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LABORATORY OF ENTOMOLOGY

Yeast-generated carbon dioxide as a mosquito attractant

A field study conducted in Mbita, at the Mbita Point Research & Training Centre of the International Centre of Insect Physiology and Ecology (ICIPE) in West Kenya.

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Preface

Upon today millions of people in the tropics are struck by several lethal diseases. Vector borne diseases are responsible for many of those deaths and for that purpose only, it is already useful to study and to investigate the diseases and its vectors. Tropical Africa has always fascinated me, as a human and as a biology student. Therefore insects with human societal relevance draw my attention. Malaria is one of the major vector borne diseases in sub-Saharan Africa, responsible for millions of deaths annually, especially under children, and yet remains a neglected disease. To prevent malaria transmission vector control remains the most generally effective measure and is therefore one of the four basic technical elements of the Global Malaria Control Strategy (WHO, 2008). By controlling the vector's population it is possible to reduce the levels of transmission and subsequently to reduce the malaria morbidity and mortality. Therefore I find it valuable to study the biology of *Anopheles gambiae s.l.*, the main Malaria vector in sub-Saharan Africa (White, 1974), and its behaviour. Ultimately studies on the mosquito vector can lead to new sustainable vector control measures to diminish the global malaria burden.

Yeast-generated carbon dioxide as a mosquito attractant

Abstract

Carbon dioxide plays an important role in the host-seeking behaviour of blood-feeding mosquitoes (Diptera: Culicidae). A new, convenient carbon dioxide (CO_2) source was tested to attract mosquitoes in field and semi-filed studies in Western Kenya. Yeast was grown on a mixture of sugar and water in a sealed off water barrel, where the fermenting yeast converts sugar into CO_2 and ethyl alcohol. The efficacy of the yeast-generated CO_2 as a mosquito attractant was tested and compared with the more conventional cylinder gas CO_2. In semi-filed dual-choice experiments, MM-X traps baited with the yeast- generated CO_2 attracted as many *Anopheles gambiae sensu stricto* mosquitoes as MM-X traps baited with cylinder CO_2. During 16 experimental nights in the field, MM-X traps baited with the two different CO_2 sources and an unbaited MM-X trap as a control, trapped 878 mosquitoes, with the mosquito species belonging to the genera *Mansonia* (58.3%), *Culex* (28.4%), *Anopheles* (10.8%) and *Aedes* (2.5%). Similarly in the field, traps baited with the yeast-generated CO_2 attracted as many mosquitoes and also as many *Anopheles gambiae sensu stricto* than the cylinder CO_2. Additional to the CO_2, yeast could positively influence the attractiveness of the CO_2 produced and with this potential yeast-generated CO_2 could be a better alternative than cylinder CO_2 to attract and sample mosquitoes in the field.

1. Introduction

Malaria is an infectious disease caused by protozoa of the genus *Plasmodium*, and is transmitted to humans by *Anopheline* mosquitoes. Mosquito species of the *Anopheles gambiae* Giles complex (Diptera, Culicidae) are responsible for much of the malaria transmission in sub-Saharan Africa. Specifically *Anopheles gambiae* Giles *sensu stricto* (hereafter termed *An. gambiae*) is the most important malaria vector in sub-Saharan Africa (White, 1974). For most female mosquitoes to develop eggs, they need one or more blood meals, obtained from vertebrate hosts. To find a host carbon dioxide, which is emitted by vertebrates, activates and attracts female mosquitoes (Gillies, 1980; Snow, 1970). Therefore carbon dioxide is used to trap and sample mosquitoes in the field, and it is used to study behavioural aspects of the mosquito like host-seeking and blood-feeding behaviour. For the purpose of interfering in the mosquito – host interaction and controlling mosquito populations, hereby reducing disease transmission and human mortality.

To help improve understanding of this behaviour, this study is focused on developing and testing a better, more sustainable and cheaper carbon dioxide source to attract *An. gambiae* mosquitoes in the field. Succeeding would not only improve and simplify field studies but lead to improved vector control measures.

The field work in this study was conducted at the Mbita Point Research & Training Centre of the International Centre of Insect Physiology and Ecology (ICIPE) in Western Kenya.

1.1 Biology of mosquitoes

Mosquitoes fall under the family Culicidae with three subfamilies: Toxorhynchitinae, Culicinae and Anophelinae. Within the subfamilies Culicinae and Anophelinae three mosquito genera, *Aedes, Anopheles,* and *Culex,* contain mosquito species that are responsible for the transmission of a wide range of human diseases, vector born diseases. Consequently the biology of these genera is important from an epidemiology point of view. The life cycle and, in value to this study, the host-seeking behaviour of *An. gambiae,* are described in this chapter.

Every mosquito exhibits a variety of behaviours appropriate to its sex, to ensure its nutritional and reproductive requirements (Clements, 1992). Both sexes require sugar from plant sources, mates and resting sites. Additionally, females require blood from a certain host, as a source of protein for egg development, and oviposition sites. Like other true flies, culicids exhibit complete metamorphosis, the juvenile form passes through both larval and pupal stages. The larvae are anatomically different from the adults, live in a different habitat and feed on a different type of food. Transformation to the adult takes place during the non-feeding pupal stage (Le Sueur 1988).

1.1.1 Life cycle

To complete a life cycle, mosquitoes go through three aquatic stages, egg, larvae and pupae, to finally emerge as an adult mosquito. Inseminated adult female mosquitoes fertilize the oocytes with spermatozoa, as they are ovipositing. After maturing a batch of eggs she will look for and respond to stimuli from suitable oviposition sites, to lay her eggs. The oviposition site can be a

wide range of different types of water bodies, depending on the mosquito species and their habitat (Clements, 1992).

1.1.1.1 Egg

The female mosquito may lay from 50 up to 500 eggs at once, depositing them on water or on sites that will be flooded. The eggs can be dropped individually to float on the water surface, as by females of *Anopheles*, or packed together to form a floating egg raft, as by *Culex* ((Clements, 1992; Le Sueur, 1988). Each egg is protected by an egg shell, which surrounds the oocyte, egg, embryo or possible the pharate larva. The egg shell is soft and flexible when laid but hardens after time. The shell is rigid and solid to provide mechanical support, protection and gas exchange while minimizing water loss.

Almost immediately after the eggs have been laid embryonic development starts and within one to two days to a week or more, depending on temperature, the embryo develops into a fully formed larva (Clements, 1992).

1.1.1.2 Larvae

When the young mosquito larva hatches from the egg it is fully adapted for living in water. The body of the larva can be differentiated in three regions: a sclerotized head, a broad thorax and a segmented abdomen (Kettle, 1995). To develop and to survive it uses atmospheric oxygen for respiration and water-borne particles as food. Because of their air-breathing habit the mosquito larvae has to live close at the air/water interface.

The larvae feed by collecting/ filtering food resources, mainly micro-organisms and plant tissues. Through regular beating of their mouth brushes, the mosquito larvae generate water current which flows towards the head.

During the larval period the mosquito larvae moult four times, passing through four instars. On the first three occasions that it leaves it cuticle the larva remains much as before. During the fourth moult the imaginal disks develop rapidly, changing the form of the larva more likely to that of an adult (Clements, 1992), and the organism leaving the fourth larval skin is a pupa.

1.1.1.3 Pupae

The pupa remains an aquatic organism. The head and the thorax are combined into a cephalothorax, which is joined to a segmented abdomen. The pupa floats at the water surface with the top of its thorax in contact with the surface membrane. The respiration is taken over by a pair of broad trumpets dorsally placed on the cephalothorax. During this final stage the larvae does not eat and completes the metamorphosis within one or two days, if the temperature is sufficiently high. When the adult is fully formed within the pupal cuticle, the insect rest at the water surface and starts to swallow air. This increases the internal pressure, forcing a split along the midline of the pupal thoracic cuticle (Clements, 1992). Finally the adult can expand out of the cuticle and emerges on the surface water.

1.1.1.4 Adult

Soon after emergence the mosquito wings and legs become extended and the body cuticle begins to harden, this within half an hour of eclosion. The adult mosquito then flies to a shelter and rests for several hours. After the male mosquito's external genitalia have turned upside down, taking about one day, copulation will take place. During mating the male deposits his spermatozoa in the bursa copulatrix of the female, from which they move to the spermathecae (Clements, 1992). The role of adult male mosquito is limited to inseminating of females, and when not resting the males are feeding on plant sugars or swarming, a behaviour pattern that is likely to bring them into contact with females. An inseminated female can carry sufficient sperm in her spermathecae to fertilise all the eggs she may produce. To start producing eggs or to replenish metabolic reserves she will search for an appropriate host for her first blood meal. The blood meal, one or several (Takken, et al., 2002), will provide the female mosquito the required proteins to start ovarian development and finally to mature eggs. Subsequently the female will look for a suitable breeding site for oviposition. In tropical regions the females will be engaged in a continuous pattern of host searching, blood-feeding, egg development and oviposition. In temperate regions the female will usually overwinter before laying her eggs.

1.1.2 The host-seeking behaviour *An. gambiae*:

Blood proteins are essential for a female *An. gambiae* mosquito to develop eggs. To be able to get a blood meal from a vertebrate hosts, she first has to find a suitable host. This results in a host-seeking behaviour. Host-seeking has been defined as the in-flight orientation of the avid female toward a potential blood-host (Bowen, 1991). Several factors influence the host-seeking behaviour; temperature, humidity and visual objects. Host location and feeding behaviour of *An. gambiae* are odour mediated (Takken, 1991; Takken and Knols, 1999).

Host emanations contain 3 different sources of olfactory cues: skin emanations, exhaled air and urine. At short distance from the host body temperature and moisture are more important elements in host-seeking (Laarman, 1958), while at larger distances visual and olfactory cues determine the behaviour of the female mosquito (Takken, 1991). When female mosquitoes detect host odour carried to them by wind, they fly upwind toward the odour source. This is called odour modulated upwind anemotaxis (Payne, et al., 1986). Two important sensory organs that contribute to the sense of smell are the maxillary palps (Omer and Gillies, 1971), which measure the level of carbon dioxide, and the antennae, which detect other host-released odours (Lu, et al., 2007; Qiu, et al., 2006). In this chapter the role of carbon dioxide and human odours in the host-seeking behaviour of *An. gambiae* is described.

1.1.2.1 The role of carbon dioxide in the host-seeking behaviour of *An. gambiae*

Carbon dioxide (CO_2) is exhaled by all vertebrates and thus an ideal stimulus that signifies the presence of a potential host. Hence CO_2 is an established host attractant for mosquitoes both in the laboratory and in the field (Gillies, 1980). Gillies also (1980) concluded that CO_2 both activates and orients mosquitoes. With activation is meant the induction of flight activity, CO_2 induces take off and sustains the female to fly upwind toward the odour source. Besides the activating and orienting role of CO_2 in female mosquitoes, Knols (Knols, et al., 1994) demonstrated that CO_2 also influences the selection of biting sites. Mosquitoes respond to

alternations of carbon dioxide concentrations and therefore to sustain flight, in absence of other host odours, intermittently CO_2 stimuli are needed (Gillies, 1980). And this is in fact what happens in an outdoor airstream, where the natural turbulence of the air causes any volatile substance to appear as short pulses rather than a continuous flow (Dekker, et al., 2005; Dekker, et al., 2001; Takken, 1991).

