambiae* mosquitoes.

The objective of this study is to characterize potential larval habitats of malaria mosquitoes in relation to land use patterns across the seasons. Specifically, the aim is to (i) identify larval breeding sites and species composition of anopheline mosquitoes, (ii) estimate mosquito larval density and productivity, (iii) assess the influence of physical characteristics of breeding sites on mosquito productivity, (iv) assess the influence of land use on mosquito larval productivity, (v) identify environmental factors associated with anopheline breeding sites, and (vi) assess the influence of rainfall on mosquito abundance.

Methodology and study design

Description of the study areas

The study was carried out in Mvomero District, which is located about 350 km west of Dar es Salaam. The study area ranges from 6º 06' to 06º 19'S and 37º 38' to 37º 32'E with a total area of 7,325 km^2 (Figure 1). The district has an estimated human population of 260,000 predominantly of the Nguu and Zigua ethnicity, and has an average growth rate of 2.6%. Rainfall is high and bimodal (March–May and October–December) with a relatively short dry spell between June and September. The average annual rainfall is over 1000 mm and the mean maximum temperature is 31 ºC, whereas the mean minimum temperature is 17 ºC. The area is characterized by five land use classes namely: farmland, settlements and combined class of woodland, shrub and thicket, continuous rain forest, and patches of rain forest. The study area has been described extensively by Mboera *et al.* (2007, 2010).

The district is located in the foothills of Nguru Mountains to the north-west and Uluguru Mountains to the South-East. The study area lies at an altitude ranging 290 to 380 m above sea level across the Wami river basin. The main economic activities are agriculture, livestock keeping and fishing. Fishing is practised mainly by the communities living along the major rivers of Wami and Divue, but also other villagers produce fish by using man-made ponds. The economy of Mvomero district depends heavily on agriculture mainly from crop production.

Crops such as cereals, legumes, fruits and vegetables are grown during the long rains (March to June) and short rains (September to November). The flat river basins are well known for irrigated rice production which takes place throughout the year by utilizing water from the existing rivers. These water bodies also serve as mosquito breeding sites which are extended throughout the year due to irrigation. The intensive crop production has facilitated population increase, human settlement and growth of towns in the district. Therefore, the demand for construction materials and related activities such as brick making has increased drastically.

Figure 1 - A map of Tanzania showing the location of Mvomero district, which has four divisions, located between 6˚06’ to 06˚ 19’’ S and 37˚38’ to 37˚32’ E and covers a total area of 7,325 km^2

Due to the above features, malaria transmission is intense throughout the year and peaks during and after the rain seasons. The major malaria vectors in the area are *An. gambiae* s.l. (58.9%) and *An. funestus* (12.0%). Malaria is prevalent throughout the year, with *Plasmodium falciparum* accounting for 97% of malaria species (Mboera 2007). The combined overall sporozoite rate for *An. gambiae* + *An. funestus* was 3.2% and the mean annual entomological inoculation rate for *An. gambiae* s.l. was 728 infective bites per person per year (Mboera 2010).

Six villages were selected for the entomological investigations based on the type of land use, topographic features and agricultural practices (Figure 2). The selected sites include the following features: i) traditional irrigated rice production at Komtonga, ii) improved rice irrigation scheme at Mkindo, iii) brick production sites at Manyinga and Hembeti villages, (v) marshes and man-made ponds at Dihombo and Bwagala sites, respectively. These parameters were correlated to the distribution of breeding sites favourable to mosquito development.

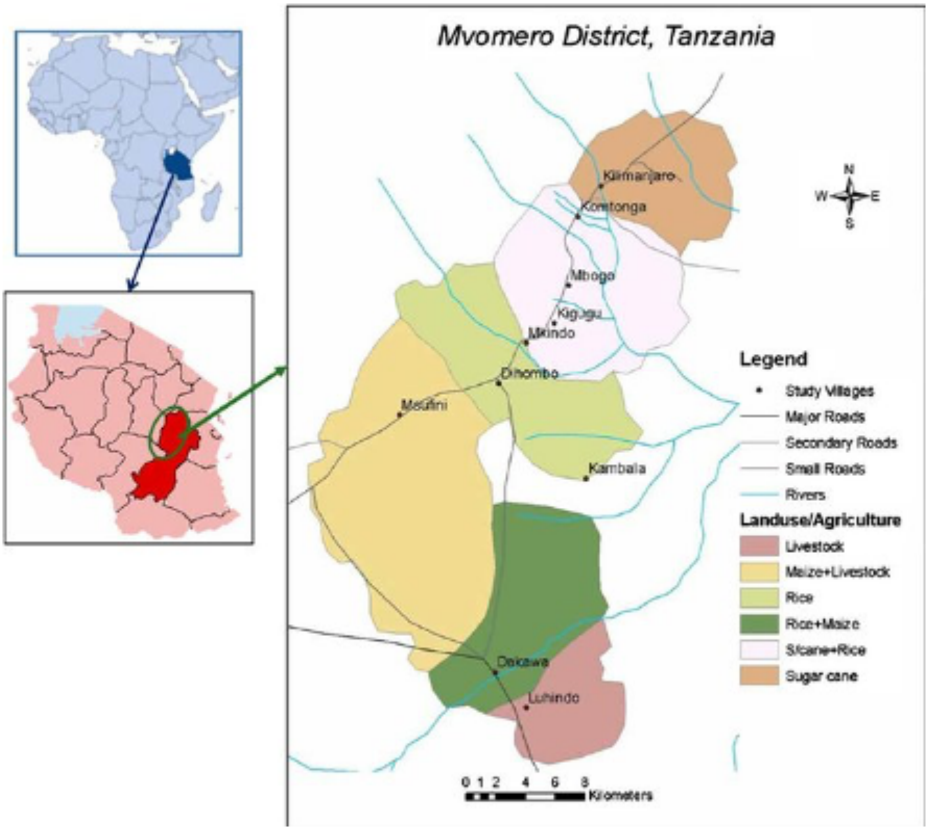

Figure 2 - A map showing the location of the study villages and their dominant types of land use within the Mvomero district, Tanzania (Source: Randel et al., 2010).

Ecological features and land use patterns in the study villages/ hamlets

a) Komtonga

The mosquito-breeding sites in this village are generally characterised as natural swamps lying on the plain basin and river banks. There is small-scale rice farming using the traditional ground flooding irrigation practices. Irrigation water comes from the Divue river which meanders along the plain basin.

b) Mkindo

There is improved rice-irrigation farming in this village where rice paddies are cultivated in bunds. Irrigation employs a gravitational water supply technique from water canals. There is a farmers' field training centre which provides farmers skills on improved water control, management and agronomic practices.

c) Manyinga

This village is situated between Madizini and Turiani towns. Due to the presence of clay soil, and availability of water from a nearby water table, it is famous for its brick production to meet the demand of fast growing bordering towns. Therefore, the majority of breeding sites consist of water-filled brick pits

d) Hembeti

In the past, this village was also involved in brick production due to the presence of clay soil. Most breeding places are natural swamps and ponds resulting from brick making. However, there is also small-scale rice production using canal irrigation.

e) Bwagala

The village is characterised by suburb environment being in vicinity to Mtibwa shanty town. The majority of breeding sites are man-made ponds that are used as drinking water reservoir for livestock and domestic used, natural ponds. However, rice is cultivated annually during the long rains.

f) Dihombo

This village is adjacent to Mkindo and sandwiched between two major trunk roads. The majority of breeding places consist of natural swamps and tyre prints. There is small-scale rice production using canal irrigation and rain.

Sampling frameworks

A monthly survey was conducted to identify and geo-locate all potential habitats in selected villages of Mvomero district (Figure 1). Villages were selected from the map, on the basis of their proximity to the Dakawa-Handeni main road (Figure 2). A regular sampling strategy by using transects was employed, whereby a point was established in which a transect was designed across the point. Each transect had a length of 1 km and a width of 40 m. All open water bodies along the designated transect were searched for potential breeding sites and Geo-referenced by a Global Positioning System (Garmin 12; Olathe, KS, USA).

Identification of potential breeding sites and mosquito species

From every water body within the designated transects, 10 dips were taken evenly with a WHO standard 350 ml white dipper. We recorded the total number of mosquito larvae per dip in every breeding site. In order to distinguish the major families of mosquitoes found in the study area, larvae were collected from the productive breeding sites and raised and hatched separately in the laboratory. Adult mosquitoes were identified morphologically by light microscopy using a morphological identification key (Gillies and De Meillon 1968). Further identification to sibling species within the *An. gambiae* complex was done using the ribosomal DNA-polymerase chain reaction according to van Rensberg et al. (1996).

Estimation of mosquito larval density

The presence of larvae at high or low densities was determined by dipping (see above for description of dipping method). If 7 to 10 dips contained mosquito larvae, the site was regarded as having a high mosquito density. Sites with 1 to 3 positive dips per 10 dips were regarded as having a low density, 3 to 4 positive dips were regarded as medium density and sites without mosquito larvae were recorded as empty.

Estimation of mosquito breeding site productivity

To quantify the relative mosquito larval productivity, a score modified after habitat characterization in Dar es Salaam (Sattler et al. 2005), whereby a number was assigned to each breeding site according to its size and mosquito density (estimated as number of positive dips per 10 dips) (Table 1). This classification was used to characterize anopheline as well as culicine mosquito breeding sites.

Monitoring of adult mosquito density

Adult mosquitoes were sampled monthly in 10 houses for two consecutive days in each of the study villages. Mosquito collections were done using Centre for Disease Control (CDC) light traps (J.W. Hock Ltd. Gainesville, Florida, USA). For operation, a total of 10 traps were set per night per village. Each light trap was hung at the top of the foot-end of the bed with at least one person sleeping under an untreated mosquito net (Mboera et al. 1997). The light traps were set at 18:00h and collected the following morning at 6:00h. Inquiries were made as to whether the trap fan and light had both worked all night long. Catches from faulty traps were not included in the score.

Ten houses were selected randomly in each village and one room in each selected house was sprayed with pyrethrum during the morning hours. Houses were selected from different locations in the village and at least one house from each hamlet was included in the survey. Prior to spraying, white sheets were covered on the floor and on top of the bed or anything placed on the floor. Two people sprayed the room for 10 min, one sprayed inside while another sprayed from outside the room through the eaves. Knocked down mosquitoes were collected and preserved in Petri dishes lined with moist cotton wool and filter paper.

Species identification

All larvae collected were examined morphologically using larval identification keys and grouped into respective families based on preliminary morphological identification. Then they were transferred to the insectary and reared until adult emergence. Newly emerged adults were identified to species level using morphological keys (Gillies and Coetzee 1987). All *Anopheles gambiae* s.l. specimens were preserved in Eppendorf tubes, which contained silica gel, for further identification to distinguish between sibling species in the *An. gambiae* complex. Genotypic identification was conducted by using the ribosomal DNA-polymerase chain reaction (PCR) to separate *An. gambiae s.s.* from

An. arabiensis (Scott et al. 1993). Culicine mosquitoes were identified as *Cx. quinquefasciatus* or other culicines.

Ecological factors associated with anopheline breeding sites

For every water body inspected, the following ecological parameters were recorded; (i) type of vegetation, (ii) presence of mosquito larvae, (iii) presence of predators of mosquito larvae, (iv) pollution of breeding site water, (v) presence of shade, and (vi) water movement. In order to identify factors influencing the presence of anopheline larvae, these parameters were correlated to the presence of anopheline larvae in each breeding site inspected.

Breeding site characteristics and mosquito productivity

To explore the relationship between breeding site characteristics and mosquito productivity, physical characteristics of surveyed water bodies i.e. (i) water depth, (ii) size of site, and (iii) stability, were recorded for all existing water bodies. These data were correlated with species of mosquito found in the site, mosquito larval productivity of the site and type of land use in the area where a water body exist.

Influence of economic activities on mosquito productivity

All potential breeding sites were identified by a unique code number that represents its location and type (rice field, brick pit, etc.). Mosquito productivity was determined for all breeding sites found in the area. The influence of economic activities on mosquito productivity was determined by comparing mosquito productivity between the major economic activities in the area.

Influence of rainfall

This was a longitudinal study, whereby larval and adult mosquito surveys were conducted monthly. The acquired information was correlated with weather data obtained from a local weather station at Mtibwa sugar factory. The frequency of sites with positive and negative anopheline larvae as well the frequency of major malaria mosquito species was monitored and compared with rainfall patterns throughout the year.

Meteorological data

The meteorological data were provided by Mtibwa Sugar cane manufacturing company located within the study area. The factory recorded mean values of temperatures, rainfall and relative humidity each month throughout the year.

Statistical analysis

In our study, various environmental factors were either positively or negatively associated with anopheline larvae. The association between the presence of anopheline larvae in breeding sites and other environmental factors has been determined by relative effect. The relative effect was estimated using an odds ratio which indicated the likelihood that a breeding site with a given environmental factor will contain anopheline larvae.

By using available larval density and productivity for each site, the proportions of variables were calculated for each land use. The differences of larval productivity between types of land use were evaluated for significance by using the Chi-square test. The contingency chi-square test was also used to compare the proportion of the different types of water bodies positive for anopheline larvae.

The effects of depth, size and stability of each breeding site as an independent predictor of number of mosquito larvae found in breeding sites were modelled using generalized linear model (GLM) with a Poisson distribution and log link function

Ethical issues

The study received an ethical approval from the Medical Research Coordinating Committee of the National Institute for Medical Research of Tanzanian and a research permit from the Tanzanian Commission for Science and Technology (CST/RCA 138/225/2008).

Results

Mosquito species composition

A total of 15,876 anopheline larvae and 38,975 culicine larvae were collected. After morphological investigation, 4,026 of the anophelines were identified as *An. funestus*, while 11,850 belonged to the *An. gambiae* complex. Molecular analysis of a subsample of 1200 adult *An. gambiae* s.l., collected in the CDC light traps, revealed that 51% were *An. gambiae s.s*. and 49% *An. arabiensis*.

Characteristics of potential breeding sites

The survey of potential breeding sites of anopheline mosquitoes was conducted from the short rainy season to the long rainy season. A total of 451 permanent potential breeding sites were identified in six villages. These belonged to brick pits (40.8%, n=184), rice fields (38.6%, n=174), hoof prints (5.1%, n=23), water wells (4.9%, n=22), streams (2.9%, n=13), ponds/swamp (2.9%, n=13), broken pots (2.7%, n=12), rock holes (0.9%, n=4), ditches (0.9%, n = 4) and tire print (0.4%, n=2) (Table 2). However, subsequent to 454 mm of rainfall during the long rains, the number of brick pits was reduced from 184 to 38, as most of the valley was temporarily flooded. The number of rice field ponds was also reduced from 174 to 83, hoof prints from 23 to 3, water wells from 22 to 3, and streams from 13 to 2. Swamps and ponds, which are considered as permanent breeding sites in the area, were reduced from 13 during the short rains to ten large water bodies after the long rains. However, several storm water ponds re-emerged, especially in the village of Kambala. Such ponds were preferred by gravid females due to their shallowness (Mwingira 2009).

Economic activities and mosquito productivity

The main economic activities that contributed to the majority of water bodies in Mvomero district are brick making and rice production. Generally, brick pits and rice fields had the highest number of breeding sites for mosquitoes during both short and long rains (Table 2). Moreover, the surface areas of rice fields are larger than the surface areas of brick pits. However, the proportion of sites with mosquito larvae was significantly higher for brick pits than for rice fields ($\chi^2 = 10.5$, $P < 0.001$). In general, Manyinga village had the largest number of water bodies per transect and highest frequency of anopheline-occupied breeding sites (data shown in figures).

Table 1 - Classification of larval productivity in potential mosquito breeding sites

Surface area of site	<1 m^2	1-10 m^2	>10 m^2
Larval Density			
Low	1	1	2
Medium	1	2	3
High	2	3	3

KEY: 1 = Low larval productivity, 2 = Medium larval productivity, 3 = High larval productivity.

Table 2 - Types of mosquito breeding sites found in Mvomero district

	Short rains N (%)	**Long rains N (%)**
Brick pits	184 (40.8)	38 (25.9)
Rice fields	174 (38.6)	83 (56.5)
Hoof prints	23 (5.1)	3 (2)
Water wells	22 (4.9)	3 (2)
Stream	13 (2.9)	2 (1.4)
Pond/Swamp	13 (2.9)	10 (6.8)
Broken pots	12 (2.7)	2 (1.4)
Rock holes	4 (0.9)	2 (1.4)
Ditches	4 (0.9)	2 (1.4)
Tire print	2 (0.4)	2 (1.4)

Productivity of breeding sites and land use

Mkindo and Komtonga, each with 38 breeding sites, were selected to represent rice producing villages (Table 3). Mkindo has modern irrigation schemes with systematic regulation of the volume of water entering or leaving specific rice fields and of the duration of water staying in the field. On the other hand, Komtonga village practices traditional irrigation methods whereby the banks are higher, allowing a large volume of water to enter the rice fields, and water remaining present for a longer time (Table 4). Both rice growing areas and brick pits were highly productive for mosquito larvae. In the rice growing area the mean numbers ± S.E. of larvae found were 130.5 ±11.8 and 92.0 ± 8.5 for early and late instars in Komtonga, and 104.5 ± 11.8 and 57.0 ± 5.3 in Mkindo. In the brick-making villages these values were 609.0 ± 47.5 and 252.0 ± 22.8 for Manyinga and 80.0 ± 3.2 and 11.0 ± 0.5 for Hembeti (Table 4). In rice-growing villages the ratio of early and late instars was 0.7 at Komtonga and 0.6 at Mkindo. In the brick-making villages this was 0.4 at Manyinga and 0.1 at Hembeti (Table 3).

Table 3 - Number (N) and distribution (%) of permanent and semi-permanent breeding sites found in Mvomero

Village/Hamlet	N	(%)	Main types of breeding sites
Bwagala	32	(15.5)	Man-made ponds
Dihombo	6	(2.9)	Natural swamps
Hembeti	16	(7.8)	Brick pits
Komtonga	38	(18.4)	Traditional rice fields
Manyinga	76	(36.9)	Brick pits
Mkindo	38	(18.4)	Modern rice fields

Table 4 - Mean number of early and late instars of anopheline mosquitoes according to development stage (Mean ± SE). N = number of sites examined.

