College of Natural and Computational Sciences

Department of Zoological sciences

THE ROLE OF CATTLE URINE AS A HOST-HABITAT CUE AND NUTRIENT RESOURCE FOR MALARIA MOSQUITOES

By Mengistu Dawit Bulo

A PhD Dissertation submitted to the Graduate Programme of Addis Ababa University in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Biology

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Abstract

Behavioural and physiological resistance of *Anopheles* mosquitoes to long lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) is an emerging challenge for the malaria vector control and elimination programmes. This necessitates the development of new tools, deriving, at least in part, from a more in-depth understanding of the behavioural ecology of these disease vectors. Olfaction is at the centre of the interaction between the mosquito and host, an interaction that is essential to the fitness of the insects. As such, the olfactory system must be able to accurately assess these resources, by the quality and quantity of odorants emitted by the hosts in a dynamic background of habitat odours within the landscape. At the broadest scale, olfactory cues emanating from both the host and from odours deposited within the landscape by the host, indicate the recent presence of potential hosts. These host-habitat cues have gained attention recently, as they have the potential to provide novel substrates for control tool development. Mammalian urine has previously been shown to act as a host habitat cue for tsetse flies, and attract the malaria vector, *Anopheles arabiensis*. Research presented in this thesis, extends these findings and identifies cattle urine as a host-habitat cue and a source for nutrients.

Host seeking and gravid *An. arabiensis* are differentially attracted to the odour of fresh and aged cattle urine. Using combined gas chromatography and electroantennographic detection as well as combined gas chromatography and mass spectrometry analyses, bioactive volatile compounds were identified in aging urine. The quality and quantity of these compounds changed with the age of the urine. In general, the number of bioactive compounds decreased with the age, whereas the total release rate of these compounds increased from 30 to 242 µg h^{-1}. Synthetic blends of the bioactive compounds in their natural ratio generally reproduced the behavioural response of host

seeking and gravid *An. arabiensis* to fresh and aging urine. The odour 24 h aged cattle urine and its synthetic blend was found to elicit a significant behavioural response in host seeking *An*. *arabiensis,* as well as in the sibling species *An. coluzzii* and *An. quadriannulatus.* To further evaluate the role of individual components within this blend, subtractive assays were conducted and showed that all compounds are required to elicit the full behavioural response in *An. arabiensis*. These results strongly suggests that host seeking *Anopheles gambiae* sensu lato use the odour fresh cattle urine as faithful indicator of the presence of potential hosts. To further evaluate the efficacy of the synthetic odour blend of 24 h aged urine, field studies were performed at a village near Meki town in Central Great Rift Valley of Ethiopia. Using indoor Centre of Disease and Control light traps, a significantly higher number of host seeking and blood fed *An. arabiensis*, as well as host seeking *Culex* spp., were collected in odour-baited traps compared to control traps. This suggests that a synthetic blend of cattle urine odour urine has the potential of attracting a wide range of physiological stages and species of mosquitoes, and thus be used as a component in integrated vector control management.

Besides acting as a host habitat cue, cattle urine, and its main nitrogenous constituent, urea, provides an important nutrient resource for host seeking and gravid *An. arabiensis*. Feeding assays, in which gravid females fed on diluted aging cattle urine revealed that most females readily imbibed the largest meals of cattle urine, when aged less than 48 h. In addition, females imbibed urea over a large range of concentrations, excepting concentrations approximating those isotonic with mosquito haemolymph. Host seeking females, which fed on cattle urine and those fed on urea were shown to exhibit similar levels of short distance flight as those fed on sugar or water in tethered flight assays. Long distance flight, however, was curtailed in those fed on cattle urine and urea compared to sugar and water. While this did not indicate a role for cattle urine in

increasing the flight energy reserves, it suggests that imbibed urine may be acting as a signal of host presence and to remain in the local area. Feeding on urine and urea also significantly affected reproduction. The size of both eggs and larvae were enhanced following the intake of 24 h aged urine and moderate concentrations of urea. The number of eggs increased after feeding on select doses of urea. In summary, cattle urine provides malaria mosquitoes with both a host-habitat cue, which attracts host seeking mosquitoes, through which novel odour baits can be developed, and a nutritional resource that enhances flight energy and reproduction.

Key words: malaria, mosquito, host-habitat, host seeking, gravid, urine, nutrient

Dedicated to

Rahel, Lehaem and Nathan

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Abbreviations/Acronyms

CDC	Centre for Diseases Control and Prevention
CRISPR -Cas9	Clustered Regularly Interspaced Short Palindromic Repeats-associated Protein9
DDT	Dichlorodiphenyltrichloroethane
dpe	day post-eclosion
EAD	Electroantennographic Detection
FID	Flame Ionization Detector
GC	Gas Chromatography
GR	Gustatory Receptor
IR	Ionotropic Receptors
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
IVM	Integrated Vector Management
KI	Kovát´s Index (Retention Index)
LLIN	Long Lasting Insecticidal Nets
MS	Mass Spectrometer
PI	Preference Index
OBP	Odorant Binding Protein
ORN	Odorant Receptor Neuron
STI	Sterile Insect Technique
PCR	Polymerase Chain Reaction
PSC	Pyrethroid Spray Catches
WHO	World Health Organizations
YPP	Yolk Protein Precursor