Carbon dioxide appears to be important for all host-seeking mosquito species, but a major disadvantage is that an insect flying upwind in a plume of CO_2 cannot determine the species of its source. The host preference of the mosquito also plays a role in its response to different levels of carbon dioxide. Thus mosquitoes with a broad host range are expected to respond commonly to carbon dioxide and their attraction will increase as the level of the compound is raised (Mboera and Takken, 1997). The degree of attractiveness to CO_2 is assumed to increase with the degree of zoophily (Costantini, et al., 1996; Dekker and Takken, 1998; Snow, 1970). Anthropophilic mosquitoes also show less dependency on carbon dioxide in their host-seeking behaviour, suggesting that they rely to a greater extent to other human emanations in addition to CO_2 (Dekker and Takken, 1998; Takken and Knols, 1999; White, 1974). *An. gambiae,* is highly anthropophilic (White, 1974) hence CO_2 is considered only to contribute partially to its attraction of humans (Costantini, et al., 1996; Mboera and Takken, 1999; Snow, 1970). However carbon dioxide acts as a synergist when offered in combination with human odours (Schmied, et al., 2008; Spitzen, et al., 2008; Takken and Knols, 1999); it increases the chance that the female mosquito will arrive at the host. But both Dekker *et al.* (2001) and Spitzen *et al.* (2008) conclude that when CO_2 is the only attractant, *An. gambiae* is deterrent from entering a trap. But Spitzen et al. (2008) also hypotize from their study that when the CO_2 release point is positioned just downwind of the trap entrance, CO_2 appears to guide mosquitoes to the vicinity of the trap, where skin emanations then become the principle attractant, causing the mosquito to enter the trap.

In another recent studies (Qiu, et al., 2007; Schmied, et al., 2008), CO_2 played a dominant role in trapping mosquitoes in the field. When Schmied *et al.* (2008) added CO_2 to a MM-X trap baited with foot odour, CO_2 increased the number of *An. gambiae s.s.* catches by 268%. And in Qiu *et al.* (2007) partly due to the design of the MM-X odour delivery system, CO_2 functioned as an extra carrier gas. CO_2 carried the odour blend to the odour release point at the bottom of the trap. This all exemplifies the importance of carbon dioxide as a mosquito attractant in field setups.

1.1.2.2 The role of human odours in the host-seeking behaviour of *An. gambiae*

As mentioned before, all warm-blooded vertebrates exhale CO_2 but it is unlikely that *An. gambiae*, which has a preference for humans, will be guided to its host by this compound alone. Besides carbon dioxide there are several human related odours identified to play a role in the host-seeking of *An. gambiae* (Braks, et al., 2001; Braks and Takken, 1999; Costantini, et al., 1996; Dejong and Knols, 1995; Mukabana, et al., 2002; Mukabana, et al., 2004; Njiru, et al., 2006; Qiu, et al., 2004; Qiu, et al., 2006); aliphatic carboxylic acids, ammonia and lactic acid (Braks, et al., 2001; Dekker, et al., 2002a; Dekker, et al., 2002b; Smallegange, et al., 2005). 2-Oxopentanoic acid, a carboxylic acids, elicited a landing response in *An. gambiae* mosquitoes (Dekker, et al., 2002a; Dekker, et al., 2002b; Healy and Copland, 2000). Lactic acid is present in

high levels in human emanation (Dekker, et al., 2002a) and for *Aedes aegypti L.*, another anthropophilic mosquito species and vector of yellow fever (Harrington, et al., 2001), lactic acid plays an important role in host-seeking (Acree, et al., 1968). Smallegange *et al.* (2005) also found a synergistic effect of ammonia, carboxylic acids and lactic acid in a bioassay with *An. gambiae*. And in The Gambia a blend of ammonia + L-lactic acid + CO_2 + 3-methylbutanoic acid proved to be very attractive for different mosquito species (Qiu, et al., 2007).

1.2 Yeast-generated carbon dioxide

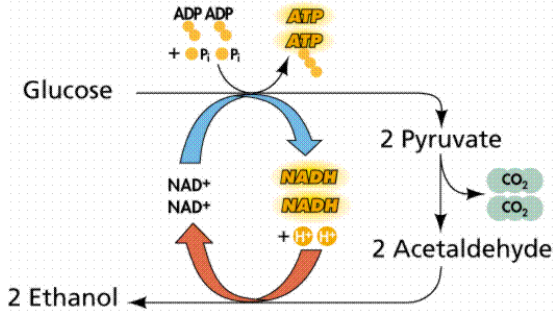
In the previous chapter the role of carbon dioxide in host-seeking behaviour was discussed. For the several mentioned reasons, CO_2 is used to trap and sample mosquitoes in the field, as it is also used to study behaviour aspects of the mosquito. To improve field studies and help understanding the behaviour of *An. gambiae*, this study is focused on developing and testing a better, more sustainable and cheaper carbon dioxide source to attract *An. gambiae* mosquitoes in the field. The production of CO_2 by yeast was analysed, optimised and compared to gas cylinder CO_2 in field experiments conducted at the Mbita Point Research & Training Centre of the International Centre of Insect Physiology and Ecology (ICIPE) in West Kenya.

1.2.1 Carbon dioxide production

Yeasts are chemoorganotrophic microorganisms which derive their chemical energy from the breakdown of organic compounds such as carbon substrates. In this study sugar was used to grow the yeast on, for the convenient reasons; most yeasts employ sugars as their preferred carbon and energy source, it is easy to use and the wide spread availability of sugar.

Yeast converts sugar into cellular energy and thereby producing water or ethanol and carbon dioxide as metabolic waste products. Through glycolysis, a sequence of enzyme-catalysed reactions, glucose is converted to pyruvic acids. In addition yeast can break down pyruvate through aerobic or anaerobic respiration. When oxygen is present yeast can oxidize pyruvate to generate energy and thereby produce water and carbon dioxide. In anaerobic conditions ethanol fermentation (Fig. 1) takes place, where pyruvate is converted first to acetaldehyde and carbon dioxide, then to ethanol (Walker, 1998).

Fig. 1: Ethanol fermentation (University of Florida 2009)



1.2.2 Volatiles

The metabolism of yeast yields besides ethanol and carbon dioxide, also a great number of other by-products, such as glycerol, acetic acid, succinic acid and lactic acid (Antonelli, et al., 1999). A part of these products readily evaporates and forms the volatile composition of the yeast, which can gradually spread out. Within yeast species, different strains differ in volatile production (Antonelli, et al., 1999). These additional volatiles carried by the yeast-generated CO_2 could augment the attractiveness of the CO_2 to *An. gambiae* mosquitoes in comparison to the conventional cylinder CO_2.

Saitoh *et al.* (2004) proved that yeast-generated carbon dioxide attracted adult mosquitoes in the field. And in Wageningen, previously to this study, Wolfgang Schmied observed, in a dual-choise experiment with yeast-generated CO_2 versus the conventional cylinder CO_2 , a tendency of *An. gambiae* s.s. mosquitoes to prefer yeast-generated CO_2. This preference might be caused by the additional volatiles produced by the fermenting yeast.

1.2.3 Benefits of yeast-generated carbon dioxide

Up to now most mosquito behavioural studies use gas cylinder bottles to deliver carbon dioxide. Gas cylinder bottles can provide CO_2 in controlled flow rates at any time and are thus very handy to use in laboratories. But for use in experiments out in the field or for wide spread vector control, gas cylinder bottles give lots of difficulties. The cylinder bottles are expensive and not widely available. Secondly they are heavy, hard to move, and thus making it almost impossible to distribute them over vast areas. Alternative carbon dioxide sources are propane driven mosquito traps (Burkett, et al., 2001) or dry ice (Xue, et al., 2008). The propane driven mosquito traps are also impractical over vast areas, expensive and hard to get in tropical Africa. Dry ice is also expensive, the rate of release can be altered because of altered weather and dry ice can be difficult to process and transport and therefore be dangerous to workers (Xue, et al., 2008).

On the other hand, the yeast-generated CO_2 setup can be made with cheap local available materials and within one hour the yeast can provide CO_2. Instead of using relatively expensive sugar, molasses or fermenting fruits, or any other carbon substrate can be used for growing the yeast. Yeast generated CO_2 might also be more attractive to the *An. gambiae* mosquitoes than the conventional CO_2 sources.

Under natural conditions, mammals release breath in pulses and therefore alternations of carbon dioxide plumes attract the mosquito (Mboera and Takken, 1997; Takken, 1991) and sustain the mosquito's flight (Gillies, 1980). Similarly, yeast produces CO_2 in an intermittent flow rate. Moisture and temperature are attractants at close range to the host (Laarman, 1958) and carbon dioxide could also have a combined action with warm, moist convection currents to attract the mosquitoes to host (Gillies, 1980). Yeast will also positively influence the moisture and temperature gradient around the setup. And maybe most importantly, additional volatiles are produced by the yeast, which can be carried by the CO_2 produced. These volatiles could augment the attractiveness of the CO_2 to *An. gambiae* mosquitoes in comparison to the conventional cylinder CO_2. To have an inside in the volatiles produced, the headspace of yeast-generated carbon dioxide was taken.

2. Goals and Research questions

2.1 Goals

To develop, test and compare yeast-generated carbon dioxide to gas cylinder carbon dioxide, as a mosquito attractant in a semi-field and field setup in western Kenya.

2.2 Research questions

2.2.1 Preliminary experiments

I. Which CO_2 flow rate is optimal for *Anopheles gambiae sensu stricto* collection?
II. Yeast- generated CO_2: Which amounts of yeast, sugar and water is needed to obtain the optimal CO_2 flow rate in question 1?

2.2.2 Screen house experiments

III. Do traps baited with yeast-generated CO_2 catch more *An. gambiae s.s.* female mosquitoes than cylinder CO_2 baited traps?

IV. Is the yeast-CO_2 production rate, after running for 24h and 48h, still attractive to female *An. gambiae* mosquitoes?

2.2.3 Field experiments

V. Does yeast-generated CO_2 attract *Anopheles gambiae s.l.* mosquitoes?

2.2.4 Malaria Sphere

VI. Can a trap baited with yeast-generated CO_2 decrease house entry response?

3. Materials and Methods

All experiments were performed at the Mbita Point Research & Training Centre of the International Centre of Insect Physiology and Ecology (ICIPE) in western Kenya.

3.1 Mosquitoes

The Mbita strain of *An. gambiae s.s.* mosquitoes used in the experiments have been maintained under laboratory conditions. Adult insects were kept in 30 cubic cm cages provided with 6% sucrose solution as maintenance diet. The cages were kept under ambient climatic conditions and females are given the opportunity to blood feed three times per week for 10 min on an arm of a volunteer. Eggs were collected on wet filter paper and transferred to plastic containers containing filtered water from Lake Victoria. Larvae were fed daily on Tetramin® fish food. Upon pupation, insects were transferred to adult cages for emergence.

3.2 Mosquito traps

The Mosquito Magnet X trap (MM-X® model; American Biophysics Corp., RI, U.S.A) an counter flow geometry trap, was used as trap and odour dispensing device (Njiru, et al., 2006). Each MM-X trap was hanging fifteen cm above the ground. In the Malaria Sphere, also a second type of trap was used, a CDC Miniature light trap (Model 512; John W. Hock Company, Gainesville, Florida, USA) (Qiu, et al., 2007) with an incandescent light bulb. The Malaria Sphere (Knols, et al., 2002) is a screen house with a hut build inside and with local vegetation planted around the hut, mimicking a field situation (further described in chapter 3.7 Malaria sphere).

After removing the caught mosquitoes, each trap was cleaned with 10% ethanol and stored in a closet to dry for the next experiment.