Land use	Villages	N	Early instars Mean ± SE (%)	Late instars Mean ± SE (%)	Instars ratio Early: Late
Rice	Mkindo	38	130.5 ± 11.8 (14.1)	92.0 ± 8.5 (22.3)	0.7
growing	Komtonga	38	104.5 ± 9.8 (11.3)	57.0 ± 5.3 (13.8)	0.6
Brick	Manyinga	76	609.0 ± 47.5 (65.9)	252.0 ± 22.8 (61.2)	0.4
making	Hembeti	16	80.0 ± 3.2 (8.7)	11.0 ± 0.5 (2.7)	0.1

Breeding sites with predators were more frequent in rice producing villages (n=59) than in brick-making villages (n=45) (Table 5). The total number of either anopheline or culicine larvae in water bodies that contained predators was lower than those in water bodies without predators (Table 5). Predators had a stronger impact on culicine larvae than on anopheline larvae.

The number of breeding sites in which anophelines and culicines co-habited was higher than breeding sites where anopheline larvae existed alone (Table 6). The numbers of sites with co-habiting anophelines and culicines or sites with culicines only were not much different. Moreover, in breeding sites where anopheline and culicine larvae co-habited the number of larvae of both anophelines and culicines was higher compared to breeding sites where either species occurred alone (Table 6).
Table 5 -

Table 5 - Mean number of anopheline and culicine larvae collected from sites with or without predators. N = total number of sites sampled

Status of sites	**Type of land use where a breeding site was found**			
	Brick making		**Rice production**	
Without predators	**N**	**Mean ± SE, (%)**	**N**	**Mean ± SE, (%)**
Anophelines	36	547 ± 54.5 (11)	12	233.5 ± 22.5 (27.7)
Culicines	81	3257.5 ± 222.5 (65.3)	11	279.5 ± 6.2 (33.1)
With predators	**N**	**Mean ± SE, (%)**	**N**	**Mean ± SE, (%)**
Anophelines	18	325 ± 20.5 (6.5)	29	142 ± 13.3 (16.8)
Culicines	27	858 ± 23.4 (17.2)	30	188.5 ± 2.6 (22.4)
Predation effect	**Percentage reduction by predators**			
Anophelines	25.5		24.2	
Culicines	58.3		19.5	

Table 6 - Mean number ± S.E. of larvae sampled in sites where anophelines and culicines co-habit or exist solitarily. N = number of sites sampled.

Status of sites	**Type of land use where a breeding site was found**			
	Brick making		**Rice production**	
Co-habitation	**N**	**Mean ± SE**	**N**	**Mean ± SE**
Anophelines	52	673.5 ± 54.3	38	180 ± 17.1
Culicines	52	3685.5 ± 299.3	38	261 ± 0.6
Co-habitation	**N**	**Mean ± SE**	**N**	**Mean ± SE**
Anophelines	2	365 ± 36.3	3	47.5 ± 2.7
Culicines	56	924 ± 88.8	3	120 ± 12.0

Anopheline population dynamics

During the cool months of June - September, breeding sites were very few (Figure 3). Only an average of 7 to 12 breeding sites was found to contain anopheline larvae in the surveyed villages. The few positive breeding sites were clustered at Mkindo and Komtonga villages which have rice irrigation schemes. Overall, the number of both positive and negative anopheline breeding sites increased with the increase in rainfall, except in the month of April when negative sites outnumbered positive sites.

Adult anophelines collected indoors, mostly *An. gambiae* s.l. and *An. funestus*, were present throughout the 12-month study period, with seasonal variation in density (Figure 4). Populations of *An. gambiae* s.l. peaked during the long rains, with a second peak during the short rains. The population of *An. funestus* became almost undetectable during the months of heaviest rains (March and April) and peaked towards the end of the short rainy season (November-December). A total of 45,702 female mosquitoes were collected in 15 houses during the 12 sampling rounds. The most important indoor host-seeking mosquito species was *An. gambiae* s.l. which accounted for 59.5% of the mosquitoes collected (Table 7). *Anopheles funestus* accounted for 10.7% of adult mosquitoes, and *Cx. quinquefasciatus* for 26.8%. Fourty six % of the man-biting mosquitoes were collected in Komtonga and most of the mosquitoes in this village were the malaria vectors *An. gambiae* s.l. and *An. funestus*.

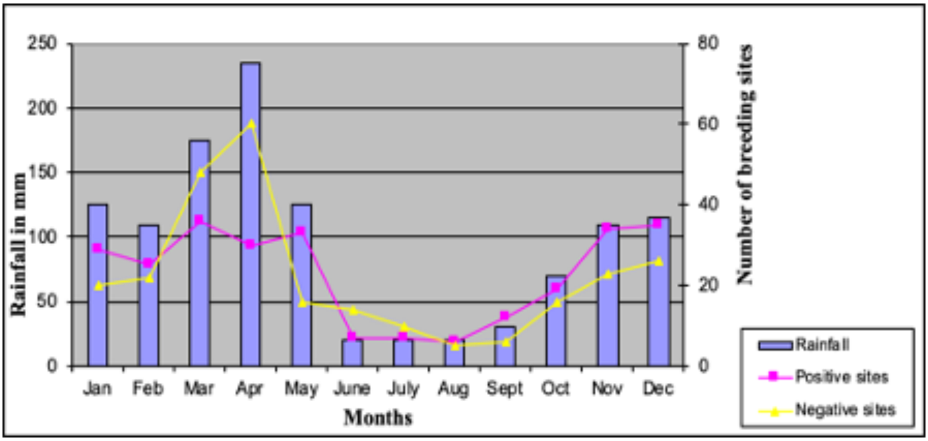

Figure 3 - The relationship between rainfall (mm) and mean number of breeding sites

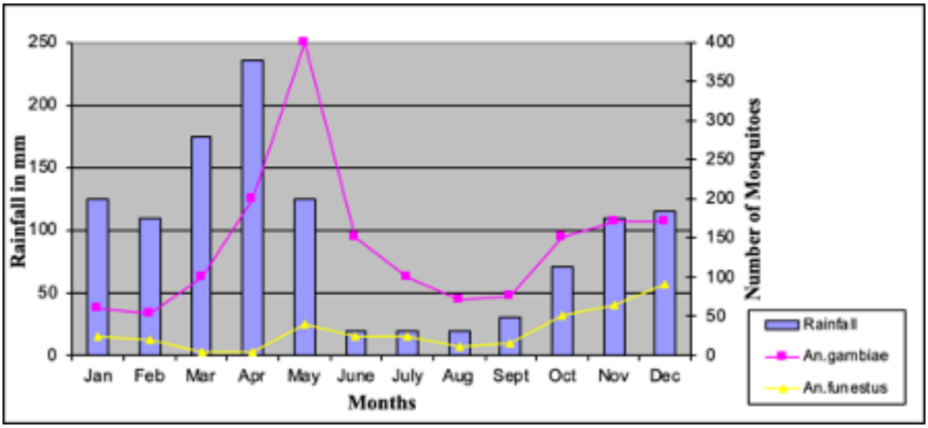

Figure 4 - The relationship between monthly rainfall (mm) and the mean number of adult *An. gambiae* s.l. and *An. funestus* mosquitoes per trap per night

Important factors associated with anopheline larvae in water bodies

Drainage, water pollution and movement, presence of predators and presence of heterospecific larvae (culicines), presence of grass, water depth and direct exposure to the sun were significantly associated with the presence/absence of anopheline larvae in the water bodies (Table 8). Water bodies that are open to sunlight (odd ratio [OR] = 33.2, 95% confidence interval [CI] = 19.72-55.85), shallow site (OR = 7.5, 95% CI = 4.7-12.08), water bodies with grass (OR = 3.2, 95% CI = 2.15-4.9) and the presence of culicine larvae (OR = 1.9, 95% CI = 1.24-2.83) were much more likely to contain anopheline larvae than when grass and culicine larvae were absent, the water had greater depth and the sites were shaded. Presence of predators (OR = 0.3, 95% CI = 0.16-0.54), drainage (OR = 0.1, 95% CI = 0.06-0.23), water movement (OR = 0.1, 95% CI = 0.04-0.16) and water pollution (OR = 0.1, 95% CI = 0.05-0.13) were negatively associated with anopheline larvae.

Table 7 - Species composition and number (%) of adult mosquitoes collected in six study sites

Hamlet/ village	***An. gambiae* s.l.**	***An. funestus***	***Cx. quinquefasciatus***	**Others**	**Total**
Bwagala	1,951 (41.3)	125 (2.6)	2,560 (54.1)	92 (1.9)	4,728
Dihombo	1,628 (49.6)	209 (6.4)	1,220 (37.1)	228 (6.9)	3,285
Hembeti	1,240 (51.8)	128 (5.3)	876 (36.6)	150 (6.3)	2,394
Komtonga	15,526 (73.7)	4,210 (20)	932 (4.4)	410 (1.9)	21,078
Manyinga	2,673 (40.8)	154 (2.3)	3,453 (52.7)	274 (4.2)	6,554
Mkindo	4,164 (54.3)	82 (1.1)	3,219 (42.0)	198 (2.6)	7,663
Total	27,182 (59.5)	4,908 (10.7)	12,260 (26.8)	1,352 (3)	45,702

Table 8 - Analysis of factors associated with presence of mosquito larvae in breeding sites

Variable	**Odd ratio (OR)**	**[95% CI]**	**P-value**
Culicine larvae	1.9	1.24 - 2.83	0.003
Predator	0.3	0.16 - 0.54	0.02
Sunlit	33.2	55.85 - 19.72	0.001
Water pollution	0.1	0.05 - 0.13	0.0001
Shallow site	7.5	12.08 - 4.70	0.0001
Drainage	0.1	0.23 - 0.06	0.007
Grasses	3.2	4.90 - 2.15	0.854
Water movement	0.1	0.16 - 0.04	0.0001

Discussion

We characterised larval habitats in two dominant land-use patterns: brick making villages (Manyinga and Hembeti) and rice-producing villages (Mkindo and Komtonga), where rice is produced throughout the year by irrigation. Our study showed that the number of breeding sites with co-habiting anophelines and culicines was higher than the number of breeding sites containing either of the species. As well, the total number of larvae in breeding sites co-habiting the two species was much higher than the total number of larvae in breeding sites containing only anophelines or culicines. This finding is in line with other studies that also observed anophelines and culicines often sharing the same breeding sites (Harris et al. 2011). Findings from laboratory assays suggest that *An. gambiae s.s* prefer to lay eggs in water containing low numbers of *Cx. quinquefasciatus egg rafts as compared to water without any egg raft* (Wachira 2010). These findings not only support the hypothesis that gravid females of both species benefit mutually when co-existing but also that they are possibly guided by similar cues when searching for breeding sites.

The odds of having anopheline larvae in a breeding site in our study area was almost two times greater for sites with culicine larvae, three times greater for sites with grassy vegetation, seven times greater for shallow sites and 33 times greater for sites that are exposed to sunlight. This finding re-emphasizes the influence of sunlight on the suitability of breeding sites and consequently the selection of such sites for oviposition by gravid *Anopheles* mosquitoes. If this concept is taken seriously, it can have significant implications for malaria control strategies. For example, locating brick pits in the shade (big trees or man-made shade) would reduce mosquito productivity compared to the existing practice of locating brick pits in an open space with maximum sunlit exposure. Other factors to consider are sites with predators, polluted water, good drainage or fast moving water, as these were significantly not associated with the presence of anopheline larvae.

Most of mosquito breeding sites in our study area that contributed to high larval productivity were the result of human activities, as was found elsewhere in East Africa (Imbahale et al. 2011). This study re-confirms that anophelines prefer shallow and relatively small sunlit habitats as previously observed (Minakawa et al. 1999, Gimnig et al. 2001, Minakawa et al. 2005). Moreover, breeding sites containing anophelines were associated with submerged aquatic vegetation (Rodriguez et al. 1993, Hernandez et al. 1997, Torres-Estrada et al. 2005). This suggests that some plants present in breeding sites may play a role in guiding gravid females to oviposit through the emission of infochemicals. This is in line with previous findings that a mixture of terpenoid and alcohol compounds identified by GC-MS of plant volatiles associated with *Anopheles* larval breeding sites mediate *Anopheles* oviposition (Rejmankova et al. 2005, Torres-Estrada et al. 2005, Eneh et al. 2016) Moreover, agronomic practices in rice fields and organic waste disposal in water ponds influence the attractiveness of sites for gravid mosquitoes (Victor and Reuben 2000, Darriet et al. 2010, Wondwosen et al. 2016) and consequently, increased larval productivity of these sites.

Another observation from our study is that small ponds such as brick pits were significantly preferred by anophelines as compared to large sites such as rice fields. This is in line with previous observations that small ponds have less predation compared to large and long lasting ones (Washburn 1995, Sunahara 2002, Fillinger et al. 2004, Mutuku et al. 2006). It has been suggested that anophelines use visual and/or chemical cues to assess potential breeding sites in terms of the presence of predators and estimate predation risk among breeding sites (Blaustein et al. 2004). Moreover, we found that sites with co-habiting anopheline larvae and predators had fewer larvae than sites without predators. This confirms earlier findings that gravid females quantify risks of predation when selecting a suitable oviposition site, therefore avoiding oviposition in sites already colonized by competitors (Mwingira et al. 2019) or predators (Blaustein et al. 2005, Silberbush and Blaustein 2011, Warburg et al. 2011) in order to maximize their fitness.

The entomological inoculation rate (EIR) profiles indicated that the risk of exposure to infected mosquitoes is highly heterogeneous and seasonal with high inoculation rates during the rainy season and with little transmission during the dry season (J.I et al. 2003). The abundance of larvae and adult mosquitoes in our study sites displayed similar temporal and spatial variation. The variation in abundance between *An. gambiae* s.l. and *Anopheles funestus* can be explained by their differences in ecological requirements. The density of *An. gambiae* peaked during the long rainy season, where transient habitats like shallow sunlit, plain valleys and human-made habitats such as brick pits and rice fields are filled with rain water. *Anopheles funestus*, however, prefers habitats providing shelter by plants such as grass and mature rice plants. They are abundant during the short rainy season, where there is intermittent rainfall. Therefore, in line with findings from other studies, we confirmed the fact that *An. funestus* prefers breeding sites that contain clear water with submerged grasses (Gillies and De Meillon 1968, Gillies and Coetzee 1987).

Several studies have shown that irrigated rice cultivation enhances population development of many malaria mosquito species especially *An. gambiae* s.l. (Ijumba and Lindsay 2001b, Briet et al. 2003, Klinkenberg et al. 2003b), and has been associated with high malaria transmission in sub-Saharan Africa (Appawu et al. 2004, Diuk-Wasser et al. 2006). This is one of the reason why there still is a large population of *An. gambiae* s.l., whereas in other parts of Tanzania these populations have declined, especially those of *An. gambiae* s.s. Worse of all, brick-making and Riceland communities are experiencing the highest mosquito densities throughout the year. Our result correlates with previous findings at Mvomero where the prevalence of malaria parasitaemia was highest in Komtonga village (66.25%) with a high EIR followed by Mkindo and Manyinga villages (Mboera et al. 2010b). Therefore, there is a strong association between land use and mosquito biting risks in Mvomero district.

Conclusion

Generally, our study confirms that the frequency of both anopheline and culicine mosquito appearance is influenced by rainfall patterns and follows a classical distribution pattern. Human activities such as brick making and rice production have a strong influence on the production of adult mosquitoes, and hence constitute a risk factor for malaria and other mosquito-borne diseases. The presence of larvae of *Anopheles* mosquitoes in breeding sites was associated with vegetation, presence of culicine larvae, shallowness and sunlight. The association between anopheline and culicine larvae is a novel finding that can be exploited to improve future control strategies for these vectors. The productivity of anopheline larvae can be reduced by introducing larval predators, pollution or water management in the field. These results can be used for designing strategies that may reduce the high level of mosquito production in Mvomero district

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Traditional rice fields at Digoma village, Mvomero. Experiments were conducted in this valley.

Chapter 6

Synergism between nonane and emanations from soil as cues in oviposition-site selection of natural populations of *Anopheles gambiae* and *Culex quinquefasciatus*

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Abstract

Olfactory cues have been shown to have an important role in guiding gravid mosquito females to selected sites for egg laying. The objective of this study was to determine the influence of soil from a breeding site and the putative oviposition pheromone nonane on oviposition-site selection of natural populations of *Anopheles gambiae* s.l. and *Culex quinquefasciatus*.

This field-based study was conducted in Mvomero District in East-central Tanzania. In a dual-choice experimental set up, clay bowls were dug into the ground and filled with one of the following treatments: (i) distilled water + autoclaved soil (control), (ii) distilled water + soil from a natural mosquito breeding site, (iii) distilled water + nonane and (iv) distilled water + nonane + soil from a natural breeding site. Soil was either left untreated or was autoclaved before use. The number of larvae present in the bowls after 10 d was used as outcome measure.

Nonane and untreated soil significantly influenced the selection of oviposition sites by both *An. gambiae* ($P< 0.0001$) and *Cx. quinquefasciatus* ($P<0.0001$). Autoclaved soil, however, had a significant effect on oviposition behaviour of *Cx. quinquefasciatus* ($P<0.005$) but no effect on *An. gambiae* ($p= 0.18$). A mixture of nonane and untreated soil caused a synergistic effect on oviposition behaviour compared to either nonane or untreated soil alone, in both *An. gambiae* (Oviposition Activity Index (OAI) = 0.56; $P< 0.001$) and *Cx. quinquefasciatus* (OAI =0.59; $P<0.0001$). The larval pheromone nonane and emanations from breeding-site soil both induced oviposition in wild *An. gambiae* s.l. and *Cx. quinquefasciatus,* with a synergistic effect when both stimuli were present simultaneously. This is the first study in which nonane is shown to cause oviposition under natural conditions, suggesting that this compound can potentially be exploited for the management of mosquito vectors.

KEYWORDS: Oviposition, pheromone, nonane, breeding-site soil, *Anopheles gambiae*, *Culex quinquefasciatus*, Tanzania

Introduction

Vector control is a fundamental element of the existing global strategy to fight mosquito-borne diseases (WHO 2017). Existing mosquito control programmes have been developed based on understanding of the behaviour and lifecycle of the vectors. The use of insecticide-treated nets (ITNs) and the recently developed toxic sugar bait technique resulted from the exploitation of blood and plant host-seeking behaviour of mosquitoes, respectively (Malima et al. 2009, Marshall et al. 2013, Qualls et al. 2014, Revay et al. 2014). Additionally, indoor residual spraying with insecticides (IRS) is based on the observation that mosquitoes rest on walls after feeding and thereby come into contact with insecticides (Curtis and Lines 1985). Larviciding and other environmentally-based interventions (Curtis et al. 2002), on the other hand, are based on observations that mosquito eggs are laid on water and immature stages develop in water. Oviposition has been considered to be an important target to exploit for the control of mosquito-borne diseases (Bentley and Day 1989, Dugassa et al. 2013, Killeen et al. 2014).

Currently, the main effective methods of mosquito-borne disease control are the use of ITNs and IRS (WHO 2018). However, these methods are challenged by the wide-spread development of insecticide resistance (Norris and Norris 2011, Ranson et al. 2011, Hemingway et al. 2016) and the observed behavioural adaptation of mosquitoes to avoid insecticides (Govella et al. 2013). Mosquitoes can detect the presence of insecticides from treated surfaces and divert their biting activities in time and space to exploit untreated resources (Takken 2002, Lindblade et al. 2004, Grieco et al. 2007).

Recently it was reported that malaria mosquitoes have altered their blood host-seeking behaviour by shifting biting time (Moiroux et al. 2012, Yohannes and Boelee 2012, Sougoufara et al. 2014, Thomsen et al. 2017) and place, i.e. outdoors vs. indoors (Govella et al. 2010, Russell et al. 2011) in response to the wide coverage of ITNs. Moreover, in certain areas malaria vectors have shifted their blood-host preference to other vertebrate species as a result of insecticide use (Mwangangi et al. 2013). In such circumstances, the core interventions of ITNs and IRS need to be supplemented by larval source management, which includes vector habitat modification, habitat manipulation, larviciding and biological control (WHO, 2018).

One of the possible options is habitat manipulation by using infochemicals derived from mosquito breeding sites to lure vectors into death traps (Herrera et al. 2014, Okal et al. 2015, Eneh et al. 2016). Already habitat-derived infochemicals have been used to enhance the trapping of gravid *Culex quinquefasciatus* (Mboera et al. 1999, Barbosa et al. 2010, Barbosa and Regis 2011) and *Aedes aegypti* (Barrera et al. 2013, Barrera et al. 2014) mosquitoes in autocidal oviposition traps. The use of infochemicals from breeding sites to lure gravid mosquitoes has the potential to target egg-laying adults (Zeichner and Perich 1999, Herrera et al. 2014). Thus, infochemicals that direct gravid mosquitoes to lay eggs in selected habitats are likely to be the focus of future vector control strategies.

These strategies are relevant because most insects express a preference for oviposition habitats that improve survival, growth and reproductive potential of their offspring, especially for species in which juveniles are incapable of migrating away from poor-quality habitats (Peckarsky et al. 2000). Sites selected for oviposition by mosquitoes are usually few and, once identified, they can easily be targeted for control measures. Oviposition-habitat selection is particularly relevant in insect vectors of medical importance as it determines the localities to which larvicidal control measures need to be targeted (Service 1993).

The search for oviposition attractants is aimed at discovering a chemical compound or blends of compounds that attract selected species (Bernier 2003). Skatole and (5R,6S)-6-acetoxy-5-hexadecanolide were discovered to be oviposition cues for *Cx. quinquefasciatus*, and a blend of these compounds caused a synergistic response in gravid mosquitoes (Mboera et al. 2000). Recently, it was reported that gravid mosquitoes of *Anopheles gambiae* are attracted to cedrol, a compound identified from a natural breeding site, as oviposition cue (Lindh et al. 2015). Cedrol was found to be derived from grass species found in breeding sites of *An. gambiae s.s.* (Eneh et al 2016). Additionally, nonane, a compound identified in the headspace from mosquito larval habitats in the laboratory, was found to be attractive to gravid *An. gambiae* (Chapter 4). Nonane is a volatile chemical compound with nine carbon atoms. A related chemical compound with nine carbon atoms similar to nonane and which acts as an attractant to mosquitoes is nonanol, which is known to attract *Cx. quinquefaciatus* (Syed and Leal 2009). Current evidence suggests that species-specific as well as habitat-derived chemicals affect oviposition behaviour of mosquitoes.

The objective of the present study was to explore the influence of habitat-derived infochemicals and nonane on the selection of oviposition sites by *An. gambiae* and *Cx. quinquefasciatus* under field conditions. Studies were done i) to establish the most effective (artificial) oviposition device for field use, ii) to examine the effect of soil-derived infochemicals and of the putative oviposition cue nonane and iii) to investigate the interaction between soil from breeding sites (Herrera-Varela 2015) and nonane on oviposition behaviour of wild *An. gambiae* and *Cx. quinquefasciatus*.

Material and Methods

Study area

The study was carried out in Mvomero District in east-central Tanzania (latitude 5°47'09''-7°23'40''S, longitude 37°11'09''- 38°01'33''E), between March and

June 2012. This area has typical tropical characteristics: temperatures oscillating between 19 and 31°C, RH >80%, and annual rainfall of 1,146mm (based on data collected from Mtibwa meteorological station, 2008-2013). The area has a bi-modal type of rainfall with long rains from March to June and short rains from October to December, with a relatively short dry spell between July and September.

Digoma village was selected for the field experiments; the village borders the Nguu mountains and receives water from rivers which flood the valleys. This enables irrigated rice production in the river basin throughout the year. In addition to rice production, therefore, the area has favourable environmental conditions for mosquito production. Malaria and lymphatic filariasis are the most common mosquito-borne diseases in the area (Mboera et al. 2011). The most abundant mosquito vectors in the area include *An. gambiae* Giles *s.s*, *An. arabiensis* Patton, *An. funestus* Giles and *Cx. quinquefasciatus* Say. *Anopheles gambiae s.s.* and *An. arabiensis* are genetically related and morphologically indistinguishable, and are here grouped as *An. gambiae* unless otherwise mentioned.

Oviposition containers

Containers used for oviposition in this experiment included clay pots, plastic bowls, aluminium pans and plates which were either blue or transparent in colour (Fig. 1). With the exception of aluminium plates, which had a diameter of 27 cm and a depth of 4 cm, all other containers were of similar size (average diameter of 25 cm and a depth of 7 cm).

Distilled water

Distilled water was used in the experiment to dilute chemicals and obtain the desired dosages, and also to dissolve oviposition substrates before setting up the experiments. Distilled water was also used for rinsing all washable items used in the experiments. It was produced and packed by LAL Laboratories, Tanga, Tanzania. Distilled water was used alone in the early experiments as oviposition substrate and in the control arm. For experiments involving nonane, a control solution was used, which consisted of 55% v/v distilled + 40% v/v methanol + 5% v/v tween20. Previous studies had not found any behavioural or larvicidal effect of this mixture (Schoelitsz et al., 2020).

Soil

Clay soil originated from a natural breeding site in Mvomero that contained early-stage larvae of *An. gambiae*. Two hundred gram of dry soil was added to each container to simulate natural conditions of breeding sites. Previous studies have shown that volatile emissions associated with microbial organisms in the soil mediate the location of potential mosquito habitats (Knols et al. 2004). Therefore, a fraction of soil samples from known breeding sites was dried, autoclaved twice for 15 min. at 130°C and 1.4 kg/cm^2 pressure and allowed to cool down to kill any organisms that might be involved in the production of volatile chemicals (Trexler et al. 2003b, Knols et al. 2004).

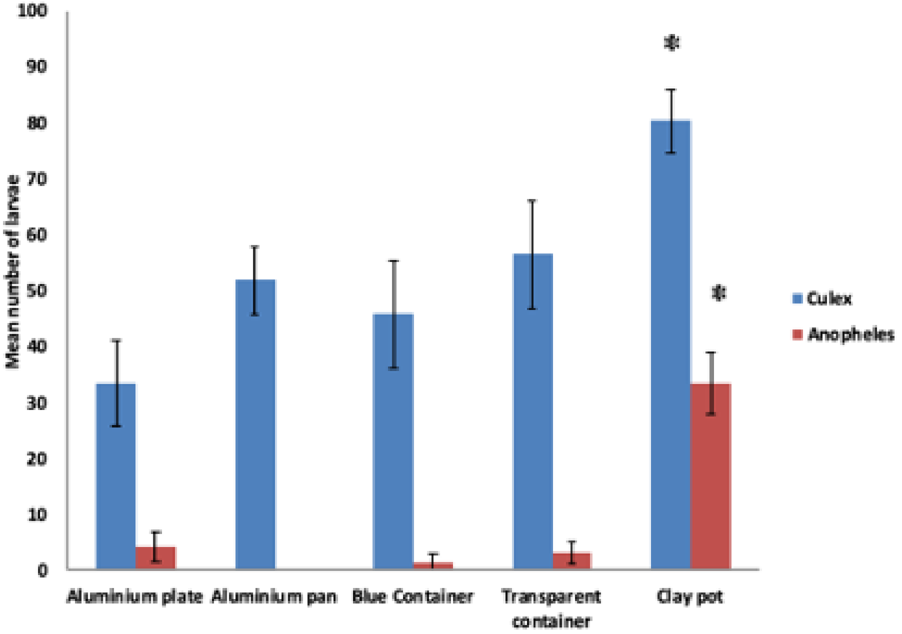

Figure 1 - The mean number ± S.E. of larvae resulting from oviposition by a natural population of mosquitoes in a multiple choice set-up of oviposition containers. Asterisks indicate statistical differences from other treatments for a given species (*: $p < 0.05$, Friedman test). The blue bars represent anophelines while the red bars represent culicines.

Chemical cues

Nonane (Lot and filling code: 132995235107188, ≥ 99.0%; Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands) was selected as chemical cue to lure gravid mosquitoes. Nonane is insoluble in water and therefore it was dissolved in methanol and tween20 in the following ratio: 55% v/v of nonane + 40% v/v methanol + 5% v/v tween20. The mixture was further diluted in distilled water with one magnitude per dilution. In experiments 3 & 4, nonane was tested at a concentration of 5.5 x 10^{-5}M and it was paired with a control solution of distilled water + methanol + tween20.

Selection of artificial oviposition containers

In our effort to simulate natural breeding sites, we conducted experiments in order to search for the most preferred artificial breeding site for a natural population of mosquitoes. We therefore evaluated a range of man-made liquid receptacles in the field in order to identify the most suitable oviposition bowl for mosquitoes in the area. These included plastic bowls (blue and transparent), aluminium plates and pans, and clay pots. To explore possible colonization of artificial habitats by wild mosquitoes, 25 containers were placed randomly in an open sunlit field. Five lines, each composed of five containers were placed 3 m from each other, in sub-soil with the top of the container being at soil surface level. Containers were filled with distilled water to capacity and were checked daily for the presence of larvae/pupae for a period of 10 days. Evaporated water was replenished with an equal amount of water in each container daily. Distilled water had already been successfully used as oviposition substrate for mosquitoes in the laboratory and semi-field environment (Schoelitsz et al., 2020). Therefore, the aim of this experiment was to test if a natural population of mosquitoes would oviposit in our simulated breeding sites containing distilled water. The container that produced the highest number of larvae was selected for the behavioural experiments.

Site selection for oviposition trial

Four sites (north, south, east and west) were selected for the dual-choice oviposition trial in an area covering a total of 4 ha. on both sides of a river near Digoma village, N.E. Tanzania. This area was chosen based on the following criteria: proximity to the river basin, presence of rice fields, absence of flooding, open to sun and proximity to human settlements. Rice growing is the main economic activity. All sites were surrounded by a wire mesh to prevent humans, animals or frogs from interfering with the experiments. In addition, a local field worker was hired to oversee the site during the entire study period.

Clay pots

As clay pots gave the best result as oviposition container (see above), there were selected for the remainder of the study. The pots had an average diameter of 200 mm and a depth of 100 mm was used as artificial breeding sites for the field trial. They were made locally from clay soil, moulded by hand to make a pot shape and left to dry, where after they were cured by fire. In our study, clay pots were positioned in the ground so that the margins of the pots were level with the surrounding ground. The pots were placed in the valley plain, within a rice field in the vicinity of a village.

Design of oviposition experiments

Clay pots were placed at selected sites in the field 72 h before the start of the experiment and filled till capacity with distilled water; water was replenished until the clay reached saturation. Prior to the start of the experiment, the clay pots were emptied and immediately filled with 1L of the oviposition substrate (treatment or control) one h before sunset. Oviposition pots were left undisturbed for five days and from the 6th day, pots were inspected every morning and larvae were collected and recorded daily from 06:30 am for the next 10 days. Whenever the water level decreased in the pots, distilled water was added to maintain the water level. Collected larvae were then transferred to a temporarily established local laboratory together with the water from the pot and reared under controlled conditions. This water was used as rearing substrate in the laboratory for the first 24 h. After that time, larvae from each oviposition pot were transferred to mosquito rearing bowls which contained distilled water. Rearing bowls were placed below light bulbs and larvae were fed Tetramin® fish food twice daily. Larval growth and development were observed and recorded until pupation and adult formation. Oviposition pots containing experimental substrates remained in the field for 15 d thereafter the substrates were removed; the pots were cleaned and replaced. For each pair of treatment and control, the pots were oriented facing East and West positions, and these positions were exchanged for each replicate.

Dual-choice tests

The effect of substrate on oviposition choice of wild mosquitoes was tested in a dual choice test, where one clay pot contained the treatment substrate and the other pot the control substrate (Table 1). Treatment and control were placed 3 m from each other. Each treatment pair was replicated 40 times, 10 pairs at four different sites (see site selection above); pots with substrate were incubated in the field for five days, and then examined for the presence of larvae for 10 days. Newly emerged larvae were collected daily. For each replicate, the positions of the treatment and control were exchanged each time to counterbalance the effects of wind direction.

Influence of autoclaved soil from a natural breeding site

We placed 200 g of autoclaved soil from a natural anopheline breeding site + distilled water in a clay pot and tested against distilled water only. Pots were each filled with 1250 mm distilled water.

Table 1 - Experimental treatments for the dual-choice tests

Treatment series	Substrate A	Substrate B	No of replicates
1	Distilled water + autoclaved soil	Distilled water	40
2	Distilled water + untreated soil	Distilled water + autoclaved soil	40
3	Distilled water + autoclaved soil + nonane	Distilled water + autoclaved soil	40
4	Distilled water + autoclaved soil + nonane	Distilled water + untreated soil	40
5	Distilled water + untreated soil + nonane	Distilled water + untreated soil	40

Influence of untreated soil from a natural breeding site

Clay pots were filled with 200 g of dried soil from a natural anopheline breeding site. To test whether soil produces chemical cues or acts only as a visual cue for gravid mosquitoes (Herrera et al. 2014), the soil was tested against autoclaved soil. The pots were each filled with 1250 ml of distilled water.

Influence of nonane

1250 ml of the nonane solution + autoclaved soil was tested against 1250 ml of distilled water + autoclaved soil. Pots were filled with either 200 g autoclaved soil and distilled water or 200 g autoclaved soil and a nonane solution.

Influence of soil from a natural breeding site and nonane

To investigate the interactive effects of breeding-site soil and nonane, combinations of both candidate stimuli were tested alone or as a mixture: (a) nonane + breeding-site soil against nonane + autoclaved breeding soil and (b) nonane + breeding-site soil against distilled water + breeding-site soil. Pots were filled with 200 g of soil and distilled water or a nonane solution until capacity.

Mosquito species composition

All larvae collected were transferred to the insectary and reared until adult emergence. Newly emerged anopheline adults were identified to species level using morphological keys (Gillies and Coetzee 1987). *Anopheles gambiae* specimens were preserved in Eppendorf tubes which contained silica gel for further identification to distinguish between sibling species in the *An. gambiae* complex. Genotypic identification was conducted by using the ribosomal DNA-polymerase chain reaction (PCR) to separate *An. gambiae* s.s. from *An. arabiensis* (Scott et al. 1993). Culicine mosquitoes were identified as *Cx. quinquefasciatus* or other culicines.

Data analysis

SPSS 14 for Windows® was used to conduct Wilcoxon signed-rank tests for paired samples in order to determine the difference in the number of larvae in each oviposition bowl as an indicator of number of eggs laid. A Friedman test for multiple samples was used to determine the oviposition preference among several containers. The preferences of mosquitoes for ovipositing on different treatments were evaluated based on container index (CI) (% bowls harbouring larva). All statistical tests were conducted by using absolute numbers of larvae in pots as a proxy for the number of eggs laid in the pot.

The larval density index (LDI) was defined as the total number of larvae found divided by the total number of oviposition containers with larvae.

The oviposition active index (OAI) was used to determine the attractiveness to the treated substrate compared to control. It was calculated according to the formula; OAI = Nt-Nc/Nt+Nc (Kramer and Mulla 1979). Where Nt = number of larvae on the test substrate and Nc = number of larvae on the control substrate. In this study, we found that anopheline eggs, which are black in colour, tend to stick to the surface of the clay pot which is also black. This poses a challenge to accurately score the number of eggs as a measure of oviposition activity of gravid females. Therefore, the number of larvae was scored as a proxy for the eggs that were laid in respective pots.

Results

Mosquito species composition

During the study on oviposition site selection and containers (see below), a total of 1,349 anopheline larvae and culicine 2,815 larvae were collected. All anopheline larvae collected in the containers consisted of *An. gambiae*. Molecular analysis (by PCR) of a subsample of 200 *An. gambiae* larvae revealed that 86% were *An. gambiae s.s*. and 16% *An. arabiensis*. Culicine larvae were identified as *Cx. quinquefasciatus*.

Mosquito oviposition-site selection between different substrates and containers

Preliminary experiments showed that natural populations of *Cx. quinquefasciatus* oviposited in containers that were filled with distilled water. By contrast, a natural population of *An. gambiae* mosquitoes did not oviposit in containers filled with distilled water only. After adding 200 g of soil from a known anopheline breeding site to each container, we observed significantly more larvae in clay pots than in other containers. On average, 80.4 ± 5.7 ($\chi^2 = 14.97$, $P=0.005$) larvae of *Cx. quinquefasciatus* and 33.4 ± 5.4 ($\chi^2 = 9.92$, $p = 0.042$) larvae of *An. gambiae* were found in clay pots containing soil (Fig. 2). It was therefore decided to use clay pots for all successive experiments as proxies for natural breeding sites.

Influence of chemical cues from soil

In a choice assay between distilled water with breeding-site soil and distilled water with autoclaved soil, the accumulated total number of *An. gambiae* larvae found in pots containing breeding-site soil left for 10 d was 254, with an average of 6.35 ± 1.16 larvae per pot. The number of *Cx. quinquefasciatus* larvae was 644, with an average of 16.1 ± 1.6 larvae per pot. The number of larvae found in pots containing breeding-site soil was significantly higher than that in pots with autoclaved soil for both *An. gambiae* and *Cx. quinquefaciatus* ($P < 0.0001$ and $P < 0.0001$ respectively). There were only few larvae of *An. gambiae* found in pots containing autoclaved breeding-site soil (Table 2).