3.3 Yeast- generated carbon dioxide setup

All the yeast-generated carbon dioxide experiments were accomplished with local available yeast and sugar: Angel® instant dry yeast, produced by Angel yeast CO., LDT in Hubei, P. R. China and Sony sugar, made from green sugar cane and manufactured by South Nyanza sugar CO., LDT in Awendo, Kenya. The yeast was grown on a mixture of sugar and tap water, in a setup based on two five litre barrels for the yeast culture and a one litre bottle, capturing the foam to prevent the foam from entering the traps. Holes were made into the screws to pass tubes through that connected the five litre barrels with the one litre bottle. The latter was connected with a second tube to a MM-X trap. The connections were sealed with teflon tape to prevent leakage of carbon dioxide. Fresh cultures were prepared one hour prior to the experiment. Yeast, sugar and water were added in the five litre barrels and once well mixed, until the whole was dissolved.

3.4 Preliminary experiments

In the literature contradictory roles of CO_2, in host-seeking behaviour, can be found. Similar to this, there is no consensus on the CO_2 flow rate to use for attracting mosquitoes. Flow rates from 230ml/min (Pates, et al., 2001; Takken, et al., 1997), 300ml/min (Mboera, et al., 2000) and 500ml/min (Njiru, et al., 2006) are used. With these preliminary experiments a good flow rate for

trapping *An. gambiae* mosquitoes was determined and which ratio yeast and sugar is needed to obtain that CO_2 flow rate.

3.4.1 Determination of the optimal CO_2 flow rate for *An. gambiae s.s.* collection

This experiment was executed during a period of thirty days from the 17th of November until the 16th of December. A mosquito cage was made of mosquito netting, 6x2x2 meters to run the experiment in. On both ends a tripod was placed that supported each a MM-X trap. A carbon dioxide cylinder, 4,5% CO_2, was lying just outside the cage and was, connected to one of the MM-X traps, alternating per day to exclude any bias. Each of the five different carbon dioxide flow rates; 25, 60, 100, 250 and 500 ml/min, were tested four times against a control, an empty trap. The three lower flow rates were selected to test if a low CO_2 flow rate is attractive and if it can trap as many mosquitoes as 250 and 500ml/min. The latter flow rates are commonly used and 250ml/min is close to 230ml/min, considered as an equivalent for a human (Pates et al 2001). On each experimental day hundred, three to seven days old, previously not blood fed female *An. gambiae s.s* mosquitoes were collected from the colony, at 13:30 to starve them for eight hours. The female mosquitoes were released at 21:30 from the centre of the newly build mosquito cage. The next morning at 6:30 the experiment was stopped by closing the two MM-X traps and the carbon dioxide cylinder, after which the traps were placed in a freezer to kill the mosquitoes to be able to count them.

3.4.2 Carbon dioxide productivity

The carbon dioxide output produced by the yeast-sugar mixture was estimated by measuring the volume of displaced water from submerged conical tubes. Two yeast quantities, 17.5 grams and 35 grams per barrel, were grown during the day on three different sugar doses, 250-, 500- and 750 grams per barrel (Table 4), to measure their specific CO_2 production. The preliminary experiment started on Thursday 20th November, each yeast and sugar rate was grown for ten and a half hours. A single measurement was done during two minutes. The first two measurements were done after half an hour and one hour after mixing of the yeast, sugar and water. Subsequently every 15 minutes for the next three hours and finally, for the remaining six hours and a half, every half hour the CO_2 production was measured. Additionally the yeast- generated CO_2 production, with 35 grams of yeast and 500 grams of sugar, was measured outside overnight to mimic field conditions. Most yeast species have an optimal growth temperature between 30 and 35°C (Walker, 1998). Of two setups, one was buried in the ground to test if the soil, surrounding the setup, could work as isolation to maintain a warmer temperature for the yeast to grow on during the night.

3.5 Field experiment

The field experiments were based in Luanda, a rural village, in the basin region of Lake Victoria, Nyanza Province, western Kenya at an altitude of 1169 m above sea level. The area has two rain seasons, a main rainy season from March to May, and a short rainy season from October to December. Experiments were conducted at the end of the short rainy season, December. Luanda is a rural village with a variety of mosquito breeding habitats (Minakawa, et al., 1999). A previous study of Seyoum *et al.* (Seyoum, et al., 2003) showed that *Anopheles gambiae sensu lato* and *An. funestus* were the main *Anopheline* species present during the study period. Of the

An. gambiae s.l. samples identified by PCR 18.5% were *An. gambiae s.s.* and 81.5% *An. arabiensis*.

With the help of local confidants four approximately similar houses were selected. The selection was based on several criteria; household, cooking spot, thatched roof, vegetation around the house and all on a walking distance (Fig. 3). Each of the selected houses would be provided with a MM-X trap, a battery and a carbon dioxide cylinder. The MM-X traps were placed outside, under the thatched roof on the window side of the house (Fig. 2) whereas the yeast-setup was placed inside, save from cattle. Each experiment ran from 20:30 hrs until 6:30 the next morning and all treatments were rotated in a Latin square design. The rarely caught male mosquitoes were discarded from the data.

Fig. 2: An MM-X trap hanged under the thatched roof on the window side of the house of one of the selected houses in Luanda.

In the first series, the attractiveness of yeast-generated CO_2, at two different yeast/sugar concentrations, rate x and y, was compared to the attractiveness of cylinder CO_2 (250ml/min) and an empty trap. 17.5 grams of yeast and 250 grams of sugar / barrel was used for rate y and 35grams of yeast and 500 grams of sugar / barrel for rate x. These yeast/ sugar ratio were chosen because they produced the two attractive flow rates, measured in the preliminary experiment. 250ml/min cylinder CO_2 flow rate was chosen also as a result of the preliminary experiment and 250ml/min CO_2 is close to correspond to a human exhaled CO_2 rate (Pates et al. 2001).

The second series determined if additional odours, collected on a worn sock, augmented the attractiveness of the yeast-generated CO_2 and the cylinder CO_2 towards female mosquitoes. The worn sock was chosen based on the role of human foot odour in the host selection of *An. gambiae s.s* (Dejong and Knols, 1995; Schmied, et al., 2008).

The first series was started on 8th December and the second series on the 16th.

First series:
- Yeast- CO2 rate x
- Empty trap
- Yeast- CO2 rate y
- Cylinder CO2 250cc

Second series:
- Yeast- CO2 rate y
- Cylinder CO2 250cc
- Yeast- CO2 + odour (sock)
- Cylinder CO2 + odour (sock)

Fig. 3: Map of Luanda with the four selected houses (Google maps).

3.6 Screen house experiment

3.6.1 Yeast-generated CO_2 as an attractant for *An. gambiae s.s.* female mosquitoes

To investigate whether the yeast-generated CO_2 is able to compete with cylinder based CO_2 thirteen different 2-choise experiments (Table 1) were conducted in a screen house. The greenhouse measures 11.4 x 7.1 x 2.5 meters, had a glass-panelled roof, gauze covered side walls, and sand on the floor (Knols, et al., 2002; Njiru, et al., 2006). The ambient conditions are also described in Njiru *et al.* (2006). In two opposite corners a tripod was supporting a MM-X trap hanging 15cm above the ground. Next to the tripods a carbon dioxide cylinder was placed to provide the MM-X traps. Experimental treatments were alternated between the two possible positions to exclude any bias. Each setup was repeated four times. Per experiment two hundred previously not blood fed, three to seven days old, female *An. gambiae s.s* mosquitoes, starved for eight hours were released. The mosquitoes were released, in the centre of the screen house, at 21:30 and the experiment was stopped the next morning at 6:30. After the experiment was ended the traps were placed in a freezer to kill the mosquitoes for counting. The yeast CO_2 setup was started at 20:30, one hour prior to the experiment, to avoid a low CO_2 production during the first hour of the experiment.

An extra odour source, a 12 hours worn sock, was used to test if yeast-generated CO_2 is able to augment the catches of that odour source. Finally the yeast was grown for respectively 24 and 48 hours, to test if the produced CO_2 still could attract *An. gambiae s.s.* mosquitoes and if it could compete with cylinder CO_2.

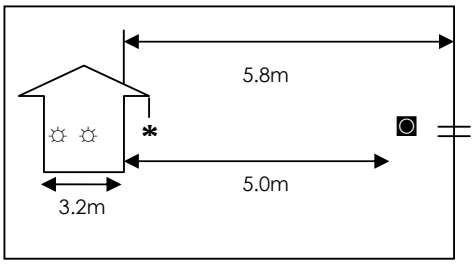
Table 1: The treatments that were tested during dual-choise trapping experiments in the screen house. The treatment on the left of the arrow (↔) was tested against the one on the right of it.

1	Empty trap ↔ Empty trap
2	Yeast rate x ↔ Empty trap
3	Yeast rate y ↔ Empty trap
4	Yeast rate y ↔ Cylinder CO_2 rate y
5	Yeast rate x ↔ Cylinder CO_2 rate x
6	Odour (Sock) ↔ Empty trap
7	Yeast rate y + Odour (Sock) ↔ Empty trap
8	Yeast rate y + Odour (Sock) ↔ Odour (Sock)
9	Yeast rate y + Odour (Sock) ↔ Cyl. CO_2 rate y + Odour
10	Yeast rate y (24h) ↔ Empty trap
11	Yeast rate y (24h) ↔ Cylinder CO_2 rate y
12	Yeast rate y (48h) ↔ Empty trap
13	Yeast rate y (48h) ↔ Cylinder CO_2 rate y

3.7 Malariasphere experiment

Experiments were carried out in the 'Malariasphere' (Knols, et al., 2002). The Malariasphere is a transformed screen house, with a local house, vegetation and breading sites build inside. To simulate the natural ecosystem of *An. gambiae* mosquitoes, in a screen-walled green house exposed to ambient climate conditions, in western Kenya (Fig. 4 and (Knols, et al., 2002). This specific screen house setting was used to test if the yeast-generated CO_2 can compete with a sleeping human inside the hut. During the overnight experiment a 27 year old male volunteer slept in the hut, protected with a non-impregnated mosquito bed net.

Fig. 4: The Malariasphere; to evaluate the response of An. gambiae *to yeast-generated CO_2 and a sleeping volunteer in the hut. Mosquitoes were released 5m from the hut. One MM-X trap was placed outside the hut under the roof and two CDC light traps were placed inside. (◙= mosquito release point, *****= MM-X trap, ☼= CDC-trap)*



Two CDC Miniature light traps were hung beside the bed net on the foot-side of the sleeping volunteer, with its shield touching the side of the net (Mboera, et al., 1998). An odour-baited MM-X trap was hung under the thatched roof, in between the mosquito releasing point and the hut. In each experimental run 200 previously not blood fed, three to seven days old, female *An. gambiae* s.s. mosquitoes, pre-starved for 8 hours, were released at 21:30. The next morning the experiment was ended at 06:30 by closing the traps. At 11:00 a recapture was done inside the hut, by way of actively searching for mosquitoes resting inside. Each experimental run had six replicates. A male volunteer aged 27, presented natural human host odours and slept inside the hut on a bed, covered with an untreated bed net. The different treatments that were tested in the

Malariasphere used the CO_2 flow rate y, 100ml/min, as a result of the findings from both the field and screen house experiments. To investigate whether yeast-generated CO_2 and cylinder based CO_2 is able to compete with a sleeping human inside the hut; five different 2-choise experiments (Table 2) were conducted.

Table. 2: The treatments that were tested during dual-choise trapping experiments in the Malariasphere. The treatment on the left of the arrow (↔) was tested against the one on the right of it.