Influence of nonane

In a choice assay between control + autoclaved soil + nonane and control + autoclaved soil only, a total of 503 larvae of *An. gambiae* were found in 32 out of 40 clay pots containing nonane, while a total of 825 larvae of *Cx. quinquefasciatus* were found in all 40 clay pots containing nonane. Larvae of *An. gambiae* were mainly found in pots containing nonane, with an average number of 12.6 ± 1.6 larvae per pot, whereas larvae of *Cx. quinquefasciatus* were found in both treated and control pots with an average of 20.6 ± 1.1 found in nonane pots and 5.7 ± 1.0 in control pots. The number of larvae found in pots containing nonane was significantly higher than the number of larvae found in the control pots for both *An. gambiae* and *Cx. quinquefasciatus* ($P < 0.0001$ and $P < 0.0001$, respectively) (Tables 2 & 3).

Table 2 - Mean number of larvae from a natural population of *Anopheles gambiae* s.l that oviposited in clay pots filled with distilled water (DW), and distilled water + autoclaved soil (AC), distilled water + untreated soil (BS) and distilled water + nonane in a dual choice set-up in the field. Larval density index (LDI), oviposition activity index (OAI) and p-values are shown.

Oviposition substrate	Pot positivity		Quantity of larvae in pots		OAI	p-value
	No.	LDI	No. (%)	Mean ± SE		
DW + AC soil	2	3.5	7 (7.1)	0.2 ± 0.8	1	0.18
DW	0	0	0 (0)	0		
DW + AC soil	2	5.5	11 (0)	0.3 ± 0.2	0.9	0.0001
DW + BS soil	22	11.6	254 (100)	6.4 ± 1.2		
DW + AC soil + nonane	32	15.7	503 (100)	12.6 ± 1.6	0.9	0.0001
DW + AC soil	3	5.3	16 (0)	0.4 ± 0.2		
DW + AC soil + nonane	22	19.5	429 (57.2)	10.7 ± 1.7	0.1	0.097
DW + BS soil	14	23	322 (42.8)	8.1 ± 1.8		
DW + BS soil + nonane	38	25.6	972 (78.3)	24.3 ± 2.0	0.6	0.0001
DW + BS soil	24	11.5	276 (21.7)	6.9 ± 1.1		

Table 3 - Mean number of larvae from a natural population of *Culex quinquefasdatus* that oviposited in clay pots filled with distilled water (DW), and distilled water + autoclaved soil (AC), distilled water + untreated soil (BS) and distilled water + nonane in a dual choice set-up in the field. Larval density index (LDI), oviposition activity index (OAI) and p-values are shown.

Oviposition substrate	Pot positivity		Quantity of larvae in pots		OAI	p-value
	No.	LDI	No. (%)	Mean ± SE		
DW + AC soil	24	10.5	252 (61.6)	6.3 ± 0.9	0.2	0.005
DW	20	7.9	157 (38.4)	3.9 ± 0.7		
DW + AC soil	22	9.6	210 (24.6)	5.3 ± 0.9	0.5	0.0001
DW + BS soil	32	20.1	644 (75.4)	16.1 ± 1.6		
DW + AC soil + nonane	40	20.6	825 (78.3)	20.6 ± 1.1	0.6	0.0001
DW + AC soil	20	14.9	229 (21.7)	5.7 ± 1.0		
DW + AC soil + nonane	40	21.2	847 (62.1)	21.2 ± 1.3	0.2	0.0001
DW + BS soil	36	14.4	517 (37.9)	12.9 ± 1.0		
DW + BS soil + nonane	40	39.9	1599 (79.4)	40.0 ± 1.7	0.6	0.0001
DW + BS soil	34	12.2	414 (20.6)	10.4 ± 0.9		

The influence of nonane and soil from a breeding site

In a choice test between distilled water with autoclaved soil + nonane against distilled water with breeding-site soil, a total of 751 *An. gambiae* larvae were found. Of these, 57.1% were found in pots containing autoclaved soil + nonane and 42.9% were found in pots containing breeding-site soil (Table 2 & Fig. 2). Also, a total of 1,364 larvae of *Cx. quinquefasciatus* were found; 62.1% of these were found in pots containing autoclaved soil + nonane and 37.9% were found in pots containing breeding-site soil (Table 3). There was no significant difference between the number of larvae found in pots containing autoclaved soil + nonane and pots with breeding-site soil for *An. gambiae* (*P*< 0.097). However, for *Cx. quinquefasciatus,* there was a significantly higher number of larvae in pots containing a mixture of autoclaved soil and nonane compared to breeding-site soil (*P*< 0.0001).

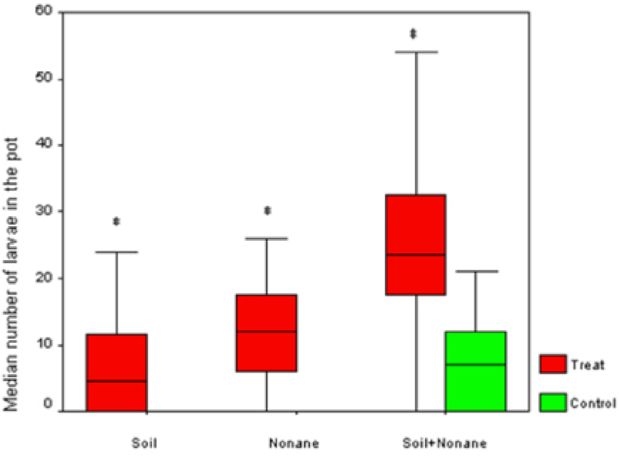

Figure 2 - Oviposition response of *Anopheles gambiae* s.l. to a substrate containing soil, nonane or soil + nonane in a dual-choice field study.
Distilled water + autoclaved soil was used as control. Median and quartiles are given; asterisks indicate statistical differences between treatment and control for a given pair (*: *p*<0.05, Wilcoxon signed rank test).

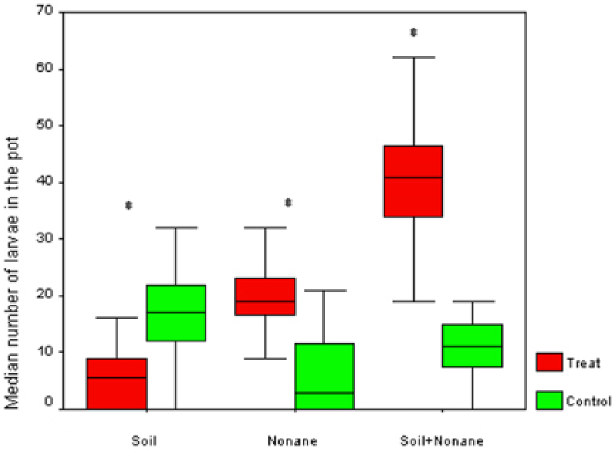

Figure 3 - Oviposition response of *Culex quinquefasciatus* to a substrate containing soil, nonane or soil + nonane in a dual-choice field study.
Distilled water + autoclaved soil was used as control. Median and quartiles are given; asterisks indicate statistical differences between treatment and control for a given pair (*: *p*<0.05, Wilcoxon signed rank test).

Influence of a mixture of nonane and soil from a natural breeding site

In a choice test between distilled water + breeding-site soil + nonane against distilled water + breeding-site soil, a total of 1,248 *An. gambiae* larvae were found. Of these, 77.9% were found in pots containing distilled water + soil + nonane while 22.1% were found in pots containing distilled water + soil (Table 2 & Fig. 2). Additionally, a total of 2,013 larvae of *Cx. quinquefasciatus* were found; 79.4% of these were found in pots containing distilled water + soil + nonane and 20.6% were found in pots containing distilled water + soil only (Table 3). The number of larvae found in pots containing distilled water with distilled water + soil + nonane was significantly higher than larvae found in distilled water + soil for both *An. gambiae* and *Cx. quinquefasciatus* ($P< 0.0001$ and $P< 0.0001$, respectively).

Discussion

The oviposition pheromone nonane and emanations from breeding-site soil both attracted wild females of *An. gambiae* and *Cx. quinquefasciatus* to oviposit in the oviposition containers under field conditions. When both stimuli were present simultaneously, they acted synergistically. These results demonstrate the role that natural products play in the oviposition behaviour of wild populations of mosquitoes. In this study, *An. gambiae* selected clay pots above plastic or aluminium bowls for oviposition. It is likely that clay pots simulate the natural conditions that mosquitoes prefer for oviposition.

Similar to our study, Herrera et al. (2014), while studying oviposition behaviour in Kenya, reported that oviposition substrates containing soil from a known breeding site produced significantly more larvae than a substrate without soil. This suggests that soil from a known breeding site contains and emits a chemical signal associated with microbial activity that attracts gravid mosquitoes and induces oviposition (Ikeshoji et al. 1975, Benzon 1988, Takken and Knols 1999, Gimnig et al. 2001, Ponnusamy 2008). The cue produced by the breeding-site soil is likely to be inter-specific because it originates from microbial interactions and attracted gravid *An. gambiae* s.l. and *Cx. quinquefasciatus* to lay their eggs in pots containing such soil. In this study, autoclaved soil, in which all micro-organisms had been killed, did not affect the oviposition behaviour of *An. gambiae*. This is consistent with other studies of mediation of oviposition-site selection by infochemicals of microbial origin (Trexler et al. 2003a, Sumba 2004).

The effect of cues from micro-organisms present in breeding-site soil appears to be much stronger on *An. gambiae* than on *Cx. quinquefasciatus*, as the latter also laid eggs in pots containing autoclaved soil and even distilled water only, and thus behaved as a generalist species, in contrast to *An. gambiae*. Nevertheless, when untreated soil from a natural breeding site was used, the number of larvae of *Cx. quinquefasciatus* collected was significantly enhanced as with *An. gambiae* s.l. (Table 2), suggesting the production of chemical oviposition cues by the soil/water substrate affecting both mosquito species. This was recently supported by work from Eneh et al. (2016) and Herrera et al. (2014), who demonstrated the emission of such cues from breeding-site soil and grasses. The latter authors suggest that these cues are derived from micro-organisms present in natural breeding sites. This suggestion is supported by our observation that with autoclaved soil few *An. gambiae* s.l. females were attracted to the oviposition bowls, while with natural, untreated, soil many females laid eggs in the bowls.

The assay with nonane as a single cue (Fig. 2) indicated the mediation of oviposition behaviour of a natural population of *An. gambiae* s.l. by an intra-specific cue, as the volatile compound is produced by larvae and it attracts conspecific gravid mosquitoes to oviposit. As described previously (Chapter 4), nonane is a pheromone that originates from *An. gambiae* larvae and attracts conspecific gravid mosquitoes. The response elicited by nonane in this study is consistent with our previous findings on the effects of infochemicals emitted by early instars of *An. coluzzii* on the oviposition behaviour of conspecific gravid females in the laboratory (Chapter 3) and in the semi-field system with *An. gambiae* s.s. (Chapter 4). A similar response was observed with *Cx. quinquefasciatus* despite the fact that nonane originated from *An. gambiae*. This strongly suggests that the two species use the same chemical cues to locate suitable breeding sites. The presence of eggs or larvae of one species can thus act as an oviposition attractant for gravid mosquitoes of another species. It has previously been found that the oviposition pheromone of *Cx. quinquefasciatus* also attracted other culicine species, and this suggests that mosquitoes use a wide range of chemical cues in their oviposition behaviour (Mboera et al. 1999, Lindh et al. 2015). The finding that two different mosquito species, which are not genetically related, have evolved to respond to the same oviposition cues, is an interesting topic for future research, and suggests that olfactory receptors associated with oviposition are widely shared between different mosquito genera.

Mosquitoes use both inter- and intra-specific cues in locating suitable breeding sites. Previous studies on the role of the two cues reported conflicting findings. Some scholars thought that the intra-specific cue (pheromone) emitted by larvae from suitable breeding sites augments the attraction to inter-specific volatiles associated with microbial activity in natural anopheline pools (Sumba 2004, Rejmankova et al. 2005, Lindh et al. 2015). Other authors reported that the presence of larvae in distilled water, even at low density, does not increase oviposition compared to distilled water without larvae (Munga et al. 2006, Sumba et al. 2008). Similarly, in our previous studies (Mwingira et al., 2019), we found that the presence of late-stage larvae, even at a low density, did not increase the oviposition response when compared to distilled water alone. However, we found that the presence of early instars increases oviposition compared to distilled water alone, suggesting that early instars emit intra-specific cues that are attractive to gravid mosquitoes. Nonane was identified from headspace volatiles collected from both early and late instars (Schoelitsz et al., 2020), and the present study shows that wild *An. gambiae* s.l. females prefer to oviposit in breeding sites emitting this compound, which, therefore, should be considered a cue signifying a suitable site for larval development.

Previous studies on the role of infochemicals emitted by larvae have cleared earlier doubts on whether the larval pheromone is stimulatory by itself (i.e., in the absence of the kairomone) or that the production of this pheromone occurs only in *An. gambiae* habitats containing suitable organic matter, microbes and algae (Merritt et al. 1992, Gimnig et al. 2001, Sumba 2004). Furthermore, in order to understand the interaction between an intra-specific signal (pheromone) and an inter-specific cue to the behaviour of *An. gambiae*, we combined nonane and soil from a natural breeding site in a choice assay and tested this combination against soil or pheromone alone. The number of larvae that were found in water with nonane and breeding-site soil was higher than the number of larvae found in water with either nonane or soil alone. This indicates that inter- and intra-specific cues act synergistically when attracting gravid mosquitoes to lay eggs. Recently, cedrol was reported as an oviposition stimulant for *An. gambiae*, derived from breeding-site soil (Lindh et al. 2015). This compound was found to originate from grass present in the breeding site (Herrera et al. 2014, Eneh et al. 2016). Additionally, gravid *An. coluzzii* and *An. arabiensis* were found to be attracted to grass volatiles (Asmare et al. 2017). It is therefore possible that in our study, plant- or soil-derived chemicals were responsible for the behavioural effect of the soil, of which cedrol to date is the only identified compound with proven activity in the field. The observed synergistic response on *An. gambiae* and *Cx. quinquefasciatus* may thus have been caused by the interaction of nonane and cedrol. The discovery of these chemical cues opens the way to the development of oviposition-based mediation/manipulation of populations of these harmful mosquito species.

Our results resemble the effect of the *Cx. quinquefasciatus* oviposition pheromone (5*R*,6*S*)-6-acetoxy-5-hexadecanolide and infochemicals derived from hay infusions, where a similar synergistic effect of both stimuli on gravid females was found (Mboera et al. 2000). This study suggests that there are several mechanisms ensuring that gravid females are guided effectively to sites that are suitable for egg laying and that the observed additive behavioural responses are a result of the perception of several interacting stimuli.

Being an infochemical of *An. gambiae* origin, nonane was expected to affect only gravid *An. gambiae* mosquitoes. In this study, significantly more culicine larvae were also found in pots containing nonane. This suggests that this compound can be used for the surveillance of other mosquito species as well. This finding is consistent with other studies which suggest that *An. gambiae* and *Cx. quinquefasciatus* share breeding sites in many cases (Minakawa et al. 2004, Munga 2006, Mutuku 2006, Mwangangi 2008, Mwingira 2009). Further studies on the interaction of inter-and intra-specific cues of *An. gambiae* in the behaviour of other species will help to understand the universal role of infochemicals among various species of mosquitoes.

The findings from our study suggest that breeding-site derived infochemicals can be used for surveillance and control of mosquito vectors. In order for ovitraps to become effective as control agents in situations of multiple alternative oviposition sites (such as rice fields), the ovitrap should be at least as attractive, preferably more attractive than existing oviposition sites (Trexler et al. 1998). In the present study, nonane has been as attractive as breeding-site soil and the mixture of nonane and breeding-site soil induced a synergistic response. Therefore, if various signals are combined and emitted from specified breeding sites, gravid mosquitoes can be manipulated to lay their eggs on designated sites, which can easily be targeted for larvicide application. Therefore, the study reveals that the above attractants have the potential for use in developing a lure-and-kill system for the control of disease vectors.

Conclusion

This study shows that nonane and volatiles from natural breeding-site soil attract gravid females of *An. gambiae* and *Cx. quinquefasciatus* to sites containing these cues, and that both stimuli, once combined, act synergistically.

Acknowledgements

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Large water bodies near human settlements at Mvomero district, treating such sites with larvicides is difficult and expensive.

Chapter 7

Attract-and-kill strategy for mosquito larval control: effect of oviposition cues on the efficacy of the larvicides Temephos and Bacillus thuringiensis israelensis on malaria and lymphatic filariasis vectors.

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Submitted to Acta Tropica

Abstract

Mosquito females detect breeding sites using volatile chemical signals emanating from these sites. Recently, olfactory attractants were identified affecting natural populations of *Anopheles gambiae* and *Culex quinquefasciatus*. These cues can be exploited for inducing the mosquitoes to lay eggs in sites that contain selected toxicants so that mosquito offspring gets killed in the immature stage. The aim of the present study was to assess whether mosquitoes can be lured to lay eggs in insecticide-treated sites baited with attractive infochemicals.

In this study, the effect of the oviposition attractant nonane mixed with the synthetic insecticide temephos or the natural biocide *Bacillus thuringens israliensis* (*Bti*) on oviposition choice and larval development of the malaria vector *An. gambiae* was determined. Studies were done in a screened semi-field habitat and in a natural mosquito habitat in Tanzania. In semi-field results were scored according to the number of eggs laid in clay pots filled with a watery solution of nonane and temephos or *Bti* tested in a dual choice situation. In the field study the effects were assessed by studying the number of larvae and pupae developing in artificial breeding sites placed in the field in sunlit and shaded areas and containing one of the treatment mixtures. We also studied the number of adult mosquitoes that emerged from pupae collected in the artificial breeding sites

In semi field, mosquitoes laid eggs in all treatments. Pots with temephos alone contained significantly fewer eggs than pots with water, water + nonane or water + nonane + temephos. On the other hand, Bacillus thuringiensis israelensis did not affect the number of eggs laid. In all cases, the presence of nonane resulted in more eggs compared to pots without nonane. In the field study, both An. gambiae s.l. and Cx. quinquefasciatus were found. Mosquitoes laid eggs in all treated sites but with significant differences between treatments. For An. gambiae, the frequency of eggs laid in pots containing temephos was lower than in pots containing nonane only or Bti. Pots with nonane + temephos produced significantly fewer larvae than those with nonane only. There was no difference in larval numbers between pots containing nonane + Bti and nonane only. Oviposition of Cx. quinquefasciatus was not affected by treatment. All mosquito larvae in sites treated with either temephos or Bti, died before reaching the pupal stage. Sites treated with nonane alone were more productive for larvae than sites treated with temephos or Bti alone. In a sun-exposed environment, An. gambiae had a higher container index than under shady conditions, while the opposite was true for Cx. quinquefasciatus.

The addition of nonane to sites treated with either temephos or *Bti*, resulted in a greater number of visits of gravid females than either insecticide alone. Both biocides were effective in preventing pupal development. Temephos has a strong deterrent effect on gravid mosquito females of *An. gambiae* s.l. but not of *Cx. quinquefascitatus*, whereas *Bti* did not affect oviposition. *It is concluded that the attract-and-kill method has high potential to be implemented as a novel tool for mosquito vector control.*

KEYWORDS: *Anopheles gambiae*; *Culex quinquefasciatus*; oviposition; attractant; lure and kill; temephos; *Bacillus thuringiesis israelensis*

Introduction

Advances in chemical ecology have led to the development of technologies where pest insects including disease vectors can be manipulated by a skillful combination of attractants and/or repellents that lead them to traps or killing devices (Takken 2010, Mafta-Nero 2018, Mbare et al. 2019). When an attractant and a repellent are used simultaneously, the strategy is called push-pull (Cook et al. 2006, Menger et al. 2015, Wagman et al. 2015). When an attractant and an insecticide are used simultaneously, the strategy is called attract-and-kill (Michaelakis et al. 2007, Ong and Jaal 2015, Mafra-Neto and Dekker 2019). These strategies work best for insects in which there is proof that their behavioral responses are mediated through olfactory cues. In mosquitoes most behavioural responses are known to be mediated by olfactory cues e.g. mating (Takken and Knols 1999, Fawaz et al. 2014), host seeking (Zwiebel and Takken 2004, Takken and Verhulst 2017, Robinson et al. 2018) and oviposition site selection (Himeidan et al. 2013, Lindh et al. 2015, Eneh et al. 2019, Mwingira et al. 2019). Consequently, the development of an efficient attract-and-kill strategy for oviposition site-seeking mosquitoes could be a novel vector control tool that is simple and cost-effective (Rapley et al. 2009, Barbosa et al. 2010, Govella and Ferguson 2012, Mafra-Neto and Dekker 2019).

In this paper, we are introducing a special type of attract and kill strategy on *Anopheles gambiae* s.l. Giles and *Culex quinquefasciatus* Say, which are the most abundant mosquito vectors in rural and sub-urban areas of northern-eastern Tanzania (Mboera et al. 2010, Malima et al. 2013) Worldwide, these mosquito species have been frequently studied because of their roles as vectors of pathogens of humans and domestic animals. In Africa, *An. gambiae* s.l is the primary vector of the malaria parasite (Mboera et al. 2015, Pimenta et al. 2015, Traoré et al. 2019), whereas *Cx. quinquefasciatus* has been incriminated as a vector of lymphatic filarial worms (Mwakitalu et al. 2013, Simonsen and Mwakitalu 2013, Derua et al. 2017). Moreover, both mosquito species occur sympatrically in breeding sites and in human settlements where they seek blood from vertebrate hosts (Minakawa et al. 2004, Mwingira et al. 2009, Kweka et al. 2012). Our aim was to attract gravid mosquitoes to lay eggs in larvicide-treated areas so as to kill larvae as they hatch. We expect that this strategy will remove the next generation of mosquitoes and therefore reduce vector abundance.

Oviposition traps (ovitraps) have been used extensively for the surveillance of populations of mosquitoes (Velo et al. 2016, Silva et al. 2018). Consequently, ovitraps located around breeding sites or within human settlements may be an effective tool for control if both species are attracted to the same source. Strategies that target gravid mosquitoes would be useful if they control vectors that rest and bite both indoors and outdoors irrespective of their state of insecticide resistance (Okal et al. 2015). However, their efficacy in surveillance and also as “egg sinks” depends on their attractiveness to gravid mosquitoes compared with other, natural oviposition sites (Reiskind and Zarrabi 2012). The sensitivity of ovitraps to detect the presence of mosquitoes may be enhanced by the use of physical cues (Kumawat et al. 2014), chemical cues (Suman 2019) or both physical and chemical cues (Dhileepan 1997). Compounds or blends of compounds that lure more than one species of mosquito vectors to lay eggs in the same ovitraps are highly desirable for integrated vector control. Such behavioural manipulation may render ovitraps to become artificial breeding sites. As a control strategy, ovitraps have been modified either physically (Mackay et al. 2013), or chemically (Anderson and Davis 2014). For example, a sticky glue (Roslan et al. 2017), or an insecticide can be added to ovitraps in order to kill adults (Quimbayo et al. 2014, Long et al. 2015) and/or their immatures (Snetselaar et al. 2014). Recently, oviposition cues for *An. gambiae* have been identified and isolated. Cedrol, which attracts gravid females, was isolated from water taken from natural breeding sites (Lindh et al. 2015), and also nonane was isolated from water containing live larvae (Schoelitsz et al., 2020). Moreover, nonane was shown to be produced only in the presence of *An. gambiae* larvae, and is considered to be a putative oviposition pheromone. Nonane attracted gravid mosquitoes to artificial breeding sites in the field, demonstrating its potential to be exploited as a mosquito oviposition attractant.

The choice of a larvicide to be used in vector control programmes remains to be a challenge after realizing the negative ecological/environmental impacts following the use of chemical insecticides (Govindarajan and Benelli 2016). However, *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* are among the eco-friendliest and efficient larvicides used to decrease mosquito populations and to reduce disease transmission intensity, especially by *Culex* and *Anopheles* mosquitoes (Derua et al. 2019). The use of an oviposition attractant in the strategy is supported by the fact that such cues induce gravid females to lay eggs in sites treated with these cues (Barbosa and Regis 2011, Llagas 2011, Ponnusamy et al. 2015). In the present study we evaluated an attract-and-kill strategy for *An. gambiae* and *Cx. quinquefasciatus* in semi-field and field settings by combining nonane and *Bti* in one arm or nonane and temephos in the other arm.

Material and Methods

Components of the attract-and-kill system

Attractant

The tested chemical attractant used to lure mosquitoes in our study is the alkane compound nonane and was purchased from Sigma-Aldrich, The Netherlands (Lot and filling code: 132995235107188, ≥ 99.0%, Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands). Nonane is a volatile chemical which contains naphtene and isoparaffin originally identified in the headspace from mosquito larval habitats and found to be attractive to gravid *An. gambiae* (Chapter 4). Nonane is insoluble in water and therefore it was dissolved in methanol and tween20 in the following ratio: 55% of nonane + 40% methanol + 5% tween20. The mixture was further diluted in distilled water with one magnitude per dilution. Nonane was tested at a concentration of 5.5×10^{-5}M and it was paired with a control solution of distilled water + methanol + tween20.

Larvicides

The larvicides used in this study were temephos (Abate) and *Bacillus thuringiensis* var *israelensis* (*Bti*). Temephos is an organophosphorus insecticide that has been used as larvicide against several species of mosquitoes and black flies (Abe et al. 2014). Temephos used in this study was in a sand granule formulation at the operational dosage of 0.005g/l. The *Bti* used (Vectobac WG), was a wettable granule formulation with 3000 International Toxic Units (ITU)/mg at a recommended concentration of 0.01g/l. *Bti* is widely used for the control of mosquitoes and is environmentally safe with few effects on non-target aquatic organisms (Marina et al. 2014).

Semi-field study design

Screen spheres

Experiments were carried out in a screened house (mosquito-sphere) in the field at the Amani Research Centre of the National Institute for Medical Research which is located at Ubwari area (05°10’ 22” S, 38°46’73” E), Muheza district, Tanzania. The mosquito sphere has a length of 12.2m, width of 8.2m, and a height of 5m, and it allows normal flow of air, temperature, relative humidity and rainfall in a similar way to outdoor environment (Figures 1&2). Each mosquito sphere has a double door system which prevents escape or entrance of foreign population of mosquitoes from outside. To mimic the outside environment, the sphere contains different types of grasses, flowers, shrubs, banana plantation and a small hut built of mud bricks and coconut thatch (Kitau et al. 2010).

Figure 1 - Semifield environment (mosquito sphere) where experiments were conducted prior to field conditions.

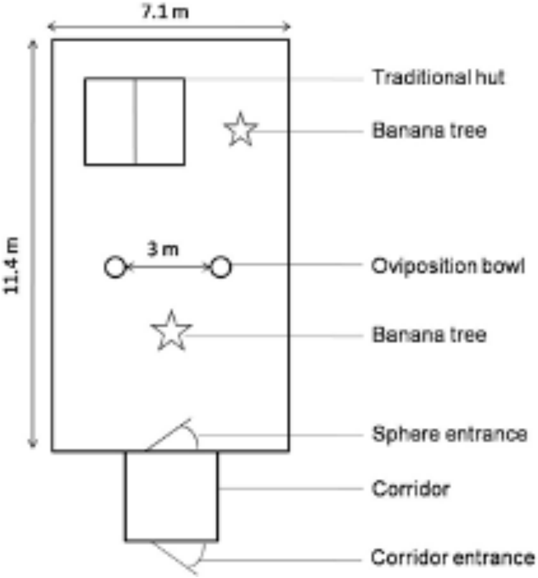


Figure 2 - Schematic top view of one of the three mosquito spheres

Mosquitoes

The mosquitoes (*An. gambiae* s.s. R70 strain) used in the semi-field experiment were reared in the laboratory of the Amani Research Centre. The original population was collected in Muheza, Tanzania, in 1990 and kept under laboratory conditions since that time.

While in the laboratory, mosquitoes were kept under 12h:12 h light: dark regime, at a temperature of 27 ± 1°C and relative humidity (RH) of 75 ± 5%. Adults were kept in 30 x 30 x 30 cm net cages and were given access to blood every other day. Moreover, in every cage, a 6% glucose solution in water was available *ad libitum*. Eggs were laid on wet filter paper and then placed in a plastic bowl with tap water for emergence. Larvae were fed on TetraMin baby fish food (Tetra, Germany) until they reach pupal stage. Pupae were collected from the bowls using a hand-held pipette and placed into plastic cups filled with tap water for emergence.

Bioassay conditions

The bioassay of oviposition attractiveness performed in mosquito spheres, utilized plastic bowls as artificial breeding sites. In each sphere, two plastic containers (with a diameter of 26 cm and a height of 10 cm) were used. The climatic conditions inside mosquito sphere were similar to ambient condition, with an average air temperature of (29 ± 1°C) and RH between 80 and 90%. These parameters were closely monitored during the entire experiment by using a Hobo® data logger. The mosquitoes intended for the experiments were placed in separate cages as pupae emerged. They had access to 6% glucose solution and received blood meals 2-3 days after emergence. On the experimental day, 5-6 day old gravid female mosquitoes were placed in release cages with access to a glucose solution.

Experimental design in screen spheres

Experiments were designed in a dual choice setup asdescribed previously (Schoelitsz et al., 2020), in which gravid mosquitoes (*Anopheles gambiae* s.s.) were allowed to choose among two identical oviposition bowls placed at the centre of sphere, 3m apart from each other. One of the bowls contained a test solution while the other a control solution. The bowls had a capacity of 5 l and each was filled with 3 l of either test or control solutions. A cage containing 250 gravid *An. gambiae* mosquitoes (5-6 d old) was placed at one side inside each mosquito sphere. At 17:30 h the cage was opened so that females could leave and select an oviposition site of their preference among the two identical oviposition bowls.

Semi-field interventions

Three screen houses were used at each experiment; the treated and control bowls were tested nine times, with three replicates per sphere, in which the position of bowls containing treated and control solution was exchanged between replicates. There were three days off between different experiments. Eggs were scored in two consecutive days, firstly after 12 and secondly after 36 h. Six dual-choice experiments were designed as follows: (i) distilled water + temephos against distilled water, (ii) distilled water + *Bti* against distilled water, (iii) distilled water + temephos + nonane against distilled water + nonane, (iv) distilled water + temephos + nonane against distilled water + temephos, (v) distilled water + *Bti* + nonane against distilled water + nonane, (vi) distilled water + *Bti* + nonane against distilled water + *Bti*. All experiments commenced during sunset, a period in which *An. gambiae* females are actively searching for oviposition sites (Sumba et al. 2004). The unit measure for all semi-field experiments was the number of eggs laid in each bowl. Eggs were counted and recorded, then transferred to the laboratory and reared until larval and/or adult stages. Any abnormal behaviour during egg hatching, larval, pupa and adult development was observed and recorded.

Field study design

Field site description

Digoma village is located Mvomero District (latitude 6°05.820'S, longitude 27°36.400'E), Morogoro region in east-northen Tanzania. It lies adjacent to the Nguu Mountains at an altitude of 1,160 m above sea level. Digoma has a mean annual rainfall of 1,146 mm and an average RH of 80%. Mean annual temperatures in the area vary between 19 - 31°C (Mtibwa meteorological station, 2008-2018). Rice cultivation is the main occupation of the inhabitants which takes place throughout the year using river water from the mountains for irrigation. There is bimodal type of rainfall in the area, whereby long rainy season ranges between April and June and a short rainy season in October – November. Due to the presence of the irrigated rice scheme, mosquito breeding sites proliferates even during the dry periods. Therefore, there are numerous breeding sites of *An. gambiae* and *Cx quinquefasciatus* throughout the year. Apart from that, maize and sugarcane plantations are among crops cultivated in the area. Other economic activities of inhabitants include keeping of domestic animal such as cattle, goats, pigs, sheep and poultry.

Site selection

Four sites (north, south, east and west) were selected in an area covering a total of 4 ha. on both sides of a river at Digoma village, (6˚ 05.820'S, 27˚ 36.400'E). This area was selected based on the following criteria: proximity to the river basin, presence of rice fields, absence of flooding, openness or presence of short grass and proximity to human settlements. Rice growing is the main economic activity. All sites were surrounded by a wire mesh to prevent humans, animals or frogs from interfering with the experiments. In addition, a local field worker was hired to oversee the site during the entire study period.

Artificial breeding sites

Artificial breeding sites used in the field were clay pots which had an average diameter of 200mm and a depth of 100mm (Fig.3). They were made locally from clay soil, moulded by hand to make a pot shape and left to dry; where after they were cured by fire. In our study, clay pots were positioned in the ground so that the margins of the pots were at level with the surrounding ground. The pots were placed in the valley plain, within a rice field in the vicinity of a village. Based on larval survey during the baseline study, clay pots were selected because they simulate natural breeding sites better than other types of artificial breeding sites.

Field experimental design

Pots were placed at selected sites in the field 72 h before the start of the experiment and filled till capacity with distilled water; water was replenished until the clay reached saturation. Prior to the start of the experiment, the clay pots were emptied and immediately filled with 1L of the oviposition substrate (treatment or control) one h before sunset. Oviposition pots were left undisturbed for five days and from the 6th day, pots were inspected every morning and larvae were collected and recorded daily from 06:30 am. Whenever the water level decreased in the pots, distilled water was added to maintain the water level. Figure 4, illustrates the schematic diagram of the experimental setup from the day of preparation till the last day of sampling.

Figure 3 - A clay pot that was used as an articial breeding site in the field

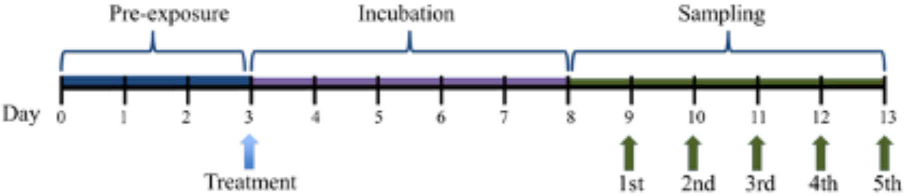


Figure 4 - The experimental timeline: for 3 days clay pots were exposed to the field with distilled water; after day 3, distilled water was removed, treatment and control substrates were poured. On day 9, the first round of larval sampling started consecutively until day 13.

Larvae were then transferred to a temporarily established local laboratory and reared under controlled conditions. Larvae were transferred together with the water in which they were found. This water was used as rearing substrate in the laboratory for the first 24 h. After that time, larvae from each oviposition pot were transferred to mosquito rearing bowls which contained distilled water. Rearing bowls were placed below light bulbs and larvae were fed Tetramin® fish food twice daily. Larval growth and development were observed and recorded until pupation and adult formation. Pots containing experimental substrates remained in the field for 10 d where after the substrates were removed; the pots were cleaned and replaced. For each pair of treatment and control, the pots were oriented facing East and West positions, and these positions were exchanged for each replicate.

Interventions in the field

The three treatments that were tested during the field experiments were: (i) the control treatment in which distilled water was mixed with nonane (5.5×10^{-11} M) in distilled water, (ii) a temephos solution (0.005g/l) mixed with nonane and (iii) a *Bti* solution (0.01g/l) mixed with nonane. Experiments in this study were designed as a dual choice set up whereby at each site two artificial breeding sites (clay pots) were placed in the soil (Fig 3). One of the pots contained the treated substrate while the other a control substrate (nonane); pots were separated 3m apart in the same procedure as in the semi-field environment. Clay pots were placed 3m away from each other (i) in an open field with exposure to direct sunlight and rain (sun-lit site); and (ii) under a cover of thatched coconut leaves (at 1m distance from the soil) to prevent direct exposure to sunlight and rain (shady site). In the field 30 replicates of treatments and controls were designed at sunlight and shaded sites to attract natural population of mosquitoes. The unit measure in field experiments was the number of larvae found in clay pots.

Scoring larval mortality

The mortality of mosquito larvae following exposure to temephos and *Bti* were observed in the semi-field and evaluated in detail during the field experiments. Laboratory studies with susceptible *An. gambiae* mosquitoes showed that exposure to a larvicide, even for one day, debilitated growth and eventually caused larval mortality. During the field studies, early stage larvae were collected from treated and control clay pots. Immediately they were counted and transferred to the local insectary to be raised in the normal procedure. Larvae from each pot were transferred to a specific rearing tray in the laboratory and were pooled according to treatment. Mortality was scored in the laboratory at six hour intervals.

Species identification

Anopheline larvae identified as *An. gambiae* s.l. were further analysed to sibling species i.d.by molecular tools according to van Rensberg et al. (1996).

Data and statistical analysis

For direct comparison between treated and control bowls within a pair; the difference in number of larvae between control and treated pots was ascertained by Wilcoxon sign ranks for the semi-field data. The Mann-Whitney U-test was used to compare between treatment and control pairs in the field. For indirect comparison between different pairs; firstly, the mean response rate was calculated as the mean proportion (%) of larvae in either or both pots during each experimental replicate. Then, independent effects of pot position, pot location, or day on larval density were tested using a generalized linear model with log link function and negative binomial distribution.

Ethical clearance

The study was conducted according to standard operating procedures approved by the Medical Research Coordinating Committee (MRCC) of the National Institute for Medical Research (NIMR). It received a research permit from MRCC with reference number NIMR/HQ/R.8a/Vol. IX/573 and a permit from the Commission for Science and Technology of Tanzania with reference number CST/RCA 138/225/2008.