1	Yeast rate y ↔ Empty hut
2	Yeast rate y ↔ Sleeping Human
3	Cyl. CO2 rate y ↔ Sleeping Human
4	Yeast rate y + Odour (Sock) ↔ Sleeping Human
5	Cyl. CO2 rate y + Odour ↔ Sleeping Human

3.8 Odour and carbon dioxide analysis

The headspace of yeast-generated carbon dioxide has been taken to collect the volatiles produced by the yeast. The experiment ran in the lab under temperatures around 22 to 25°C. The yeast setup was connected to a MM-X trap to disperse the carbon dioxide, similar to the experimental setups. Samples were collected with Personal Air Samplers (PAS) from Supelco, which pumped the headspace with 100 ml/min into Tenax tubes. The Tenax tubes were placed under the MM-X trap, 10cm under the and each measurement took 45 minutes. First, 4 control measurements were taken to measure the background, any in the lab present volatiles. Given that there were only three PAS pumps two tubes were measured at the same time. Three hours post starting up the yeast setup, the first three measurements from the yeast treatment were taken. 45 minutes later another three measurements were taken. Finally after 28 hours additional 6 measurements were taken, first three and 45 minutes after the remaining three. All 16 Tenax tubes were send to Wageningen for analysis.

In Wageningen the yeast generated carbon dioxide concentration was measured using a Xentra 4100 analyser (Servomex, The Netherlands) (Spitzen, et al., 2008). Similar to the method above, a tube from the yeast setup was connected to a MM-X trap. 10 Cm under the MM-X trap another tube was placed, connected with the carbon dioxide analyser. A second tube was placed away from the setup and connected to the analyser to measure the background carbon dioxide concentrations. When the yeast was growing for three hours, continuous CO_2 measurements were taken during twelve minutes. Every 3 minutes during one minute, 60 successive readings were taken from the yeast treatment and secondly from the background. The next days, 28 and 52 hours post mixing the yeast, in identical way the CO_2 concentrations were measured. Unfortunately the analyser could only measure over a range of 0 – 1030 parts per million (ppm).

4. Data analysis

4.1 Data collection

For every screen house experiment the total number of trapped mosquitoes were recorded per experiment, trap number and treatment. The mosquitoes caught in the field experiment were counted and identified. The *Anopheline* mosquitoes were identified up to species level using the Orstom identification key (1998), the numbers of *An. gambiae s.l., An. funestus* and remaining other *Anopheline* mosquitoes species were registered. The remaining mosquito species, *Culex, Mansonia and Aedes,* were counted and identified to family level.

4.2 Statistical analysis

The statistical analysis was performed with SPSS 15.0 and in all tests a confidence interval of 5% is used. In the preliminary, the screen house and Malariasphere experiments the attractiveness of a stimulus in a two-choice test was determined, by number of mosquitoes caught by that stimulus. Two unpaired groups were compared with both not normally distributed data. Therefore a non parametric test with two independent samples; Mann-Whitney test was applied. In the field experiment, when the attractiveness of the houses or the treatments were compared to one another, three or more groups were compared with all not normally distributed data. Here for a non parametric test with K independent samples; Kruskal-Wallis test is used. To compare the two series, two houses or two treatments with one another again the Mann-Whitney test was applied. To test if the variables series, houses and treatments interfere with each other, a general linear model, univariate analysis of variance was used.

A second analysis of the field experiment was executed with a general linear model, univariate analysis of variance, to secure the results of the previous tests.

5. Results

5.1 Preliminary experiments

5.1.1 Which CO_2 flow rate is optimal for attracting *Anopheles gambiae s.s.* mosquitoes

Five different carbon dioxide concentrations were tested against a control, an empty trap. Each concentration was tested four times. Flow rates of 25, 60 and 500 ml/min of CO_2 did not catch significantly more mosquitoes than the control trap did (P= 0.767, P= 0.081 and P= 0.309 respectively) (Table 3). The flow rates 100 and 250 ml/min were significantly more attractive than the control (P= 0.020 for both comparisons). Hence these flow rates, respectively cylinder CO_2 rate y and x, were used in the experiments. Although 100ml/min attracted on average more mosquitoes, 44.25, than 250ml/min did, 31.5, this was not significantly different from one another (P= 0.191).

Table 3: The number of An. gambiae s.s *mosquitoes trapped in a 2-choise experiment with different carbon dioxide flow rates and a control, empty trap. The P-values of a Mann-Whitney test between the number of mosquitoes caught with CO_2 and the number caught with the control trap are given in the last column.*

CO_2 flow rate (ml/min)	**Total Number of Mosquitoes released/ Exp.**	**N**	**Average Number of Mosq. ± S.D.**		**Total catch rate (%)**	**P-values**
			CO_2	**Control**		
25	100	4	21.5 ± 15.6	16.0 ± 14.1	37.5	0.767
60	100	4	25.0 ± 20.0	5.5 ± 4.1	30.5	0.081
100	100	4	44.25 ± 17.9	10.5 ± 8.4	54.8	0.020
250	100	4	31.5 ± 13.2	8.0 ± 4.2	39.50	0.020
500	100	4	17.75 ± 6.7	11.75 ± 12.1	29.50	0.309

Per CO_2 flow rate the average number of mosquitoes attracted for the CO_2 and the control ± their Standard Deviation (S.D.), the percentage of the total catch and the P-values of a Mann-Whitney test are given. N= the number of repeats.

5.1.2 Yeast-generated CO_2 productivity

In all eight different mixtures (Table 4), the yeast carbon dioxide production reached 70ml/min or more after one hour. With 17.5 grams of yeast + 500 grams of sugar, 35 grams of yeast + 500 grams of sugar and 35 grams of yeast + 750 grams of sugar the CO_2 productivity increased to a maximum of 375 and 380ml/min, respectively. The average CO_2 productions per treatment are listed in table 4. When the yeast setup was placed outside and run overnight, the average CO_2 production with 35 grams of yeast + 500 grams of sugar was 167 ml/min above ground and 169ml/min when the barrels were buried.

To obtain an equivalent CO_2 flow rate of 100 and 250ml/min with yeast, respectively the mixtures 17.5 grams of yeast + 250 grams of sugar (yeast CO_2 rate y) and 35 grams of yeast + 500 grams of sugar (yeast CO_2 rate x) were used.

Table 4: The average CO_2 production ± standard deviation and the maximum CO_2 production, for each treatment (Y= yeast, S= sugar, W= water and On= overnight).

Treatment	Average CO_2 production ml/min ± S.D.	Maximum CO_2 production ml/min
17,5 gr Y + 250gr S + 2.5l W	136.2 ± 38.1	200
17,5 gr Y + 500gr S + 2.5l W	242.3 ± 74.1	375
17.5 gr Y + 750gr S + 2.5l W	144.8 ± 50.1	240
35 gr Y + 250gr S + 2.5l W	220.2 ± 50.1	285
35 gr Y + 500gr S + 2.5l W	303.5 ± 39.7	380
35 gr Y + 750gr S + 2.5l W	298.1 ± 70.2	380
35 gr Y + 500gr S + 2.5l W On	167 ± 40.0	220
35 gr Y + 500gr S + 2.5l W On Berried	169 ± 43.3	230

5.2 Field experiment

Analysis is done for all the caught mosquitoes then for all *Anopheline* mosquitoes, all *Anopheles gambiae* mosquitoes, all *An. funestus* mosquitoes, all other *Anopheline* mosquitoes and finally for all non *Anopheline* mosquitoes. With 'other *Anopheline* mosquitoes' is meant; *Anopheline* mosquitoes besides *An. gambiae* and *An. funestus.* The non *Anopheline* mosquitoes group contained *Culex, Mansonia* and *Aedes* mosquitoes. In total 878 female mosquitoes were caught over 16 nights in the four selected houses, an overview of all data collected from the field is given in table 9. All mosquitoes were identified and of the 878 mosquitoes 95 were Anopheline and 783 were non-*Anopheline* mosquitoes. Out of 95 *Anopheline* mosquitoes, 45 *An. gambiae s.l.* were caught*,* 10 *An. funestus* and of the remaining 45, described as other *Anopheline* mosquitoes, 43 were *An. coustani* and 2 were *An. pharoensis*.

Initially, the two series, each consisting of eight experimental nights, are analysed and subsequently the series are compared with each other. The second analysis was conducted to see whether there is any difference between the four houses, where upon each house is compared with the other houses for differences in number of trapped mosquitoes. The same is done in the third analysis for the six specific treatments. Finally the fourth analysis answers if there is an interaction between the two variables house number and treatment.

5.2.1 Does the total number of caught mosquitoes decrease over time?

The field experiments were executed during 16 continuous days, from the 7th of December until the 23th of December. The short rainy season normally ends at the end of December but this year it stopped raining from the 3th of December.

To test if the total number of mosquitoes caught decrease over time, the total number of mosquitoes caught by the treatments was compared per day per treatment. And the total number of mosquitoes caught by the treatments, yeast-generated CO_2 at rate y and cylinder CO_2 250cc, in series one and two are compared to one another. In both series no significant difference was found between the mean number of mosquitoes caught per day (Table 5) (P= 0.923 and P= 0.487 series 1 and series 2, respectively). And no significant difference in mean number of mosquitoes caught was found between the two series (P=0.063).

The total number of *Anopheline* mosquitoes and the number of *An. gambiae* mosquitoes and *An. funestus* mosquitoes caught did not significantly change over time for series 1 and 2 (P= 0.938 and P= 0.902, P= 0.602 and P= 0.966, P= 0.130 and 0.728, respectively).

Neither did the total number of mosquitoes caught by the treatments, yeast-generated CO_2 at rate y and cylinder CO_2 250cc, in series one and two differ significantly (P= 0.323, P= 0.174 and P= 0.710, respectively). Similarly the number of trapped mosquitoes of other *Anopheline* species and the number of non *Anopheline* mosquitoes did not significantly change over time for series 1 and 2 (P= 0.809 and P= 0.174, P= 0.774 and P= 0.519, respectively). The series did not differ from each other in number of mosquitoes of other *Anopheline* species (P= 0.937), but in series 2 significantly more mosquitoes of non *Anopheline* species were caught than in series 1 (P= 0.035).

Table 5: Summary of the P-values of the Kruskall-Wallis test and the Mann-Whitney test for respectively; if the number of trapped mosquitoes changes over time, in series 1 and 2 and if the series significantly differ from one another in the number of caught mosquitoes by the treatments yeast-generated CO_2 at rate y and cylinder CO_2 250cc.. This was calculated for respectively the total number of mosquitoes, the total number of Anopheline mosquitoes, An. gambiae mosquitoes, An. funestus mosquitoes, other Anopheline mosquitoes and the total number of non Anopheline mosquitoes.

	Total number of mosq.	**Total number of *Anopheline* mosq.**	***An. gambiae***	***An. funestus***	**Other *Anophelines***	**Total nr of non *An. mosq.***
Series 1	0.923	0.938	0.602	0.130	0.809	0.774
Series 2	0.487	0.902	0.966	0.728	0.406	0.519
Series 1-2	0.063	0.323	0.174	0.71	0.937	0.035

5.2.2 Do the houses differ in the number of mosquitoes caught?

The MM-X traps hang at the houses differ significantly in the total number of caught mosquitoes (P= 0.001) (Table 6). The mean total number of mosquitoes caught by the trap, hang just outside house one, was significantly higher (21.56) (Table 9), than the mean total number of mosquitoes trapped at house 2 (6.06), and 3 (11.50), but did not significantly differ with the mean total number of mosquitoes caught in house 4 (15.75) (P= 0.001, P= 0.036 and P=0.266, respectively). House two's number of caught mosquitoes was almost significantly different from the number caught in house three (P= 0.051), but house two did catch significant less mosquitoes than house four (P= 0.004). House three and four did not significantly differ in caught mosquitoes (P= 0.206).

The four traps placed at the selected houses did also significantly differ in the mean number of caught *Anopheline* mosquitoes (P< 0.001). At house one, on average, significantly more *Anopheline* mosquitoes were caught (3.88) than at house two (0.44), three (0.63) and four (1.00) (P< 0.001, P= 0.001 and P= 0.003 respectively). The houses two, three and four do mutually not significantly differ in attracting the *Anopheline* mosquitoes (P= 0.381, P= 0.445 and P= 0.926 for house two and three, house two and four and house three and four respectively).