Results

Semi-field experiments

Impact of attractants on the efficacy of larvicides (*Bti* and Temephos)

Anopheles gambiae preferred to oviposit in containers with distilled water over containers treated with temephos. Containers with temephos had three times fewer eggs as compared to containers with distilled water (OAI = -0.58, p = 0.008, Mean $_{[temephos]}$ = 40.3 ± 5.2, Mean $_{[dwater]}$ = 150.4 ± 17.6) (Fig. 5). When temephos was mixed with nonane and tested against nonane only, this effect disappeared and eggs were distributed equally among the two containers (OAI = -0.01, p = 0.86, Mean $_{[temephos+nonane]}$ = 452.1 ± 51, Mean $_{[nonane]}$ = 464.8 ± 62.1). Moreover, when a mixture of temephos and nonane was tested against a control of temephos, five times more eggs were found in the container containing temephos + nonane (OAI = 0.68, p = 0.008, Mean $_{[temephos+nonane]}$ = 462.7 ± 56.9, Mean $_{[temephos]}$ = 87.8 ± 12.4) (Fig. 5).

Containers with *Bti* had more eggs compared to containers with distilled water (OAI = 0.14, p = 0.011, Mean $_{[Bti]}$ = 370.3 ± 30.4, Mean $_{[dwater]}$ = 277.4 ± 18.7). However, when *Bti* was mixed with nonane and tested against nonane, eggs were distributed equally among the two containers (OAI = 0.03, p = 0.3, Mean $_{[Bti+nonane]}$ = 833.7 ± 46.2, Mean $_{[nonane]}$ = 780 ± 38.6). Moreover, when the mixture of *Bti* and nonane was tested against *Bti*, the number of eggs found in the mixture container was fivefold higher than the *Bti* container (OAI = 0.68, p = 0.008, Mean $_{[Bti+nonane]}$ = 1014 ± 34.4, Mean $_{[Bti]}$ = 192.9 ± 30.3) (Fig. 5).

Field experiments

In total 3,487 immatures of *An. gambiae* and 7,931 immatures of *Cx. quinquefasciatus* were collected. Two hundred specimens of *An. gambiae* s.l. larvae collected in the field study were identified to species by PCR. Results shows that 86% were *An. gambiae s.s*. and 14% *An. arabiensis*.

Impact of attract-and-kill strategy on abundance of larvae

The temephos + nonane pots had fewer *Anopheles* larvae compared to pots containing nonane only. The number of larvae found in control pots was significantly higher than in treated pots (OAI = -0.8, $p < 0.05$, mean difference for shade, M$_{[shade]}$ = 82.5%, mean difference among sunlit, M $_{[sunlit]}$ = 83.6%), (Tables 1a and 1b). Similarly, for *Culex*, the number of larvae found in control pots was significantly higher than those found in treated pots (OAI = -0.2, $p <$ 0.05; M $_{[sunlit]}$ =22.3%, M $_{[shade]}$ = 25%), (Tables 2a and 2b). Among the pots that were in the shade, the sex ratio of *An. gambiae* s.l. deviated significantly from

50% ($p<0.05$, χ^2test) in favour of females (Table 3a). With *Culex* the sex ratio did not significantly differ from the 1:1 assumption ($p > 0.05$; χ^2 test) (Table 3b) Generally, the pots with *Bti* + nonane contained more larvae compared to the temephos + nonane pots. For *An. gambiae*, there was no significant difference between the number of larvae found in nonane and *Bti* + nonane treated pots (OAI = 0.09, $p> 0.05$; $M_{[sunlit]} = 8.7\%$, $M_{[shade]} = 23.2\%$). For *Culex*, there was a significant difference between the number of larvae found in control and treated pots (OAI = 0.2, $p < 0.05$; $M_{[sunlit]} = 24\%$, $M_{[shade]} = 18\%$). All larvae found in treatments containing either *Bti* or temephos died before reaching the pupal stage.

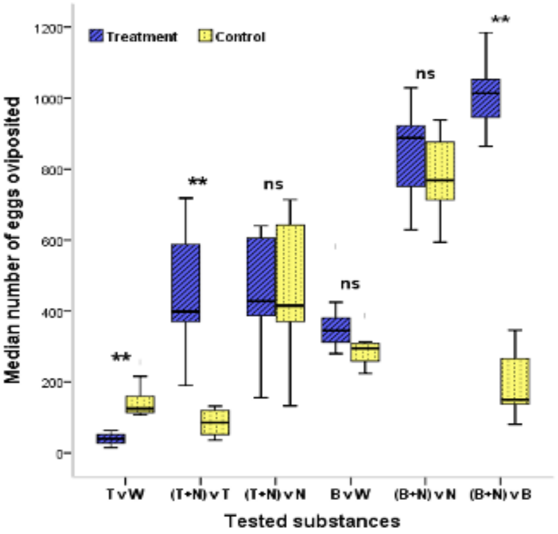
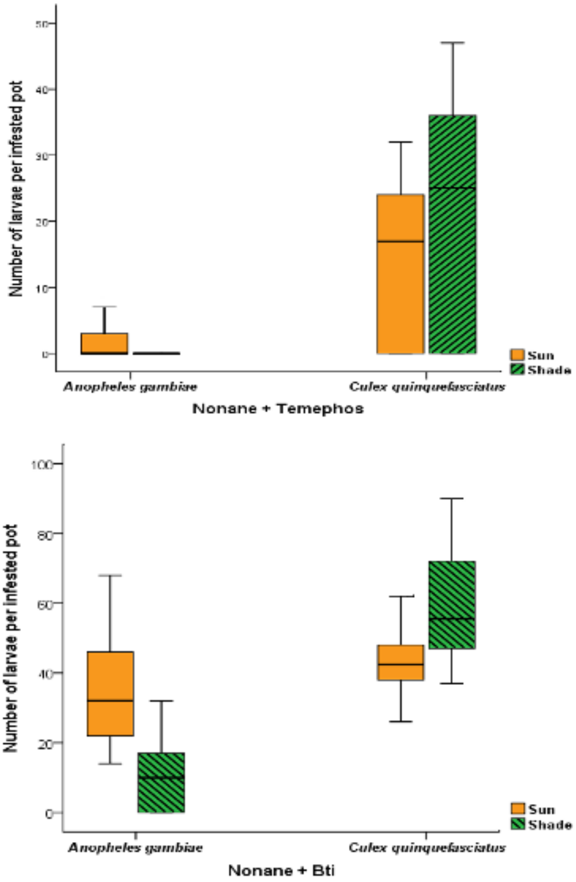


Figure 5 - Oviposition preference of *An. gambiae* to substrate containing attractants and/or toxicants in a dual choice semi-field setup. Asterick indicates statistical differences between treatment and control for a given pair (*: $p<0.05$, Wilcoxon signed rank tests). T stands for Temephos, W stand for distilled water, N stand for nonane, and B stand for *Bti*

Effects of sunshine and shade on the attract-and-kill strategy

Seventy percent of all *An. gambiae* larvae were found in pots located at sunlit sites while only about 30% were found under the shade (Fig. 6). On the other hand, about 40.5% of all *Cx. quinquefasciatus* larvae were found in pots located at the sunlit sites while about 59.5% were found under the shade. In dual choice setup, the control pots which were adjacent to the *Bti* + nonane intervention had more larvae than control pots which were adjacent to a temephos + nonane intervention, regardless of being exposed to the sun or shade (Table 1a). When *Bti* and temephos were compared indirectly, the mean ratio of larvae was higher for *Bti* than for temephos for all species ($p<0.01$, GLM) (Tables 1a, 1b, 2a and 2b). Within the same lighting condition, larvae of *Cx. quinquefasciatus* were more abundant than larvae of *An. gambiae*. The presence of a larvicide did neither alter the preference of *An. gambiae* to lay eggs in sunlit pots, nor the preference of *Cx. quinquefasciatus* to lay eggs in pots located under the shade. Also, lighting conditions had no effect on the outcome of the attract-and-kill intervention for both *An. gambiae* and *Cx. quinquefascitus*.



Figures 6a & b - The oviposition response of gravid mosquitoes when provided with breeding sites containing attractant nonane mixed with larvicides in sunlit and shaded area.

Table 1a - Oviposition response of a natural population of *An. gambiae* to oviposition pots treated with nonane and nonane + temephos or nonane + Bti in different light regimes

Treatment	Pot with larvae		Quantity of larvae			Test of significance	
	No.	P. Index	No.	(%)	Mean ± SE	OAI	P-value
Sunlit habitats							
Nonane + temephos	8	0.27	45	8.2	1.5 ± 0.5	- 0.84	0.0001
Nonane	23	0.77	504	91.8	16.8 ± 2.4		
Nonane + Bti	30	1.00	1036	54.4	34.5 ± 2.7	+ 0.09	0.13
Nonane	29	0.97	870	45.6	29.0 ± 2.8		
Shaded habitats							
Nonane + temephos	6	0.2	20	8.7	0.7 ± 0.3	- 0.83	0.0001
Nonane	14	0.47	210	91.3	7.0 ± 1.6		
Nonane + Bti	18	0.6	308	38.4	10.3 ± 1.8	- 0.23	0.077
Nonane	17	0.57	494	61.6	16.5 ± 3.4		

Table 1b - Oviposition response of a natural population of *Cx. quinquefasciatus* to oviposition pots treated with nonane and nonane + temephos or nonane + Bti in different light regimes

Treatment	Pot with larvae		Quantity of lervae			Test of significance	
	No.	P. Index	No.	(%)	Mean ± SE	OAI	P-value
Sunlit habitats							
Nonane + temephos	19	0.63	422	37.8	14.0 ± 2.14	- 0.24	0.0001
Nonane	22	0.73	693	62.2	23.1 ± 2.89		
Nonane + Bti	30	1.00	1302	62	43.4 ± 1.85	+ 0.24	0.0001
Nonane	25	0.83	799	38	26.6 ± 2.73		
Shaded habitats							
Nonane + temephos	20	0.67	650	37.6	21.7 ± 3.1	- 0.25	0.0001
Nonane	24	0.80	1079	62.4	36.0 ± 4.1		
Nonane + Bti	30	1.00	1765	59.1	58.8 ± 2.7	- 0.18	0.001
Nonane	27	0.90	1221	40.9	40.7 ± 3.4		

Table 2a - The number of *Anopheles gambiae s.l.* larvae, pupae and adults (mean ± s.e.) collected from clay pots treated with nonane + temephos or nonane + Bti

Treatment value	larvae	Pupae	Adults		Sex ratio	P-
			males	females		
Sun lit habitats						
Nonane + temephos	1.5 ± 0.5	0	-	-	-	-
Nonane	16.8 ± 2.4	16.4 ± 2.3	7.8 ± 1.1	8.5 ± 1.2	47.8	0.34
Nonane + Bti	34.5 ± 2.7	0	-	-	-	-
Nonane	29.0 ± 2.9	28.1 ± 2.6	13.1 ± 1.2	14.5 ± 1.3	47.5	0.15
Shaded habitats						
Nonane + temephos	0.7 ± 0.3	0	-	-	-	-
Nonane	7.0 ± 1.6	6.7 ± 1.5	2.8 ± 0.6	4.0 ± 0.9	41.1*	< 0.01
Nonane + Bti	10.3 ± 1.8	0	-	-	-	-
Nonane	16.5 ± 3.4	16.0 ± 3.2	5.8 ± 1.2	10.1 ± 2.0	36.6**	< 0.001

Sex ratio was calculated based on the number of individual that emerged. The ratios that deviated significantly form 0.5 are indicated by asterisks (*P<0.05, **P<0.01, $\chi 2$-test).

Table 2b - The number of *Culex quinquefasciatus*. larvae, pupae and adults (mean ± s.e.) collected from clay pots treated with nonane + temephos or nonane + Bti

Treatment value	larvae	Pupae	Adults		Sex ratio	P-
			males	females		
Sun lit habitats						
Nonane + temephos	14.1 ± 2.1	0	-	-	-	-
Nonane	23.1 ± 2.1	22.9 ± 2.9	11.1 ± 1.4	11.8 ± 1.5	48.5	0.445
Nonane + Bti	43.4 ± 1.9	0	-	-	-	-
Nonane	26.6 ± 2.7	26.4 ± 2.7	12.9 ± 1.3	13.5 ± 1.4	48.9	0.522
Shaded habitats						
Nonane + temephos	21.7 ± 3.1	0	-	-	-	-
Nonane	36.0 ± 4.1	35.0 ± 3.9	17.1 ± 1.9	17.1 ± 2.0	49.1	0.557
Nonane + Bti	58.8 ± 2.7	0	-	-	-	-
Nonane	40.7 ± 3.4	39.6 ± 3.2	19.5 ± 1.9	20.6 ± 1.7	48.6	0.341

Discussion

The presence of the insecticides temephos or *Bti* did not prevent mosquitoes from laying eggs in clay pots. Both insecticides were highly effective in killing larvae, and this occurred much faster with temephos than with *Bti*. Pots with temephos or *Bti* did not produce adult mosquitoes. Nonane was highly attractive to gravid *An. gambiae* and *Cx. quinquefasciatus* and stimulated oviposition in semi-field and field situations. The effect of nonane was not affected by temephos or *Bti*. Shade had a different effect on the oviposition behaviour of *An. gambiae* than on *Cx. quinquefasciatus*.

Most insect attractants are volatile odorants and therefore evaporate easily with increasing sunlight and temperature. Moreover, direct sunlight has an effect on most insecticides causing deterioration and loss of activity (Mountacer et al. 2014, Peck et al. 2014). This makes sunlight to be an important aspect to be considered when designing an attract-and-kill strategy. In this study, oviposition pots in the open space had twice as many larvae of *An. gambiae* s.l. than those under shade, both with temephos and *Bti*, suggesting that sunlight and shade both influence oviposition behaviour and/or treatment effect of the larvicide on this mosquito species. In contrast to *An. gambiae* s.l., *Cx. quinquefasciatus* females laid more eggs in the shady sites than in sunlight. This is not surprising, as the latter species is known to oviposit in latrines and dark holes (Maxwell et al. 1990). This observation is supported by studies where Napier grass covered sites did not produce *An. gambiae* larvae, which was ascribed to the effect of deep shade (Wamae et al. 2010). Analysis of the control group also shows that oviposition pots that were placed in an open, sunlit area had twice as many larvae than pots that were placed in the shade. This means that, mosquitoes lay more eggs in their preferred sites and they locate these sites by the use of physical and/or chemical cues. They are able to discriminate between different oviposition substrates (Mwingira et al., 2019), by detecting colour, microclimate (Huang et al. 2005, Okal et al. 2013), and even microorganisms (Huang et al. 2006, Lindh et al. 2008). The sex ratio among the emerged *An. gambiae* was also affected by light condition. In the shade, more females were produced than in the open space (Table 3a), suggesting that a shift towards more females was a response due to the absence of sunlight. In contrast to *An. gambiae* s.l., shade was more attractive to ovipositing *Cx. quinquefasciatus*, which deposited more eggs in shady sites than in sunlit sites.

From these results we conclude that any mosquito control intervention that targets larvae, must consider such preferential selection of oviposition sites in order to be successful.

Attract-and-kill as a new vector control strategy

An important requirement for an attract-and-kill system be successful, is the effectiveness of attractants to lure mosquitoes into designated breeding sites equally or more than existing natural breeding sites (Trexler et al. 1998). Our study demonstrates that nonane and/or odour from natural breeding sites can be used to enhance the number of eggs deposited in Temephos and *Bti*-treated oviposition pots. The chemical nonane, which originally was identified from the headspace of *An. gambiae* larvae, attracted gravid females of both *Anopheles* and *Culex* mosquitoes. Earlier studies from rice fields show that *An. gambiae* s.l. and *Cx. quinquefasciatus* share the same aquatic habitats (Mwangangi et al. 2008, Mwingira 2009, Mboera et al. 2011) and therefore it is not surprising that *Culex* mosquitoes seem to use odorants produced by *Anopheles* for orientation and selection of oviposition sites, as these serve as a reliable cue signaling the presence of a suitable breeding site. With the advent of odorants (or their blends) that attracts multiple species of mosquito vectors, an attract-and-kill strategy became practical towards improving an integrated vector management programme.

A stable attractant in the field

In a successful attract-and-kill system, the attractant should not affect the killing effect of an insecticide, i.e. an attractant should be able to continue luring mosquitoes after it has been mixed with insecticides. In this study, the oviposition attractive index for *An. gambiae* at a sunlit breeding site and under shade with temephos and *Bti* indicates that *Bti* did not influence the choice of oviposition site, while temephos appeared to deter *An. gambiae*, but not *Cx. quinquefasciatus*, from ovipositing. This deterrent effect was overruled when nonane was present in the oviposition bowl. This result suggests that both larvicides when mixed with the oviposition cue nonane are not deterring gravid mosquitoes away from breeding sites. This is similar to a previous study with *Cx. quinquefasciatus* where the entomopathogenic *Bacillus sphaericus* (Bs) did not influence the choice of an oviposition site when used in combination with grass infusion (Barbosa et al. 2007). In this way eggs are continually being deployed in the medium containing a larvicide and thus, the attract-and-kill strategy is successful.

The choice of an insecticide and the efficacy of a strategy

A successful attract-and-kill strategy requires an insecticide which kills the target organism when it is applied together with an attractant with no adverse effects on non-target organisms. In this study, the efficacy of both larvicides was 100%. However, *Bti* acts relatively slowly on larvae as the larvae ingest the bacteria and die because the endotoxins are released in the midgut, where they inhibit transition from larvae to pupae. *Bacillus thuringiensis israelensi*s is the preferred larvicide above temephos, as it is of natural origin and unlikely to induce resistance due to its operation with multiple endotoxins, unlike temephos which acts on one toxin only. The *Bacillus*-based insecticides are known for their ability to attract mosquitoes, and therefore have dual roles which signifies their position within an attract-and-kill system (Poonam et al. 2002). In the assay with larvicides, mosquitoes laid their eggs in both treated and untreated oviposition pots. However, the highest number of larvae in the treated pots was found on the *Bti*–control pairs.-Therefore, *Bti* is an efficient alternative to chemical insecticides not only when a metabolic resistance to chemicals has been developed by mosquitoes (Araujo et al. 2013, Tetreau et al. 2013), but also because of its dual role in attracting and killing of mosquitoes.

In this study, we found a significant difference between the number of larvae found in pots with temephos and their controls for both categories of breeding sites. This suggests that temephos acts on eggs or young larval instars and therefore reduces larval populations before they reach second or higher developmental stages. Temephos is deposited as a thin film over the water surface where its solubility results in a high concentration at the surface where eggs and larvae of anophelines exists, and it kills by disrupting normal neurotransmission through inhibition of acetylcholinesterase (Lacorte 1996). This accounts for the observed rapid killing effects observed in our study. The toxic effect of *Bti* is more slowly, as the pathogen needs to be ingested by the insect, and the endotoxins that have the killing action are only released in the midgut (Palma et al. 2014).

Potential of lure-and-kill strategy and its sustainability

The observed 100% reductions of larvae in this study is likely to affect disease transmission such as malaria and lymphatic filariasis. However, the success of such stratergies will largely depend on their applicability and cost effectiveness (Mafra-Neto and Dekker 2019). The attract and kill system used in this study involves clay pots, larvicides and an attractant, which are effective, affordable and applicable in an African rural settings. Such effective interventions need to be scaled up and added to the existing vector control tool in order to benefit the integrated vector control programmes (Russell et al. 2011, Cotter et al.

2013, WHO 2018). Consequently, our resultssuggest upscaling of the study to community level by developing a treat-yourself-kit containing an attractant and a biolarvicide that can easily be formulated in the evening at the backyard. Such a system can be initiated and maintained by house owners under the supervision of community health officers.

Conclusion

This study provides evidence that gravid females of *An. gambiae* and *Cx. quinquefasciatus* can be manipulated by oviposition stimuli to lay eggs in oviposition containers containing larvicides. This is the first time that the African malaria vector *An. gambiae* is affected successfully by an attract-and-kill system. These findings are novel and timely, due to the fact that existing vector control methods (ITN and IRS) are challenged by insecticide resistance, change in biting behaviour (Moiroux et al. 2014, Cooke et al. 2015)and change in composition of vector populations. Therefore the attract and kill strategy offers a posible alternative or an additional intervention to current vector control methods.

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Chapter 8

General discussion

New ideas on an old strategy

Adult mosquito control using long-lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) has lowered the burden of malaria and renewed hopes for eliminating the disease in sub-Saharan Africa (WHO 2017). The decline in malaria burden is a result of reduction in biting rates as well as in mosquito abundance. Nevertheless, malaria transmission persists due to the ability of mosquitoes to develop resistance to existing insecticides, divert biting and resting activities outdoors, and change their biting time as well as seek blood from other vertebrates. These challenges make malaria elimination elusive in the region and reveal a growing need for supplementary vector monitoring and control methods (WHO 2018). It was therefore, the main objective of this study to identify mosquito oviposition infochemicals and explore their potential in the surveillance and control of malaria vectors in Tanzania.

This study is based on evidence that gravid mosquitoes are guided by infochemicals to select specific breeding sites which are preferred due to their suitability. These preferred larval habitats are usually few in number and therefore can easily be targeted for the application of appropriate interventions. Identification of infochemicals that mediate mosquito oviposition behaviour can help in the development of novel control and surveillance tools. Historically, many successful mosquito eradication campaigns relied heavily on management of larval habitats (Killeen et al. 2002b). However, due to environmental concerns and the focus on adult mosquito control methods, habitat-based programmes have been mostly abandoned. As an integral component of integrated vector management, the importance of larval interventions has recently regained the attention of public health experts after long being obsolete in malaria control (Killeen et al. 2002a, Tusting et al. 2013, Williams et al. 2018). The renewed interest in larval interventions has been accompanied with the development of environmentally friendly and powerful biolarvicides such as *Bacillus thuringiensis israeliensis* (*Bti*) (Caragata and Walker 2012, Nartey et al. 2013, Dambach et al. 2014, Derua et al. 2019) and increasing understanding of larval ecology and systematic distribution of aquatic habitats (Minakawa et al. 2004, Koenraadt et al. 2006, Mala and Irungu 2011, Ndenga et al. 2011).

The discovery of infochemicals mediating oviposition behaviour in mosquitoes (Walker and Lynch 2007, Newman et al. 2013) is likely to increase the utility of larval source management as a tool for mosquito-borne disease control. Mosquito control and surveillance strategies must be augmented with olfactory cues in order to increase their effectiveness (Logan and Birkett 2007, Watentena and Okoye 2019). Already there has been a considerable interest in potentially exploiting mosquito olfaction to manipulate vector populations to our advantage (Pickett et al. 2010, Carey and Carlson 2011, Schorkopf et al. 2016). The identification of infochemicals that mediate oviposition behaviour in mosquitoes, such as those identified in this study, will pave a way to the development of novel, cost-effective and environmentally friendly control techniques.

Four main research objectives were formulated in the introduction of this thesis (Chapter 1), based on the following questions i) Which factors influence the productivity of malaria vectors?; ii) Does the mosquito *An. gambiae* discriminate between breeding sites and select suitable sites preferentially?; iii) Which chemical compounds are emitted by larval habitats and mediate oviposition behaviour in *An. gambiae*?; iv) Can oviposition attractants be used to enhance surveillance and control of malaria mosquitoes?

The impact of productive mosquito breeding sites on malaria transmission

The findings of this study have demonstrated clearly that there is heterogeneous distribution of larvae among habitats in natural breeding sites. This skewed distribution of larvae among breeding sites is in line with various studies conducted in elsewhere (Wondwosen et al. 2016, Eneh et al. 2019). In the current study, potential habitats were identified, characterized and quantified as highly, moderately, and non-productive sites. Mapping and identification of productive breeding sites is a pre-requisite for larval source management operations. In principle, larviciding should be considered as a tool for disease control when mosquito breeding sites are few, fixed, and findable (WHO 2012). As mosquito abundance decreases due to extensive use of LLINs and IRS programmes, the productivity of breeding sites also decreases. In this case, it is relatively easy and cheap to identify, treat and monitor the few breeding sites. Such informed larval interventions can play a critical role in combating malaria in Africa (Gu and Novak 2005). Prolific habitats have a great epidemiological significance, as they contribute to the existence of malaria hotspots. Such areas emit infochemicals that inform gravid mosquito the presence of suitable habitats for their offspring, and they support the growth and development of immature stages of mosquito vectors.

The ability of mosquitoes to discriminate between breeding sites and preferentially select suitable sites

The current study indicated that the presence of early-stage larvae in a breeding site attract conspecific gravid *Anopheles gambiae* sensu stricto (hereafter referred to as *An. gambiae*) females in the laboratory. However, the presence of late-stage larvae deters gravid *An. gambiae*. Moreover, the magnitude of attractiveness or deterrence increased with increased number of larvae. When larvae were masked and excluded from visual and tactile cues, conspecific gravid females still laid more eggs in sites containing early-stage larvae, indicating the involvement of olfactory cues (Mwingira et al. 2019). From these results, it was concluded that breeding sites containing conspecific larvae produce volatiles that mediate the oviposition-site seeking behaviour of *An. gambiae* and that the females could discriminate between sites based on olfactory cues (Chapter 3). This discriminatory behaviour of gravid females was further confirmed in the semi-field and field studies, in which female *An. gambiae* were significantly attracted to sites baited with nonane or soil-associated volatiles. Interestingly, gravid females of the sympatric *Culex quinquefasciatus* were also attracted to these stimuli, confirming results from studies elsewhere that demonstrate mediation of oviposition by infochemicals associated with the breeding sites (Mboera et al. 2000, Turnipseed et al. 2018, Mafra-Neto and Dekker 2019). The novelty of this research is that a specific chemical cue, nonane, was identified as a strong oviposition attractant for both *An. gambiae* and *Cx. quinquefasciatus*. Despite the apparently powerful behavioural effects of chemicals, it is generally accepted that the behaviour of mosquitoes is not governed by a single external chemical stimulus, but mediated by a large number of external and internal stimuli (Navarro-Silva et al. 2009, Brown 2015). It can be concluded therefore that, *An. gambiae* and *Cx. quinquefasciatus* share some breeding sites because they use similar semiochemicals to locate and select suitable breeding sites.

Elucidation of habitat-derived compounds that mediate oviposition behaviour in mosquitoes

In this study, 16 putative attractants were found in the headspace of a bowl that contained live larvae. Out of these, four compounds were selected for further testing: dimethyldisulfide, dimethyltrisulfide, nonane and 2,4-pentanedione. Testing them one by one resulted in the identification of compounds that either attracted or deterred gravid *An. gambiae* (Chapter 4). Synthetic versions of these four compounds were tested on gravid *An. gambiae* and were shown to mediate oviposition behaviour in laboratory and semi-field experiments (Chapter 4). Nonane was the most promising oviposition attractant candidate. Studies under natural conditions in central-eastern Tanzania showed that this compound was a strong attractant for wild *An. gambiae* as well as for the sympatric *Culex quinquefasciatus* (Chapter 6).

The studies described in this thesis demonstrate that identification of emanations from a larval habitat is an effective method for the identification of compounds that mediate mosquito oviposition behaviour. The study has led to the identification of new compounds that influence the behaviour of mosquitoes, and that compounds emitted by one species, can affect other mosquito species as well. The study has shown that nonane induces *An. gambiae s.s.*, *An. arabiensis* and *Cx. quinquefasciatus* to oviposit in the same breeding site. Such a finding is crucial in the development of an integrated vector control programme that targets different species of mosquitoes.

Oviposition attractants in surveillance and control of malaria mosquitoes

The identification of mosquito infochemicals, and the possibility of developing an ovitrap for gravid mosquitoes that contains a blend of infochemicals and environmentally-friendly insecticide, would be of great benefit to mosquito surveillance and control programmes. In Chapter 6, the oviposition infochemicals were shown to be of great promise for surveillance and control of *An. gambiae* and *Cx. quinquefasciatus*. The data suggest that if breeding containers/sites are baited with nonane, they can serve as reliable surveillance tools to monitor adult mosquitoes. This is particularly relevant in programmes such as the Global Vector Control Programme, where enhanced surveillance is one of the four pillars of action (WHO 2017). Furthermore, a mosquito population can potentially be reduced by using oviposition-modifying chemicals. Therefore, nonane and such chemicals were used to develop an attract-and-kill strategy, which has the potential to supplement currently available vector control tools (Chapter 7). It is interesting to note that two mosquito species can be controlled by using attract-and-kill techniques, when nonane is applied as an attractant.

Socio-economic implications of the findings

For a long time, much research on the development of mosquito vector control tools relied on exploitation of mosquito host-seeking behaviour (Gibson and Torr 1999). This was because details of other mosquito behaviours were not fully understood. For example, it was not fully known how females locate suitable breeding sites, and/or why a female lay her eggs in one habitat and not others. This current study has explored oviposition behaviour, and specifically how it can be exploited to strengthen existing control strategies. Such a knowledge base is critical for decision and policy making in mosquito control programmes.

The spatial distribution of mosquito breeding sites is a result of the behaviour of the females when searching for a suitable site to lay eggs. Therefore, knowledge on mosquito oviposition site selection may aid in the appropriate and timely identification of prolific breeding sites, which can then be targeted using cost-effective environmentally friendly interventions. Such gains in mosquito vector control can help to reduce the burden of malaria and other mosquito-borne diseases.

The attract-and-kill system reported in this thesis can be deployed at household level, and is a relatively simple method of mosquito control compared to other vector control tools. Community-based mosquito control programmes have already shown considerable potential for sustained, rapidly responsive, data-driven and affordable application (Lindsay et al. 2004, Fillinger et al. 2008, Dongus et al. 2011).

One of the major challenges in promoting environmental management programmes is the blanket treatment of all aquatic habitats, which is clearly beyond the reach of most resource-deprived communities in sub-Saharan Africa (Gu et al. 2008). However, results from habitat-based programmes show that it is not necessary to manage all aquatic habitats to obtain significant reductions in the incidence and prevalence of malaria in situations of low and intermediate levels of transmission (Gu and Novak 2005). Ideally, larval control activities should be targeted at sites that generate the most adult vectors, thereby reducing operational costs (Fillinger et al. 2009) and reducing insecticide costs. Consequently, evidence of the effect of control strategies that exploit mosquito olfactory cues and focus on only prolific habitats or intended species has increased in recent years (Eneh et al. 2016, Mbare et al. 2019).

It is now clear that larval-based control interventions can have a significant impact on disease incidence and prevalence. For example, application of the larvicide temephos to the aquatic breeding sites of *Aedes aegypti* has been a key part of the dengue control strategy (Grisales et al. 2013, Mazigo et al. 2019). Moreover, combining a fertilizer and larvicides has been described as a novel anti-mosquito tool in community-based mosquito vector control (Darriet 2016, Mazigo et al. 2019). Furthermore, a model combining insecticide-treated mosquito nets and larval control has predicted that a 50% reduction in vector emergence from breeding sites could contribute to an overall 15- to 25-fold reduction in entomological inoculation rate (EIR), even in highly endemic areas (Killeen et al. 2000). A further reduction can be achieved when tactics such as attract-and-kill and other behaviour-mediating interventions are incorporated into integrated mosquito management. The work presented in this thesis clearly shows that that the attract-and-kill techniques strongly affect the development of *An. gambiae* and *Cx. quinquefasciatus* in their natural settings, and therefore is likely to complement the existing integrated vector control tools for the management of mosquito-borne diseases.

Future research

The origin of nonane for the oviposition in mosquitoes

The highly selective response of *An. gambiae* to breeding sites is mediated by infochemicals emitted by different stages of conspecific larvae (Chapter 3). Among all chemical compounds identified from the headspace of larval habitats, nonane proved to be one of the best in terms of the number of gravid mosquitoes it attracts and having simple preparation methods. Nonane is a linear alkane hydrocarbon compound, which undergoes a combustion reaction in the presence of sufficient oxygen, to form carbon dioxide and water. Most pheromones comprise a hydrocarbon skeleton (Ryan 2007, Symonds et al. 2012, Fleischer and Krieger 2018) and it is becoming increasingly clear that a major function of cuticular hydrocarbons in arthropods is to serve as recognition signals between two or more individuals. In fact, one or more compounds of the complex mixture of hydrocarbons found on the cuticle of almost all arthropods is often the primary chemical cue that helps to communicate with others (Blomquist et al. 2018). The compounds identified in this study as oviposition modulators were trapped from larval headspaces; however, within the larval microcosm there might be bacterial and/or fungal populations responsible for emission of volatile chemicals. Therefore, further studies are required to ascertain the exact origin of volatile compounds identified in this study.

Involvement of plants in the mediation of oviposition behaviour in mosquitoes

During the current study, *Anopheles* breeding sites were found to be associated with grasses, floating plants, rice, etc. It is known that certain grass species have played a role in habitat selection in *An. albimanus* through the release of infochemicals (Torres-Estrada et al. 2005). Plants can influence the oviposition-site selection process directly by emitting infochemicals which may either attract or deter gravid female intending to oviposit. Indirectly, plants and their root network may harbour microorganisms such as bacteria, fungi and nematodes, which produce infochemicals also affecting mosquito behaviour. Recent findings in this area suggest that olfactory cues released from an infusion of Bermuda grasses deter *An. gambiae* mosquitoes from laying eggs (Eneh et al., 2016), whereas volatiles from the grass species *Echinochloa puyramidalis* and *E. staginina* are attractive (Asmare et al. 2017). It is, therefore, necessary to identify the species of plants that are associated with and facilitate mosquito breeding. Identification of plants which either deter gravid females from laying eggs on breeding sites in which they are grown or produce toxins that kill larvae, would greatly benefit mosquito control programmes.

The role of microorganisms

Oviposition-site selection behaviour in *An. gambiae* is known to be mediated by volatile chemicals emitted by bacterial communities in larval habitats (Huang et al. 2006, Lindh et al. 2008, Ponnusamy et al. 2008, Busula et al. 2017). The need to identify bacterial species which emit volatile chemicals that might attract both host-seeking and egg-laying mosquitoes is paramount (Verhulst et al. 2011). Recent findings in this area suggest that olfactory cues released from certain grasses in association with fungi, might be used by gravid *An. gambiae* to locate suitable breeding sites (Eneh et al., 2016; 2019). However, the complex interaction of microorganisms, plants and aquatic fauna and their roles in emission of infochemicals still needs elucidation (Eneh et al. 2019). Studying the link between larval microcosm, microbial communities and the volatiles they produce will provide information on the extent to which microbial derived infochemicals are used in mosquito site-selection process.

Modes of action of infochemicals

This study confirms that *An. gambiae* uses chemical cues from various sources to locate suitable breeding sites for oviposition. Moreover, this study also suggests the existence of several mechanisms that ensure that gravid females are guided effectively to sites that are suitable for egg laying and survival of their offspring. The observed additive/synergistic behavioural responses are a result of the interactions of several stimuli. It is therefore necessary to identify putative oviposition chemical compounds from various sources and test them alone and as a blend to determine their additive, synergistic or antagonistic effects. Recent advances in analytical chemistry, coupled with more definitive behavioural analysis have allowed elucidation of chemical compounds responsible for mediation of important resource finding processes (van Loon et al. 2015, Suh et al. 2016, Verhulst et al. 2016). If various signals are combined and emitted from specified breeding sites, gravid mosquitoes can be attracted to concentrate their eggs on designated sites, which can easily be targeted for larvicide application. Among the infochemicals identified in this study, two of them, DMDS and DMTS, foster significant aversive effects on the oviposition site selection of gravid females. On the other hand, nonane and 2,4 pentenedione were attractive and stimulating to ovipositing females respectively. This gives a possibility of conducting other behavioural assays in which various oviposition modulators are combined with each other or with selected insecticides. I propose that more research be done on the roles of inter-and intra-specific signals as attractants and deterrents modulating oviposition behaviour in *An. gambiae*. This will allow the development of a strategy that is more robust than existing tools for surveillance and control of mosquitoes to be added to the integrated vector control tools.

Responses of other mosquitoes to infochemicals of anopheline origin

The findings of this study have confirmed that nonane, which is an infochemical originating from an *An. gambiae* habitat, also attracts gravid *Cx. quinquefasciatus* mosquitoes. Therefore, more research is needed to show if this compound can be used for the surveillance of other mosquito species as well. Moreover, most infochemicals have multiple roles of attracting or deterring insects depending on the emission dose. In this study, GC-MS analysis identified 16 compounds that potentially mediate the oviposition behaviour of *An. gambiae* mosquitoes (Chapter 4). However, only four compounds were selected for dose-response experiments. Future research needs to be directed on the remaining 12 chemicals, either singly or as a blend.

In this era of newly emerging and re-emerging mosquito-borne diseases, studying the effect of habitat-derived volatiles on the oviposition site preference of mosquitoes within the *An. gambiae* complex will reveal the mechanisms underlying oviposition behaviour of the most important malaria vectors. This knowledge may also be used to understand similar mechanisms most likely present in other mosquitoes. Thus, more studies on the role of infochemicals in the behaviour of different mosquito species will help to understand the universal role of info-chemical signals among various species of disease vectors.