When the analysis is only focused on the number of *An. gambiae s.l.* mosquitoes caught at the houses, 40 in total, a similar pattern was found for the mean number of caught *Anophelines*. The four houses caught significantly different numbers of *An. gambiae* mosquitoes

(P= 0.006). At house one, on average, significantly more *An. gambiae* mosquitoes (1.81) were caught than in the three other selected houses (0.25, 0.13 and 0.31 at the houses two, three and four respectively) (P= 0.013, P= 0.005 and P= 0.017 are the P-values of the comparison of house one with house two, three and four respectively). The traps at houses two, three and four did not trap significantly more or less *An. gambiae* mosquitoes than one another (P= 0.591, P= 0.978 and P= 0.591 for house two and three, house two and four and house three and four respectively).

Only 10 *An. funestus* mosquitoes were caught, from which eight were caught at house 1. There-for the houses do significantly differ in the number of *An. funestus* mosquitoes caught (P= 0.006). The MM-X trap at house one trapped, on average, significantly more *An. funestus* mosquitoes (0.50) than the trap at house two (0) but did not significantly differ with the catches at house three and four (0.06 at both house three and four) (P= 0.017, P= 0.067 and P= 0.067 respectively). The houses two, three and four do mutually not significantly differ in attracting the *An. funestus* mosquitoes (P= 0.317, P= 0.317 and P= 1 for house two and three, house two and four and house three and four, respectively).

In total 45 other *Anopheline* mosquitoes were caught. The four traps at the selected houses caught significantly different numbers of other *Anopheline* mosquitoes (P= 0.007). At house one, on average, significantly more other *Anopheline* mosquitoes (1.56) were caught than at house two (0.19), house three (0.44) and at house four (0.63) (P= 0.002, P= 0.038 and P= 0.027 respectively). The houses two, three and four do mutually not significantly differ in attracting other *Anopheline* mosquitoes (P= 0.135, P= 0.364 and P= 0.611 for house two and three, house two and four and house three and four respectively).

The traps at the houses also significantly differed in the total number of caught non *Anopheline* mosquitoes (P= 0.003). At house one significantly more *Culex, Mansonia* and *Aedes* mosquitoes (17,69) were caught than at house 2 (5.63) (P= 0.001). But house one did not significantly differ with the total number of mosquitoes caught at house 3 (10.88) and at house 4 (14.75) (P= 0.062 and P=0.533, respectively). At house two no significantly less mosquitoes were caught than at house three (P= 0.056), but at house two significant less non *Anopheline* mosquitoes were caught than at house four (P= 0.005). The traps at house three and four did not significantly differ in number of trapped mosquitoes (P= 0.250).

Table 6: Summery of P-values of the Kruskall-Wallis test and the Mann-Whitney test for respectively; if the traps at the 4 selected houses differ in number of mosquitoes trapped and if the houses differ mutually. This was calculated for the same parameters as in table 5.

	Total number of mosq.	**Total number of Anopheline mosq.**	***An. gambiae***	***An. funestus***	**Other Anophelines**	**Total nr of non An. mosq.**
House 1-2-3-4	0.001	< 0.001	0.006	0.022	0.007	0.003
House 1-2	0.001	< 0.001	0.013	0.017	0.002	0.001
House 1-3	0.036	0.001	0.005	0.067	0.038	0.062
House 1-4	0.266	0.003	0.017	0.067	0.027	0.533
House 2-3	0.051	0.381	0.591	0.317	0.135	0.056
House 2-4	0.004	0.445	0.978	0.317	0.364	0.005
House 3-4	0.206	0.926	0.591	1	0.611	0.250

5.2.3 Do the treatments differ in mosquito attractiveness?

The five treatments and the control, an empty trap, did significantly differ in attracting mosquitoes ($P< 0.001$) (Table 7). Every treatment attracted also a significant higher total number of mosquitoes than the control did (2.13) (Table 9) ($P= 0.008$, $P< 0.001$, $P< 0.001$, $P= 0.001$ and $P= 0.001$, for 17.5 grams of yeast, 35 grams of yeast, cylinder CO_2, 35 grams of yeast + sock and cylinder CO_2 + sock respectively). But the treatments did not significantly differ from each other. The treatment 17.5 grams of yeast (8.38) did not attract, on average, significant less mosquitoes than the treatments 35 grams of yeast (14.81) ($P= 0.110$) and 250cc cylinder carbon dioxide (16.81) ($P= 0.061$). Similarly the CO_2 produced by 35 grams of yeast did not significantly attract less mosquitoes than the cylinder CO_2 ($P= 0.571$), the CO_2 of 35 grams of yeast + sock (13.00) ($P= 0.654$) and the treatment cylinder CO_2 + sock (20.75) ($P= 0.168$). The remaining comparisons, 35 grams of yeast + sock and cylinder CO_2 + sock ($P= 0.268$), cylinder CO_2 and cylinder CO_2 + sock ($P= 0.408$) did not significantly differ.

The five treatments and the control did significantly differ in attracting the *Anopheline* mosquitoes ($P= 0.049$). Every single treatment was also significant more attractive to the *Anopheles* mosquitoes than the control (0) was ($P= 0.010$, $P= 0.011$, $P= 0.032$, $P= 0.004$ and $P= 0.004$, for 17.5 grams of yeast, 35 grams of yeast, cylinder CO_2, 35 grams of yeast + sock and cylinder CO_2 + sock respectively). The treatment 17.5 grams of yeast was on average not significant less attractive to the *Anopheles* mosquitoes (1.13) than the treatment 35 grams of yeast (1.88) ($P= 0.699$) and the cylinder carbon dioxide (1.50) ($P= 0.570$). The treatment 35 grams of yeast did not significantly attract more mosquitoes than respectively the cylinder CO_2 ($P= 0.362$), the CO_2 of 35 grams of yeast + sock (1.75) ($P= 0.727$) and the treatment cylinder CO_2 + sock (2.25) ($P= 0.568$). The treatments 35 grams of yeast + sock and cylinder CO_2 + sock ($P= 0.268$), cylinder CO_2 and cylinder CO_2 + sock ($P= 0.408$) did not significantly differ in attracting the *Anopheles* mosquitoes.

During 16 days 40 *An. gambiae* mosquitoes were caught in the traps. These *An. gambiae* mosquitoes responded significantly different to the 5 treatments and the control ($P= 0.011$). The treatments with an odour source, a worn sock, attracted on average significant more *An. gambiae* mosquitoes than the control (0) did ($P= 0.004$ and $P= 0.027$, for 35 grams of yeast + sock and cylinder CO_2 + sock respectively). The treatments lacking a sock did not attract significant more *An. gambiae* mosquitoes than the control trap did ($P= 0.317$, $P= 0.201$ and $P= 0.201$ for the treatments 17.5 grams of yeast, 35 grams of yeast and cylinder CO_2 respectively). When the treatments are compared mutually, there was only one comparison that was significant different. 35 Grams of yeast + sock caught on average (1.5) significantly more mosquitoes than 35 grams of yeast without sock (0.44) ($P= 0.018$). The other treatments did not significantly differ from one another. 17.5 grams of yeast did not attract significant less *An. gambiae* mosquitoes (0.38) than 35 grams of yeast (0.44) and cylinder CO_2 (0.50) ($P= 0.741$ for both equations). The CO_2 produced by 35 grams of yeast did not significantly attract less *An. gambiae* mosquitoes than the cylinder CO_2 ($P= 0.978$) and the treatment cylinder CO_2 + sock (1.25) ($P= 0.126$). In the same way, 35 grams of yeast + sock did not significantly differ with cylinder CO_2 + sock ($P= 0.476$) and cylinder CO_2 with cylinder CO_2 + sock ($P= 0.126$). When the latter equation was analysed again with only the data of series 2, cylinder CO_2 + sock did attract significant more *An. gambiae* mosquitoes than just cylinder CO_2 ($P= 0.027$). The outcome of other comparisons with the specific data, series 1 or series 2 only, did not differ with the above results.

In total ten *An. funestus* mosquitoes were caught, these *An. funestus* mosquitoes did not have a significant preference for one of the treatments (P= 0.717). The five treatments did not significantly differ with the control in number of caught *An. funestus* mosquitoes (0) (P= 0.317, P= 0.307, P= 0.408, P= 0.317 and P= 0.143, for the treatments 17.5 grams of yeast, 35 grams of yeast, cylinder CO_2 and cylinder CO_2 + sock respectively). Neither did the treatments 17.5 grams of yeast (0.13) and 35 grams of yeast (0.25) (P= 0.957), 17.5 grams of yeast and cylinder CO_2 (0.60) (P=0.609), 35 grams of yeast and cylinder CO_2 (P= 0.978), 35 grams of yeast and 35 grams of yeast + sock (0.25) (P= 1), 35 grams of yeast and cylinder CO_2 + sock (0.25) (P= 0.508), 35 grams of yeast + sock (0.25) and cylinder CO_2 + sock (P= 0.643), cylinder CO_2 and cylinder CO_2 + sock (P= 0.200) differ from one another in the average number of caught *An. funestus* mosquitoes.

The 'other *Anopheline* species' mosquitoes hade a significant preference for one of the treatments (P= 0.020). The treatments 17.5 grams of yeast (0.63), 35 grams of yeast (1.19) and cylinder CO_2 + sock (0.75) caught on average significantly more other *Anopheline* species than the control (0) did (P= 0.027, P= 0.011 and P= 0.010 respectively). The number of 'other *Anopheline* species' mosquitoes attracted by the treatments, cylinder CO_2 (0.94) (P= 0.084) and 35 grams of yeast + sock (0) (P= 1) did not significantly differ with the control. Both 35 grams of yeast + sock and the control did not attract a single 'other *Anopheline* mosquito'. Therefore the treatment 35 grams of yeast + sock was significant less attractive than the treatments 35 grams of yeast and cylinder CO_2 + sock (P= 0.011 and P= 0.010 respectively). The remaining treatments did not significantly differ from one another in the number of caught individuals of other *Anopheline* species; 17.5 grams of yeast and 35 grams of yeast (P= 0.544), 17.5 grams of yeast and cylinder CO_2 (P= 0.595), 35 grams of yeast and cylinder CO_2 (P= 0.233), 35 grams of yeast and cylinder CO_2 + sock (P= 0.844) and cylinder CO_2 and cylinder CO_2 + sock (P= 0.334).

In total 783 *Culex, Aedes* and *Mansonia* mosquitoes were caught. These mosquitoes were attracted significantly different by the treatments and the control (P< 0.001). All the treatments caught significant more of these non *Anopheline* mosquitoes than the control (2.13) did (P= 0.020, P= 0.001, P< 0.001, P= 0.001 and P= 0.001, for 17.5 grams of yeast, 35 grams of yeast, cylinder CO_2, 35 grams of yeast + sock and cylinder CO_2 + sock respectively). Cylinder carbon dioxide did attract on average significant more of *Culex, Aedes* and *Mansonia* mosquitoes (15.31) than 17.5 grams of yeast did (7.25) (P= 0.032). The remaining treatments did not significanlty differ from one another in number of caught *Culex, Aedes* and *Mansonia* mosquitoes 17.5 grams of yeast and 35 grams of yeast (12.94), 35 grams of yeast and cylinder CO_2, 35 grams of yeast and 35 grams of yeast + sock (13.5), 35 grams of yeast and cylinder CO_2 + sock (18.5), 35 grams of yeast + sock and cylinder CO_2 + sock, cylinder CO_2 and cylinder CO_2 + sock.

Table 7: Summery of P-values of the Kruskall-Wallis test and the Mann-Whitney test for respectively; if the 6 treatments are differently attractive to mosquitoes in the field and if the treatments differ mutually. This was calculated for the same parameters as in table 5.

	Total nr of mosq.	**Total nr of *Anopheline* mosq.**	***An. gambiae***	***An. funestus***	**Other *Anophelines***	**Total nr of non *An. mosq.***
All treatments	< 0.001	0.049	0.011	0.717	0.020	< 0.001
17.5gr Y - Control	0.008	0.010	0.317	0.317	0.027	0.020
35gr Y - Control	< 0.001	0.011	0.201	0.307	0.011	0.001
Cyl CO_2 - Control	< 0.001	0.032	0.201	0.480	0.084	< 0.001
35 gr Y + Sock - Control	0.001	0.004	0.004	0.317	1.000	0.001
Cyl CO_2 + Sock - Control	0.001	0.004	0.027	0.143	0.010	0.001
17.5 gr Y - 35 gr Y	0.110	0.699	0.741	0.957	0.544	0.208
17.5 gr Y - Cyl CO_2	0.061	0.570	0.741	0.609	0.595	0.032
35 gr Y - Cyl CO_2	0.571	0.362	0.978	0.526	0.233	0.336
35 gr Y - 35 gr Y + Sock	0.654	0.727	0.018	1.000	0.011	0.462
35 gr Y - Cyl CO_2 + Sock	0.168	0.568	0.126	0.508	0.844	0.149
35 gr Y + Sock - Cyl CO_2 + Sock	0.268	0.913	0.476	0.643	0.010	0.268
Cyl CO_2 - Cyl CO_2 + Sock	0.408	0.170	0.126*	0.200	0.334	0.443

* = 0.027 When only serie 2 data is used

5.2.4 Is there an interaction between the variables house number and treatment?

No interaction was found between the house number and the treatments for the total number of mosquitoes and the total number of *Anopheles* mosquitoes caught (P= 0.719 and P= 0.957 respectively).

5.2.5 General linear model

A second analysis of the field experiment was executed with a general linear model, univariate analysis of variance, to secure the results of the previous tests. When the total number of trapped mosquitoes was analysed for the 4 houses (21.56, 6.06, 11.50, 15.75 are the average numbers of trapped mosquitoes at house one, two, three and four respectively, table 9) a significantly different preference was found (P= 0.001) (Table 8). Similarly, significant differences were found among the 6 treatments (P= 0.002) (2.13, 8.38, 14.81, 16.81, 13.00 and 20.75 for respectively the control and the treatments 17.5 grams of yeast, 35 grams of yeast, cylinder CO_2, 35 grams of yeast + sock and cylinder CO_2 + sock). No significant difference was found between the series (P=0.048, only analysed with the data of the treatments that were used in both series,

17.5 grams of yeast and cylinder CO_2). Among the three factors, house, treatment and series, no interaction was found.

Focused on the total number of *Anopheles* mosquitoes and only the *An. gambiae s.l.* mosquitoes, the houses were significant different attractive to the mosquitoes (P=0.001 and P<0.001). But the total number of *Anopheles* mosquitoes were not significantly different attracted to the different treatments (P= 0.155). The *An. gambiae s.l* mosquitoes were significantly different attracted to the treatments (P= 0.044) (0, 0.38, 0.44, 0.50, 1.50 and 1.25 for respectively the control and the treatments 17.5 grams of yeast, 35 grams of yeast, cylinder CO_2, 35 grams of yeast + sock and cylinder CO_2 + sock).

Table 8: Summery of P-values of the univariate analysis of variance test for respectively; the four houses, the six treatments, the two series and if there is an interaction or not. This was calculated for respectively the total number of mosquitoes, the total number of Anopheline mosquitoes and An. gambiae mosquitoes.

	Total number of mosquitoes	**Total number of *Anopheline* mosq.**	***An. gambiae***
Houses	0.001	0.001	<0.001
Treatments	0.002	0.155	0.044
Series	0.141		
Interactions	No		

Table 9: Overview data collected from the field experiment: The mean number of mosquitoes caught in the specific houses (left) and attracted by the specific treatments (right), their standard deviation and the minimum and maximum values.

House Nr	Total number of mosquitoes (Mean)	St. Dev.	Min.	Max.	Treatment	Total number of mosquitoes (Mean)	St. Dev.	Min.	Max.	Total number of mosq.
All mosquitoes					**All mosquitoes**					878
House 1	21.56	14.94	3	61	17.5 gr Y	8.38	6.95	3	22	
House 2	6.06	5.05	0	18	35gr Y	14.81	11.76	2	43	
House 3	11.50	10.14	0	43	Cyl CO_2	16.81	13.89	3	61	
House 4	15.75	10.21	2	34	Control	2.13	1.80	0	5	
					35 gr Y + Sock	13.00	9.90	4	31	
					Cyl CO_2 + Sock	20.75	11.94	6	35	
***Anopheline* mosquitoes**					***Anopheline* mosquitoes**					95
House 1	3.88	3.69	0	14	17.5 gr Y	1.13	1.64	0	5	
House 2	0.44	0.81	0	2	35 gr Y	1.88	2.53	0	8	
House 3	0.63	0.81	0	3	Cyl CO_2	1.50	3.48	0	14	
House 4	1.00	2.03	0	8	Control	0	/	0	0	
					35 gr Y + Sock	1.75	2.25	0	7	
					Cyl CO_2 + Sock	2.25	2.71	0	7	
***An. gambiae* mosquitoes**					***An. gambiae* mosquitoes**					40
House 1	1.81	2.11	0	6	17.5 gr Y	0.38	1.06	0	3	
House 2	0.25	0.58	0	2	35 gr Y	0.44	1.03	0	3	
House 3	0.13	0.34	0	1	Cyl CO_2	0.50	1.50	0	6	
House 4	0.31	0.79	0	3	Control	0	/	0	0	
					35 gr Y + Sock	1.5	1.6	0	5	
					Cyl CO_2 + Sock	1.25	1.83	0	5	
***An. funestus* mosquitoes**					***An. funestus* mosquitoes**					10
House 1	0.50	0.89	0	3	17.5 gr Y	0.13	0.35	0	1	
House 2	0	/	0	0	35 gr Y	0.25	0.78	0	3	
House 3	0.06	0.25	0	1	Cyl CO_2	0.60	0.25	0	1	
House 4	0.06	0.25	0	1	Control	0	/	0	0	
					35 gr Y + Sock	0.25	0.71	0	2	
					Cyl CO_2 + Sock	0.25	0.46	0	1	
Other *Anopheles* mosquitoes					**Other *Anopheles* mosquitoes**					45
House 1	1.56	2.07	0	8	17.5 gr Y	0.63	0.74	0	2	
House 2	0.19	0.54	0	2	35 gr Y	1.19	1.56	0	5	
House 3	0.44	0.63	0	2	Cyl CO_2	0.94	2.08	0	8	
House 4	0.63	1.41	0	5	Control	0	/	0	0	
					35 gr Y + Sock	0	/	0	0	
					Cyl CO_2 + Sock	0.75	0.71	0	2	
Non- Anopheles mosquitoes					**Non- Anopheles mosquitoes**					783
House 1	17.69	11.8	3	47	17.5 gr Y	7.25	6.27	2	21	
House 2	5.63	4.46	0	16	35 gr Y	12.94	11.49	2	42	
House 3	10.88	9.93	0	42	Cyl CO_2	15.31	10.81	3	47	
House 4	14.75	10.14	2	33	Control	2.13	1.80	0	5	
					35 gr Y + Sock	13.5	8.1	4	26	
					Cyl CO_2 + Sock	18.5	10.14	6	33	

5.3 Screen house experiments

To investigate whether the yeast-generated CO_2 is able to compete with cylinder based CO_2 thirteen different 2-choise experiments (Table 1) were conducted in a screen house. Two different yeast-generated CO_2 production rates, two different cylinder based CO_2 flow rates and a worn sock were tested. The two yeast-mixture, y and x, produced an estimated 100 and 170ml/min of CO_2, during the night. The cylinder CO_2 flow rate y and x were set on respectively 100 and 250 ml/min.

In the first experiment two empty MM-X traps were tested against each other to test for a bias in the setup. The 200 released *An. gambiae s.s.* mosquitoes did not have any preference, both traps caught approximately the same number of mosquitoes (P= 0.538) (Table 10) and only 5.13% of all released mosquitoes were trapped.

5.3.1 Yeast-generated CO_2 as an attractant for *An. gambiae s.s.* mosquitoes and can it compete with the cylinder based CO_2?

Both yeast-generated CO_2 rates, y (62.5) and x (62,75) attracted significantly more mosquitoes than the empty trap ((P= 0.020 for both rate y and x) (Table 10). When traps baited with yeast-generated CO_2, rates y (68.5) and x (81.5), were tested against traps baited with cylinder CO_2, respectively rate y (47.5) and x (61.0), both were able to compete with the cylinder based CO_2. Yeast-generated CO_2 rate y and x did not attract significant less mosquitoes than respectively cylinder based CO_2 rate y and x (P= 0.663 and P= 0.081, respectively).

5.3.2 Does adding yeast-generated CO_2 to an odour source augment the catch rates?

A trap baited with a worn sock was significantly more attractive (72.0) than an unbaited trap (12.0) (P= 0.021) (Table 10). When yeast-generated CO_2 at rate y was added to the worn sock, the trap attracted significantly more *An. gambiae s.s.* mosquitoes (102.75) than the empty trap (3.25) (P= 0.020). Yeast-generated CO_2 rate y does augment the catch rates of an odour source, as yeast-generated CO_2 rate y and a worn sock attracted significant more mosquitoes (145.25) than the worn sock did alone (13.75) (P= 0.021). Yeast-generated CO_2 rate y with the worn sock (85.5) could also compete with cylinder based CO_2 rate y with a worn sock (66.25): no significant difference in mosquito catches was found between both treatments (P= 0.149).

5.3.3 Can the yeast-generated CO_2 setup still attract *An. gambiae* mosquitoes when running for 24 hours and 48 hours and can it still compete with cylinder based CO_2?

When the yeast rate y was growing for 24 hours, it was still producing sufficient CO_2 to attract significant more mosquitoes (33.0) than an empty trap (4.5) (P= 0.020) (Table 10). The yeast CO_2 was also able to compete with cylinder based CO_2 rate y since no significant difference was found in number of caught mosquitoes between cylinder based CO_2 rate y (96.0) and yeast rate y (24 h) (60.0) (P= 0.564). When growing for 48 hours, the yeast rate y was not attracting significant more mosquitoes (14.25) than the empty trap (10.25) (P= 0.773). After 48 hours the yeast was not able to compete with cylinder based CO_2 rate y anymore: the cylinder based CO_2 caught significant more *An. gambiae* mosquitoes (85.5) than the yeast rate y (48 h) (7.5) (P= 0.021).

Table 10: Summery of the data collected in the screen house. The mean number of mosquitoes attracted by the treatment and the control ± their Standard Deviation (S.D.).The total catch over the 4 repeats and it's percentage and in the most right column the P-values are listed of the Mann-Whitney tests (P<0.05).