Developing an attract-and-kill test kit

The attract-and-kill strategy is likely to be one of the best methods to be included in integrated vector management programmes because gravid mosquitoes are known to be attracted to lay eggs in habitats with conspecific eggs present in breeding sites (Wachira et al. 2010) and larvae (Wong et al. 2011). In fact, conspecific immatures emit infochemicals that inform a gravid mosquito about the presence of a suitable breeding site (Chapter 4). Synthetic infochemicals can be used in combination with an appropriate larvicide to develop an attract-and-kill system (Chapter 7); (Mbare et al. 2014, Ong and Jaal 2015, Mafra-Neto and Dekker 2019). Apart from killing the larvae, the system may also remove adults from the population, if insecticides are applied to the sides of pots in such a way that when a mosquito lands there, it comes into contact with insecticides.

In this study, all hatched larvae were removed from the population pool using larvicides (Chapter 7). Only a small quantity of an attractant and a larvicide was used in a relatively small area. This is an additional benefit of an attract-and-kill system; only a small amount of the chemical is necessary to attract the target species. Most people are capable of following a pest management strategy that uses insecticides that are safe for the environment, such as *Bacillus thuringiensis israelensis*. Following the results reported in this thesis, I propose further studies on the development and use of an attract-and-kill kit in a do-it-yourself form. Do-it-yourself mosquito control products are becoming common, as people are becoming aware of such programmes (Yates et al. 2005). With the advent of infochemicals that attract more than one species of mosquitoes, an attract-and-kill tool is highly desirable to be added to integrated vector control tools (Michaelakis et al. 2007, Paz-Soldan et al. 2016, Schorkopf et al. 2016).

General conclusion

This thesis cut across behavioural and chemical assays in a holistic approach relevant for entomological investigations. The study started by observing larval habitats in the field, then simulated field conditions in the laboratory and observed the behaviours in a controlled environment. That led to the identification of infochemicals used to mediate oviposition behaviour and establishing of synthetic copies of identified chemicals. The synthetic copies of the infochemicals underwent a series of testing in the laboratory, semi-field environment and actual field sites. The study demonstrated extensively that it is possible to lure gravid females to lay eggs in habitats treated with selected larvicides. Two chemicals identified from larval habitats (nonane and 2,4-pentanedione) attracted more *An. gambiae* mosquitoes in the semi-field. Nonane proved to be attractive in the field as well. In addition to this, nonane also attracted wild populations of *Cx. quinquefasciatus*, a sympatric species to *An. gambiae*.

Existing mosquito control strategies in Africa are focusing on indoor mosquito control tools such as LLINs, IRS and spatial repellents with a limited type of insecticides as recommended by the WHO. This allows outdoor biting and resting, and allows insecticide-resistant mosquitoes to survive and sustain the burden of malaria throughout the year. This thesis has examined this problem by exploiting oviposition, which is an outdoor behaviour, and designing control strategies that complement existing control tools. Therefore, this thesis provides a useful framework to understand the oviposition behaviour of mosquitoes and exploit this knowledge in order to improve outdoor mosquito control tools. Moreover, based on this study I propose the use of a toolkit which composes of an attractant and a biocide for monitoring and control of mosquitoes. Adoption of such a toolkit for use at a community or household level would greatly benefit monitoring and control of mosquito vectors in Africa

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Summary

Summary

Mosquito-borne diseases are among the most serious infectious diseases in the world caused by parasites and pathogens. The African Mosquito *Anopheles gambiae* transmits malaria parasites as well as filarial worms. The mosquitoes feed on humans inside and outside houses, which complicates control strategies. Current control strategies focus mostly on reducing contact with host-seeking mosquitoes through the use of long-lasting insecticide-impregnated nets (LLINs) and indoor residual spraying (IRS). Despite the fact that these tools have lowered the burden of malaria in many parts of the world, transmission is sustained by vectors that bite outdoors. This thesis proposes a new strategy for malaria vector control to be added to existing control tools. It goes beyond blood-feeding processes and it targets all mosquitoes which are feeding indoors and outdoors. The proposed strategy is based on manipulation of oviposition behaviour using chemical cues that mimic olfactory cues emitted by breeding sites. Mosquitoes are attracted to lay their eggs on selected water bodies with specific biotic and abiotic factors. Organic chemical cues, emitted by these sites, are presumed to act as infochemicals enabling these mosquitoes to find suitable breeding sites. Identification of the odours that mediate the site-seeking behaviour of mosquitoes can lead to the development of novel intervention methods for mosquito-borne disease control.

My study marked an important progress in the development of mosquito surveillance and control strategies using infochemicals by: (1) generating knowledge on the biology and ecology of immature stages of *Anopheles* mosquitoes in the study area, (2) providing evidence that mosquitoes select breeding sites preferentially and lay eggs on suitable sites while avoiding unsuitable ones, (3) identifying and characterizing volatile organic chemicals that mediate oviposition behaviour in mosquitoes, (4) generating evidence that oviposition infochemicals can be used to divert oviposition activities to specific breeding sites, which can be targeted by an environmentally safe larvicide: a lure-and-kill method.

The research aimed to understand the reasons that prevent early and late larval stages from sharing breeding sites, and also why breeding sites with late stage larvae are not immediately succeeded by early stage larvae **(Chapter 5).** Several studies have indicated a possible role of habitat materials in the production of volatiles chemicals which either attract or deter egg-laying mosquitoes. A review of recently published studies showed that oviposition attractants and deterrents are produced by biotic factors present in breeding sites **(Chapter 2).** In my study it was found that breeding sites which are occupied by larvae play an important role in the production of odours that mediate oviposition behaviour of mosquitoes **(Chapter 3)**. These infochemicals can potentially be used for manipulation of mosquito behaviour by making protected resources unsuitable for mosquitoes, while luring them towards attractive sources. Such

push and pull strategies using ovipostion infochemicals for other mosquitoes were reviewed in order to develop similar surveillance and control strategies for malaria vectors. Among other larviciding products reviewed, bacterial products such as *Bacillus sphaericus* (*Bs*) and *Bacillus thuringiensis israelensis* (*Bti*) emerged with the potential to be combined with attractive infochemicals in the development of a lure and kill strategy **(Chapter 2).**

To understand the factors influencing oviposition behaviour of *Anopheles* mosquitoes, a series of dual-choice behavioural experiments were conducted in the laboratory using larval habitats materials. Results show that the presence of larvae in breeding sites mediates oviposition behaviour of *Anopheles* mosquitoes **(Chapter 3)**. The presence of early-stage larvae in oviposition cups attracted conspecific gravid mosquitoes to lay eggs, whereas the presence of late-stage larvae in the cups deterred conspecific gravid mosquitoes to lay eggs. The response was density dependent: as larval density increased so did the response, until reaching a threshold. Moreover, this behaviour was manifested more profoundly in the presence of larvae than larval-conditioned water. To test whether larval habitats produce volatiles that mediate oviposition behaviour, the oviposition cups containing larvae were covered by a filter paper to prevent gravid females from seeing the larvae in the cups. Results show that both olfactory and visual cues are involved in mediating the oviposition behaviour of anopheline mosquitoes.

To confirm the findings that larval habitats emit infochemicals that mediate oviposition behaviour of gravid mosquitoes, larval habitats containing early- or late-stage larvae were placed in containers in the laboratory. Headspace volatiles of the larval microcosm were entrained and analyzed by gas chromatography - mass spectrometry (GC-MS). Analysis of the volatiles emitted by larvae in-vitro led to the identification of 16 compounds, and four of them, when tested with *An. gambiae*, had an effect on oviposition behaviour **(Chapter 4)**. Volatile chemicals extracted from headspace collections of larvae mediated similar behaviour; volatiles entrapped from young larvae attracted conspecific gravid mosquitoes, whereas those from late-stage larvae deterred conspecific gravid mosquitoes to lay eggs. Nonane and 2,4-pentenedione, which were isolated from headspace collection of larvae, enhanced the attractiveness of a synthetic solution and could be used to increase mosquito egg deposition for monitoring or vector control purposes. Dimethyldisulphide (DMDS) and dimethyltrisulphide (DMTS), which were isolated from headspace collection of late-stage larvae only, decreased mosquito egg deposition in a synthetic solution and may act as a spatial deterrent. Laboratory and semi-field experiments in Tanzania showed similar results and suggested that nonane and 2,4-pentanedione can be used as mid-range cues for oviposition.

The study described in **Chapter 5** provided ecological characteristics of the field site where observations on the behaviour and distribution pattern of larvae were done. This is the field site where initial observations on succession of larvae in breeding sites and the actual testing of infochemicals took place. The factors influencing the selection of breeding sites by gravid females and distribution patterns of mosquito larvae were explored. Human activities such as brick-making and rice production were found to influence oviposition site selection and hence constitute a risk factor for malaria and other mosquito-borne diseases. The presence of larvae of *Anopheles* mosquitoes in breeding sites was associated with vegetation, presence of culicine larvae, shallowness and sunlight. The association between anopheline and culicine larvae is a vital finding that suggests that gravid mosquitoes of the two species use similar cues in selecting suitable breeding sites. Moreover, results from the study suggested that productivity of anopheline larvae in the field can be reduced by introducing larval predators, water management, or water pollution with rotting vegetation. The findings obtained after careful observation of the field site helped to design surveillance and larval control strategies using synthetic infochemicals identified in **Chapter 4**.

Field experiments in east-central Tanzania showed results similar to the laboratory and semi-field studies and suggested that larval habitat volatiles also attract other disease vectors **(Chapter 6)**. This study showed that infochemicals from selected sites can induce an oviposition response in mosquitoes under natural conditions, and that soil from a breeding site and nonane both induced oviposition in *An. gambiae* and *Cx. quinquefasciatus* with a synergistic effect caused by nonane + soil in *An. gambiae*. Therefore, the study also revealed that the oviposition-site-selection process of gravid mosquitoes is mediated by a combination of infochemicals derived from natural breeding sites. This knowledge can be exploited to develop a control strategy based on the push-pull or attract-and-kill concept. Furthermore, the knowledge can be utilized to develop other surveillance tools for mosquito vectors of human and animal diseases.

In order to explore the potential of using a push-pull system for mosquito control, the attractants were combined with environmentally friendly biolarvicides in order to achieve more effective control of their aquatic larval offspring **(Chapter 7)**. As an attractant for gravid mosquitoes, nonane, which originated from *An. gambiae* larvae, was selected. As toxicant for mosquito larvae, *Bacillus thuringiensis var israeliensis* (*Bti*) and Temephos were selected. Mosquitoes were successfully attracted to lay eggs in water-filled clay pots treated with attractants and combined with larvicides. Therefore, a lure-and-kill system was successfully implemented; mosquitoes were attracted to lay eggs in intervention pots containing toxicants, consequently, all emerged larvae died before reaching the pupal stage.

The results presented in this thesis create new opportunities for applied research, especially on the development of novel vector-borne disease control strategies using infochemicals **(Chapter 8)**. The volatiles produced by the larval habitats play an important role in the site-selection behaviour of *An. gambiae* and *Cx. quinquefasciatus* mosquitoes. The attractiveness of the synthetic chemicals identified in this study may be improved by making blends of attractants or in combination with other infochemicals identified elsewhere. Identified compounds can be used in push-pull and/or lure-and-kill strategies for the manipulation of mosquitoes; thereby reducing the abundance of mosquitoes, the human-biting frequency, and the intensity of pathogen transmission. Research on the role of oviposition-based infochemicals in the site-seeking behaviour of mosquitoes may lead to a better understanding of vector behaviour and contribute to the fight against vector-borne diseases.

The main conclusions from this thesis can be summarized as follows:
Volatiles produced by larval habitats play an important role in the mediation of oviposition behaviour of *An. gambiae* and in the dispersal of mosquito species. The analysis of the headspace from larval microcosms led to the identification of 16 compounds. Out of these, four (DMDS, DMTS, nonane and 2,4-pentanedione) had behavioural effects on *An. gambiae*. Nonane acts as an oviposition cue for *Cx. quinquefasciatus* as well. Mosquitoes are governed by multiple cues in the selection of suitable breeding sites, thus further research on the role of combined infochemicals to optimize selection of breeding sites should be initiated. These infochemicals can be used in push-pull or attract–and-kill strategies for surveillance and control of malaria mosquito larvae. Thus, odour-based technologies can improve the surveillance, sampling and control strategies for disease vectors. In this way the burden resulting from mosquito-borne diseases such as morbidity, mortality and economic losses will be decreased.

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Curriculum Vitae

Victor Mwingira was born in 1974 in Songea District, southern Tanzania. In 1988, he obtained his primary school education at Majimaji Primary School and in 1992, he obtained his secondary school education, followed by high school education in 1995 at Songea boys' secondary school, Tanzania.

His research career started in 2001 when he joined a diploma programme and later MSc programme in advanced studies in Biology specializing on Human Ecology at Vrije Universiteit Brussels, Belgium. He worked on diarrhoeal infections in children and developed a thesis on "Non-vaccine intervention for the prevention of diarrhoeal diseases in Tanzania". He graduated in 2003 and returned to Tanzania where he was offered a position of Training Officer at the Ministry of Health Vector Control Training Centre in Muheza, Tanzania. It was there, where he met various scientists working on mosquito and mosquito-borne diseases and developed a keen interest in studying mosquito behaviour.

In 2004, he joined the Amani Research Centre of the National Institute for Medical Research (NIMR), based at Muheza. At first, he worked with the vector control project on the development of alternative products for malaria control and personal protection. Then, he participated in many other projects including a project on establishment of a WHO reference center for evaluation of novel vector control tools at Muheza. To-date he is involved in the process of accreditation of the Amani Research Centre to a Good Laboratory Practice standard, with support from the Innovative Vector Control Consortium (IVCC).

In 2005, he joined a project titled System-wide Initiative on Malaria and Agriculture (SIMA). The project's main objective was to determine the impact of land-use and agricultural practices on malaria burden in Mvomero District, Tanzania. While working with the SIMA project, he carried studies on mosquito breeding site characteristics and factors leading to skewness in larval productivity among the different sites. The observations made during this project formed the basis of research ideas for his PhD project. In his attempt to understand why some breeding sites are favoured more often by gravid mosquitoes than others, he applied for sandwich PhD grant at Wageningen University and Research under supervision of Prof. Willem Takken.

List of publications

Schoelitsz, Bruce, **Victor Mwingira**, Leonard E.G. Mboera, Hans Beijleveld, Sander C.J.M. Koenraadt, Jeroen Spitzen, Joop J.A. Van Loon & Willem Takken. (2020) Chemical mediation of oviposition by *Anopheles* mosquitoes: a push-pull system driven by volatiles associated with larval stages. *Journal of Chemical Ecology,* 2020; https://doi.org/10.1007/s10886-020-01175-5

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Mwingira, Victor S., Willem Takken, Leonard E.G. Mboera & Marcel Dicke, Exploiting the chemical ecology of mosquito oviposition behaviour in mosquito surveillance and control – a review.

Mwingira, Victor S., Leonard E Mboera, and Willem Takken. Characterisation of larval habitats for malaria mosquitoes in relation to land use patterns in Mvomero district, Tanzania.

Mwingira, Victor S., Leonard EG Mboera, and Willem Takken. Synergism between nonane and emanations from soil as cues in oviposition site selection of gravid *Anopheles gambiae* and *Culex quinquefasciatus*

Mwingira, Victor S., Leonard EG Mboera, Niels O. Verhulst, Marcel Dicke and Willem Takken. Attract-and-kill strategy for mosquito larval control: effect of oviposition cues on the efficacy of the larvicides Temephos and *Bacillus thuringiensis israelensis* on malaria and lymphatic filariasis vectors.

PE&RC Training and Education Statement

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

Review of literature (6 ECTS)

- Exploiting the chemical ecology of mosquito oviposition behaviour in mosquito surveillance and control

Writing of Project proposal (4.5 ECTS)

- Olfactory mediation of oviposition in Anopheles mosquitoes

Post-graduate courses (15 ECTS)

- Molecular aspects of biointeractions; Entomology, the Netherlands (2007)
- Ecological aspects of biointeraction; Entomology, the Netherlands (2007)
- Advanced statistics; PE&RC, the Netherlands (2007)
- Statistical analysis with SPSS and STATA for PhD students; University of Dar es Salaam, Tanzania (2017)

Laboratory training and working visits (6 ECTS)

- Molecular aspects for identification of insects; National Institute for Medical Research, Tanzania (2012-2014)
- Mass spectrometry and gas chromatographic techniques for identification of volatile chemicals; University of Dar es Salaam, Tanzania (2014-2015)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Bulletin of Health Research: entomology (2008)
- Mass spectrometry and gas chromatographic techniques for identification of volatile chemicals: molecular parasitology (2019)

Deficiency, Refresh, Brush-up courses (4.3 ECTS)

- Basic statistics; PE&RC, the Netherlands (2006)
- Endnote advanced; Library, the Netherlands (2006)
- Analysis and prevention of health risks in the tropics; PE&RC, the Netherlands (2006)
- Vector control and ecology; National Institute for Medical Research, Tanzania (2012)
- Malaria vector control & insecticide resistance management; National Institute for Medical Research, Tanzania (2016)

Competence strengthening / skills courses (2.9 ECTS)

- PhD Competence assessment; PE&RC, the Netherlands
- The art of writing; CENTA, the Netherlands
- Reporting and publishing in a journal; online: Tropical Medicine and International Health
- Evidence based health training workshop; MUHAS/ITOHA, Tanzania
- Imaging science: video/audio content in scientific communication; PE&RC, the Netherlands

PE&RC Annual meetings, seminars and the PE&RC weekend (2.7 ECTS)

- PE&RC Weekend (2007)
- PE&RC Days (2006-2010)
- PE&RC Seminar (2014)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)

- National lymphatic filariasis elimination program publication workshop, the Netherlands (2009)
- Monthly NIMR Amani and Tanga Centres local scientific meeting, Tanzania (2012)
- Annual joint scientific meeting of the National Institute for Medical Research, Tanzania (2016)

International symposia, workshops and conferences (8.8 ECTS)

- Symposium on Insect; Plant Association, Sweden (2007)
- Multilateral Initiative in Malaria, Nairobi, Kenya (2009)
- East African Health and Scientific Conference; Dar es Salaam, Tanzania (2019)
- Pan African Mosquito Control Association; Younde, Cameroon (2019)

Lecturing / supervision of practicals / tutorials (3 ECTS)

- Ecology and control of arthropod vector (2009)
- Basic entomology (2016)
- Principles of pest management (2012)

Supervision of BSc students (9 ECTS)

- Chemical ecology of mosquitoes
- Insecticide testing for mosquitoes
- Biological control of mosquitoes

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