Experiment	N	Total nr of mosq. released	Mean Number of Mosq. Caught ± S.D.		Total catch	Total rate (%)	P - values
			Treatment	Control			
Empty trap vs Empty trap	4	800	5.5 ± 2.38	4.75 ± 3.78	41	5.13	0.538
Yeast rate y vs Empty trap	4	800	62.5 ± 23.30	4 ± 0.82	266	33.25	0.020
Yeast rate x vs Empty trap	4	800	62.75 ± 20.95	2.75 ± 1.89	262	32.75	0.020
Yeast rate y vs Cylinder CO_2 rate y	4	800	68.5 ± 53.92	47.5 ± 26.15	464	58	0.663
Yeast rate x vs Cylinder CO_2 rate x	4	800	81.5 ± 15.61	61 ± 13.93	570	71.25	0.081
Odour (Sock) vs Empty trap	4	800	72 ± 15.85	12 ± 1.83	336	42	0.021
Yeast rate y + Odour (Sock) vs Empty trap	4	800	102.75 ± 51.18	3.25 ± 1.50	424	53	0.020
Yeast rate y + Odour (Sock) vs Odour (Sock)	4	800	145.25 ± 4.27	13.75 ± 8.54	636	79.5	0.021
Yeast rate y + Odour (Sock) vs Cyl. CO_2 rate y + Odour	4	800	85.5 ± 15.76	66.25 ± 10.56	607	75.88	0.149
Yeast rate y (24h) vs Empty trap	4	800	33 ± 12.00	4.5 ± 3.11	150	18.75	0.020
Yeast rate y (24h) vs Cylinder CO_2 rate y	4	800	60 ± 30.63	96 ± 46.14	624	78	0.564
Yeast rate y (48h) vs Empty trap	4	800	14.25 ± 11.35	10.25 ± 8.02	98	12.25	0.773
Yeast rate y (48h) vs Cylinder CO_2 rate y	4	800	7.5 ± 3.70	85.5 ± 12.61	372	46.5	0.021

5.4 Malaria sphere

In the malaria sphere only rate y, as a result of previous experiments, was used for both yeast-generated CO_2 and the cylinder based CO_2, a CO_2 output of 100 ml/min. In the analysis two different counts were used. At first the treatments catch was compared to the sum of catches from CDC one and two inside the hut (P- value 1). A second comparison was made by comparing the treatments catch against the sum of the two CDC traps and the mosquitoes recaptured inside the hut (P- value 2).

In a control experiment the yeast-generated CO_2 was tested against an empty hut. In both counts, the yeast-generated CO_2 attracted significant more mosquitoes than an empty hut (P= 0.020 for both counts). All P-values are listed in table 11.

5.4.1 Can yeast-generated CO_2 and the cylinder based CO_2 attract more mosquitoes than a sleeping human inside a hut?

On average the yeast-generated CO_2 attracted 28.75 mosquitoes, this was significant less than the number of mosquitoes that were caught in the two CDC light traps (55.25) (P= 0.021) and consequently also significant less than the total of CDC light traps and the recapture (66.75) (P= 0.021).

Cylinder based CO_2 attracted on average 42.25 mosquitoes, this was not significant different from the catches of the CDC light traps (53.0) (P= 0.248) and not significant different from the catches of the CDC light traps and the recapture combined (64.75) (P= 0.248).

5.4.2 Can yeast-generated CO_2 and the cylinder based CO_2, added with a worn sock, attract more mosquitoes than a sleeping human inside a hut?

When the yeast-generated CO_2 was added to a worn sock, it attracted significant more mosquitoes (113.5) than the CDC light traps (18.0) (P= 0.020) and significant more than the CDC light traps and the recapture combined (24.0) (P= 0.021).

Without adding the number of recaptured mosquitoes, cylinder based CO_2 attracted significant more mosquitoes (101.75) than the CDC light traps (39.25) (P= 0.043). But when the number of the recapture mosquitoes (6.75) is added to the number of mosquitoes caught by the CDC light traps, cylinder based CO_2 did not attract significant more mosquitoes than the sleeping human inside the hut (P= 0.083).

Table 11: Summery of the data collected in the Malaria sphere per experiment. The mean number of mosquitoes attracted by the treatment, the CDC light traps and the recapture ± their Standard Deviation (S.D.).The second part of the table lists the total catch, it's percentage and in the two most right columns the P-values of the Mann-Whitney tests (P<0.05).

Experiment	N	Total nr of mosq. released	Mean Number of Mosq. Caught ± S.D.		
			Treatment	CDC 1 + 2	Recapture
Yeast rate y vs Empty hut	4	800	91.75 ± 25.08	9 ± 5.71	16.25 ± 4.57
Yeast rate y vs Sleeping Human	4	800	28.75 ± 5.62	55.25 ± 19.05	11.5 ± 2.08
Cyl. CO_2 rate y vs Sleeping Human	4	800	42.25 ± 2.50	53 ± 21.37	11.75 ± 2.63
Yeast rate y + Odour (Sock) vs Sleeping Human	4	800	113.5 ± 30.62	18 ± 8.98	6 ± 0.82
Cyl. CO_2 rate y + Odour vs Sleeping Human	4	800	101.75 ± 38.77	39.25 ± 15.33	6.75 ± 3.10
	Total catch	**Total rate %**	**P - values 1**	**P - values 2**	
Yeast rate y vs Empty hut	468	58.50	0.02	0.02	
Yeast rate y vs Sleeping Human	382	47.75	0.021	0.021	
Cyl. CO_2 rate y vs Sleeping Human	550	68.75	0.248	0.248	
Yeast rate y + Odour (Sock) vs Sleeping Human	428	53.50	0.02	0.021	
Cyl. CO_2 rate y + Odour vs Sleeping Human	591	73.88	0.043	0.083	

N= Number of repeats, P-value 1= the treatments catch was compared to the sum of catches from CDC one and two inside the hut, P-value 2= the treatments catch was compared against the sum of the two CDC traps and the mosquitoes recaptured inside the hut.

5.5 Odour and carbon dioxide analysis

The headspace of yeast-generated carbon dioxide sampled in Mbita, to collect the volatiles produced by the yeast, was send to Wageningen for analysis. Unfortunately the split ratio was unclear and water vapour was overwhelming and troubling the analysis so no clear results came out.

The concentrations of the background CO_2 and the yeast-generated CO_2, dispersed trough a MM-X trap, were measured 3, 28 and 52 hours post preparing the yeast mixture. Unfortunately the CO_2 analyser had a limited measuring range, 0 – 1030 ppm and both the measurements after 3 and 28 hours exceeded this maximum level. Out of which we only can conclude that the yeast-generated CO_2, distribuated by a MM-X trap, has a higher CO_2 level than 1030 ppm. The mean ± SD background CO_2 concentration were 610.10 ppm ± 72 after 3 hours and 735.30 ppm ± 111 after 28 hours. As a reference, Spitzen *et al.* (2008) measured 514 ± 65 ppm background CO_2 levels, in a flight chamber using the same carbon dioxide analyser, Xentra 4100. When measurements were undertaken 52 hours post mixing the yeast, the CO_2 concentration sampled under the MM-X trap dropped to 924.02 ppm ± 142 and the background CO_2.level was 561.04 ppm ± 145.

6. Discussion

In search of a vertebrate host, for a blood meal, female mosquitoes fly upwind attracted by host related cues (Bowen, 1991; Takken, 1991; Takken and Knols, 1999). This positive anemotaxis is governed by several factors including temperature, humidity and visual objects. The key factor in host location of *An. gambiae* are, however, host related odours (Braks and Takken, 1999; Costantini, et al., 1996; Dejong and Knols, 1995; Mukabana, et al., 2002; Mukabana, et al., 2004; Njiru, et al., 2006; Takken, 1991; Takken and Knols, 1999). One of the attractive host related odour components is carbon dioxide (Gillies, 1980; Snow, 1970) and therefore used in host-seeking behavioural studies and to attract female mosquitoes in the field. Carbon dioxide is conventionally delivered through gas cylinder bottles or in the form of dry ice. But both these CO_2 sources are expensive and hard to distribute in vast field studies (Burkett, et al., 2001; Xue, et al., 2008). Therefore a cheaper and more sustainable CO_2 sources, yeast-generated CO_2, was tested against gas cylinder CO_2 in field en semi-field conditions in western Kenya.

Throughout the experiments in the screen house, Malariasphere and in the field carbon dioxide proved to be a mosquito attractant. In the preliminary experiment, conducted with cylinder CO_2, was observed that only the traps baited with 100 and 250 ml/min CO_2 caught significant more female *An. gambiae s.s.* mosquitoes than an unbaited trap. To obtain equivalent CO_2 flow rates using yeast, respectively rate y and x, certain amounts and proportions of sugar, water and yeast were mixed in two sealed off barrels. This preliminary dual-choice experiment, to measure the optimal CO_2 flow rate to attract *An. gambiae s.s.* mosquitoes, was conducted in a rather small mosquito cage of 6x2x2 meters. The CO_2 released by the trap could easily disperse close to the other trap, which served as a control, and change the CO_2 level around the unbaited control trap and consequently influence the attractiveness to *An. gambiae s.s.* mosquitoes. Especially with higher CO_2 flow rates, which was the case for the treatment 500ml/min. The *An. gambiae s.s.* mosquitoes did not significantly prefere the trap baited with 500ml/min of CO_2 above the unbaited trap. Conducting this dual-choice experiment in a larger setup and with more CO_2 flow rates could result in a better and more trustable insight of the optimal CO_2 flow rate to attract *An. gambiae s.s.* mosquitoes.

In the screen house, if the traps were baited with one of the yeast-generated CO_2 rates, it attracted significant more *An. gambiae s.s.* mosquitoes than an unbaited trap, in dual-choice experiments. When both yeast CO_2 rates were tested against their respectively equivalent cylinder CO_2 flow rate, the yeast CO_2 was also able to attract as many mosquitoes as cylinder CO_2. After growing for 24 hours, the yeast-sugar solution producing CO_2 at rate y was still producing sufficient CO_2 to attract significant more mosquitoes than an empty trap and the yeast CO_2 was attracting as many mosquitoes as cylinder based CO_2 at rate y. Unfortunately this was not the case anymore after 48 hours. When the yeast-generated CO_2 was added to a trap baited with a worn sock, yeast CO_2 augmented the catches of the traps baited with a worn sock. And also here traps baited with the yeast CO_2 and a worn sock were able to attract as many mosquitoes as traps baited with cylinder CO_2 and a worn sock.

Analysing the yeast-generated CO_2, dispersed trough a MM-X trap, pointed out that the CO_2 concentration produced during the first 28 hours exceeds 1030 ppm. In the semi-field experiments this was sufficient to attract *An. gambiae s.s.* mosquitoes, the *An. gambiae* neurones are sensitive to a CO_2 concentration span of approximately 0-4000 ppm, the CO_2 concentrations

likely to be encountered during host seeking (Qiu, et al., 2007; Spitzen, et al., 2008; Takken and Knols, 1999). But after 48 hours, the CO_2 flow rate is very low and the yeast-generated CO_2 concentration droped to 924 ppm. This lower flow rate and CO_2 concentration could have elicited the lower catch rates after growing the yeast for 52 hours.

In the Malariasphere, traps hanging outside the hut, baited with yeast-generated CO_2 at rate y and added with a worn sock, were able to compete with a human volunteer sleeping inside the hut. Yeast-generated CO_2 at rate y and a worn sock attracted significant more *An. gambiae s.s.* mosquitoes than the CDC light traps, hanging besides a non-impregnated mosquito bed net protecting a sleeping human, and the number of mosquitoes recaptured inside the hut. When the same was done with cylinder CO_2 at rate y and a worn sock, the number of mosquitoes trapped with the CDC light traps and recaptured was not significant different from the number of mosquitoes trapped outside the hut. Hence yeast-generated CO_2 prevented more *An. gambiae s.s.* mosquitoes from entering the hut than cylinder CO_2.

To test the efficacy of yeast-generated carbon dioxide as a mosquito attractant, and for epidemiological purpose specifically for the attraction of Malaria vectors, *An. gambiae s.l.* and *An. funestus* mosquitoes, a field experiment was completed in Luanda. During 16 nights MM-X traps were baited with yeast-generated CO_2 and tested against traps baited with cylinder CO_2. A total of 878 female mosquitoes were trapped just outside the four selected houses. Out of the 878 mosquitoes, 95 were *Anopheline;* containing 40 *An. gambiae s.l.,* 10 *An. funestus* and out of the remaining 45, described as other *Anopheline* mosquitoes, 43 were *An. coustani* and 2 were *An. pharoensis*. In a previous study conduted by Seyoum et al. (2003) in Luanda, the ratios of *An. gambiae s.l.* and *An. funestus* mosquitoes were respectively 79.5% and 20.5%. In this field experiment 42% of the anopheline mosquitoes were *An. gambiae s.l.* , 10.5% were *An. funestus* and 47.4% were other *Anopheline* mosquitoes. *An. gambiae s.l.* mosquitoes in proportion to *An. funestus* mosquitoes is almost identical, 4 *An. gambiae s.l.* to 1 *An. funestus.* The molecular identification of *An. gambiae s.l.* mosquitoes to determine the percentages of mosquitoes being *An. gambiae sensu stricto* or *An. arabiensis* has still to be done.

In Luanda, the four houses were specifically selected based on a number of criteria. Although the four houses had common features, the trap placed at house one caught significantly more mosquitoes (345) than the traps placed at house two (97) and three (184). More remarkable is that the trap placed at house one caught 29 (72.5%) out of a total of 40 *An. gambiae s.l.* mosquitoes, significant more than at all other houses. Eight of ten *An. funestus* mosquitoes were also trapped at house one. On the other hand, for the non-anopheline mosquitoes there is no notable difference found between the trap catches at the different houses. This difference could be resulted by, firstly, the more dominant presence of cattle and goats near the houses three and four. Therefore *An. gambiae s.l.* and *An. funestus* mosquitoes may have been less attracted to the domestic animal odours present around house three and four (2 and 5 *An. gambiae s.l.* mosquitoes trapped, respectively). Whereas these antrophophilic species (White, 1974), may have been more attracted to the relatively higher presence of human odours around house one, where fewer cattle were present. Secondly, there is a small swamp at approximately 150 meters at the right side of house one, more distant to the other houses (Fig 3). The proximity to a breeding site therefore favours house one above the other houses for all mosquito species.

In the first series traps baited with two different yeast CO_2 flow rates, y and x with during the day respectively 136 and 304 ml/min average CO_2 production, were tested against traps baited with cylinder CO_2 (250 ml/min) and an unbaited trap, as a control. In the second series yeast CO_2 rate x and cylinder CO_2 are tested against yeast CO_2 rate x and cylinder CO_2 added to a worn sock. All five treatments attracted significantly more mosquitoes and also more Anopheline mosquitoes than the control (unbaited trap) did. Specifically for *An. gambiae s.l.* mosquitoes, only traps baited with CO_2 and a worn sock attracted significantly more *An. gambiae s.l.* mosquitoes than the unbaited trap. The yeast and cylinder CO_2 odour sources without a worn sock did attract *An. gambiae s.l.* mosquitoes but not significantly more than an unbaited trap. Since *An. gambiae s.l.* is highly anthropophilic (White, 1974) and thus attracted to human specific odours (Takken and Knols, 1999), in this case a worn sock. And recently, both Spitzen *et al.* (2008) and Schmied *et al.* (2008) concluded that CO_2 augmented the attractiveness of human odours to *An. gambiae s.s.* mosquitoes. This may have caused the larger number of *An. gambiae s.l.* mosquitoes caught in the traps baited with CO_2 added to a worn sock.

Unfortunately only 40 *An. gambiae s.l.* mosquitoes were trapped, these low catches might also explain why yeast and cylinder CO_2 did not attract significantly more *An. gambiae s.l.* mosquitoes than an unbaited trap. Also only 10 *An. funestus* mosquitoes were trapped during the 16 experimental nights. This may have resulted in no preference of *An. funestus* mosquitoes for any of the treatments. *An. funestus* is like *An. gambiae s.l.* mosquitoes anthropophilic (Mboera, et al., 1997; White, 1974) and therefore expected to also be more attracted to the treatments with a worn sock than to traps baited with only CO_2. The relatively low number of trapped *An. gambiae s.l.* and *An. funestus* mosquitoes could be resulted because the traps were placed outdoor and indoor sampling would presumably have yielded larger numbers of anthropophilic mosquitoes as shown by Mboera *et al.* (1996) in Tanzania. For ethical reasons the odour baited traps were placed outside, but with more repeats or conducting the field experiment in the long rainy season, more *An. gambiae s.l.* and *An. funestus* mosquitoes could be trapped and subsequently result in more clear data.

When the five specific CO_2 treatments are compared to one another, two groups of mosquitoes, the total number of mosquitoes and the Anopheline mosquitoes, did not show any significant preference for one of the treatments. Both yeast-generated CO_2 rates were able to compete with the conventional cylinder CO_2. Moreover, yeast CO_2 was attracting as many mosquitoes as CO_2 for all distinct groups, except for the non-Anopheline mosquitoes. So also for *An. gambiae s.l.* mosquitoes, no significant difference was found in de trap catches of traps baited with yeast CO_2 at rate y, yeast CO_2 at rate x and cylinder CO_2. Cylinder CO_2 attracted only significant more non-Anopheline mosquitoes than yeast CO_2 at rate y did. The trapped non-Anopheline mosquitoes are considered to be opportunistic blood-feeders or zoophilic. And since the degree of attractiveness to CO_2 is assumed to increase with the degree of zoophily (Costantini, et al., 1996; Dekker and Takken, 1998; Snow, 1970), the non- Anopheline mosquitoes were significantly more trapped in the traps baited with the higher CO_2 flow rate of the cylinder bottle (250ml/min) compared to yeast CO_2 at rate y (estimated on 100ml/min at night). Whereas cylinder CO_2 did not attracted significantly more non- Anopheline mosquitoes than yeast CO_2 at rate x (estimated on 170ml/min at night), probably because of the smaller difference in CO_2 flow rate.

With regard to the goal of this study, to test yeast-generated carbon dioxide as a CO_2 source to attract *An. gambiae* mosquitoes in the field, the traps baited with the yeast CO_2 attracted as many *An. gambiae s.l.* mosquitoes as the conventional gas cylinder CO_2. *An. gambiae s.s.* mosquitoes in the semi-field experiments and *An. gambiae s.l.* and *An. funestus* mosquitoes in the field. In previous lab-experiments in Wageningen, conducted by Wolfgang Schmied, *An. gambiae s.s.* mosquitoes significantly prefered yeast-generated CO_2 above cylinder CO_2 baited traps. Similar tendency was also found in the screen house experiments, although the difference was not significant, more *An. gambiae s.s.* mosquitoes were trapped in the MM-X traps baited with yeast CO_2 than the traps baited with cylinder CO_2. This tendency of *An. gambiae s.s.* mosquitoes preferring yeast-generated CO_2 over cylinder CO_2 might be caused by several factors of yeast, which influence the attractiveness of yeast CO_2. At first, yeast does not produce CO_2 at a constant flow rate but at an intermittent flow rate and this is in fact what happens under natural conditions, mammals release breath in pulses and therefore alternations of carbon dioxide plumes attract the mosquito (Mboera and Takken, 1997; Takken, 1991). In the absence of other host factors, these alternations of carbon dioxide concentrations sustain the mosquito's flight (Gillies, 1980; Omer, 1979). Secondly, in proximity of the host, mosquitoes respond to non-olfactory cues such as convection heat and body moisture (Laarman, 1958). Besides carbon dioxide, the fermenting yeast also produces water and heat (Walker, 1998). So the yeast-setup might also positively influence the moisture and temperature gradient around the setup to attract mosquitoes, which should be measured and tested in the prospect. Thirdly, the additional volatiles produced by the yeast could augment the attractiveness of the yeast generated CO_2 to *An. gambiae s.l.* mosquitoes. Yeast volatiles such as lactic acids (Antonelli, et al., 1999), which play a role in the host-seeking behaviour of *An. gambiae s.s.* mosquitoes (Braks, et al., 2001; Smallegange, et al., 2005). To have an inside in the volatiles produced, the headspace of yeast-generated carbon dioxide was taken. Unfortunately no clear result came out the analysis because of water vapour overwhelming the volatiles. To prevent this from happening again, the headspace will be sampled and analysed at the Technical University of Braunschweig in Germany. Where the volatiles will be absorbed on a filter, whereupon they will be blow out for analysis.

In laboratory experiments, a cylinder CO_2 bottle can be stationed at a fixed place and consequently the availability and the ability of turning a controlled CO_2 flow rate on and off is very useful. But in a field situation, the yeast-generated carbon dioxide setup is easier to transport over the vast Malaria endemic areas and can be made with almost entirely local available materials thus cheaper and handier to use. Widely available water or soft drink bottles for example can be used, to grow local yeast on fermenting fruits to produce CO_2. Together with the fact that yeast CO_2 has more potential of being more attractive to *An. gambiae* mosquitoes, because of the above mentioned influences of yeast on the CO_2 produced. It all demonstrates the advantages of using yeast-generated CO_2 over gas cylinder CO_2 to sample or to control *An. gambiae* mosquitoes in the field.

With further research, yeast strains with a more attractive CO_2 concentration and volatile production may be found and used to attract *An. gambiae s.l.* mosquitoes in the field. In addition to this malaria vector, the yeast setup could also be used to trap and control other vectors or even haematophagous insects in general, since almost all haematophagous insects respond to CO_2

emmissions (Clements, 1963; Gillies, 1980). Different mosquito species respond differently to CO_2 (Mboera and Takken, 1997), in relation to the mosquito's host preferences (Reeves, 1953).

By studying and experimenting with the yeast setup, to obtain the optimal growth conditions for the yeast, a wider range of CO_2 flow rates and a longer CO_2 production may be achieved. By means of adding oxygen to the barrels, for the yeast to grow aerobically and therefore produce more CO_2. By isolating the yeast-barrels, surrounding the yeast barrels with materials like linen or dry vegetation like hay, to ensure and maintain optimal growth temperatures during the night. And by enlarging the whole yeast-setup, more yeast, sugar and water in a bigger barrel, a higher CO_2 flow rate can be obtained, measured by Wolfgang Schmied in Wageningen. Using a 2-bottle system (Saitoh, et al., 2004), with one of the barrels containing relatively more yeast per gram sugar to obtain an early CO_2 production and in the other barrel relatively more sugar per gram yeast, so less yeast can grow at first but later on the yeast has grown and expanded and consequently postponed the higher CO_2 production. These studies could improve the yeast setup, for it to produce sufficient amounts of CO_2 to attract mosquitoes for more than two experimental nights. This could be useful to lower the labour and costs in large and wide spread vector control programs.

Throughout these field and semi-field experiments, yeast-generated carbon dioxide proved to attract *An. gambiae s.l.* mosquitoes and to be a good alternative for the conventional cylinder CO_2 bottles. By firstly optimizing the yeast-generated carbon dioxide and its setup, the attractiveness of the yeast CO_2 to malaria vector species can be improved. Therefore the yeast-generated CO_2 will be better than cylinder CO_2 in attracting *An. gambiae s.l.* mosquitoes. Consequently in the future fermenting yeast will be used as the CO_2 source to attract and sample mosquitoes in the field. Because of this great potential, the low costs and its practicality in the field, further study on yeast-generated CO_2 as a mosquito attractant in the field is encouraged. The yeast setup could help improve and simplify mosquito sampling and host-seeking behavioural field studies. This could lead towards improved vector control measures to ultimately reduce disease transmission and mortality.

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