Chapter 1 - General Introduction

1.1. Background

Despite concerted efforts to control malaria, human sufferings from the disease continue, with unbearable number of cases and deaths reported each year around the globe (Becker *et al*., 2010; WHO, 2016). Current control strategies are failing to reduce the infectious bites by mosquitoes, partly due to growing behavioural and physiological resistance of the vectors (Knox *et al.*, 2014; Hemingway *et al*., 2016). With almost half a million deaths in Sub-Saharan Africa, and with more than two million cases of malaria in Ethiopia, caused by *Plasmodium falciparum* and *P. vivax* parasites, there remain a long way to contain or eliminate the disease (MoH, 2016; WHO, 2017b). A major reason for this is the limited abilities of current control strategies to target exophilic and exophagic phenotypes with opportunistic feeding tendencies (Kitau *et al*., 2012). One of the most severe vectors in Ethiopia, and in large regions of Sub-Saharan Africa, is *Anopheles arabiensis*, a member of the *Anopheles gambiae* species complex (Coetzee *et al*., 2000). This species is of particular concern as it benefits greatly from current climate change, habitat manipulation and intensification of agriculture (Bouma *et al*., 2016; Chen *et al*. 2006; Jaleta *et al*., 2013; Kibret *et al*., 2015), which create excellent conditions for enhanced disease transmission (Sougoufara *et al*., 2017; Zalucki and Furlong, 2017).

Increasing the general understanding of the biology of *An. arabiensis*, can significantly increase the development of safe and reliable novel control strategies. Of specific interest are behaviours that regulate fitness, predominantly of female mosquitoes. Larval and adult nutrition is an important aspect, as it regulates flight activity, mating, egg development and oviposition (Attardo *et al*., 2005; Maïga *et al*.

2014; Vantaux *et al*., 2016). The fitness of adult mosquitoes is determined by the availability of nutrients during the aquatic stages (Becker *et al*., 2010; Stone *et al*., 2011; Oliver and Brooke, 2013; Shapiro *et al*., 2016). Moreover, adults greatly rely on the ability of finding and feeding on nectar for their survival and reproduction (Vrzal *et al*., 2010). Whether adult stages of mosquitoes make use of additional nutrients to increase their fitness is currently unknown, but could, like the sugar seeking behaviour, be targeted for the development of novel control strategies (Gary and Foster, 2006; Stone and Foster, 2013). Once mosquitoes have developed the capacity to blood feed, and are ready to develop their eggs, they start searching for available hosts in the habitat, a behaviour which is innately regulated (Takken and Verhulst, 2013). This host-seeking behaviour has been the focus of considerable attention, as it is intimately linked to the vectorial capacity of mosquitoes (Majeed *et al*., 2014; Killeen *et al*., 2017). There is, however, still a need to increase our understanding of this sequential behaviour.

To locate resources, such as nectar and blood, mosquitoes rely heavily on olfaction (Gary and Foster, 2004; Stone and Foster, 2013; Takken and Verhulst, 2013). The release of both attractive and repellent volatiles assist mosquitoes in discriminating between plants and hosts, which directly translates into survival and fecundity benefits (Manda *et al*. 2007a; Nyasembe *et al*. 2012). The preference of mosquitoes for specific resources is innate (Dekker *et al*. 2001), but may be regulated by their physiological need to secure survival and reproduction (Takken and Knols, 1999; Takken *et al*., 2002; Kitau *et al*., 2012; Rivera-perez *et al*., 2017). Several resources release similar volatile compounds, including aldehydes, alcohols, ketones, phenols and terpenes, which govern the attraction to not only sugar sources but also blood hosts (Stone, 2011; Lutz *et al*., 2017; Webster and Cardé, 2017). The identification of behaviourally active

volatiles has led to the realization of odour mediated control tools, which have been shown to be effective in reducing malaria prevalence (Homan *et al*., 2014; 2016).

Odour-mediated control tools are believed to become an important supplement in the current Integrated Vector Management (IVM) strategy (Dent, 2000; Berg and Takken 2008), which aims to keep the level of disease vectors at a low level (Matthews, 2011). The current IVM strategy includes both chemical and non-chemical based control tools, including indoor residual spraying (IRS), insecticidal treated nets (ITNs), larvicides, environmental modifications, sterile insect techniques (SIT), as well as different biological control tools (Poopathi and Tyagi, 2006; Mogi, 2007; McGraw and ONeill, 2013). Of these, chemical control has played the most significant role, as it has reduced the prevalence of the disease and even eradicated it in some parts of the world (Enayati and Hemingway, 2010). The need for alternative control strategies are, however, urgently required due to the development of physiological resistance to the insecticides, the behavioural resistance of some vectors, as well as the detrimental effects on non-target organisms (Balkew *et al*., 2010; Enayati and Hemingway, 2010; Hamusse *et al*., 2012; Coleman *et al*., 2017). Diverting vectors away from humans and attracting them to odour baited traps, using e.g. push-pull technology, is one potential avenue for the development of alternative control (Menger *et al*., 2014). The success of this technology relies on the successful understanding of the chemical ecology of the mosquitoes (Pickett *et al*., 2010).

This PhD study aims to address whether female *An. arabiensis* make use of host-habitat cues, cattle urine, to locate potential blood hosts, and/or if these cues are used directly for the location of nutrients, which may be used as energy for flight and for reproduction. Using a classical chemical ecology approach, through the combination of behavioural, electrophysiological and chemical analyses, the response of host-seeking

and gravid *An. arabiensis* to fresh and aged cattle urine is analysed. These studies reveal that the response to cattle urine odour can be reproduced by blends of synthetic volatile compounds, both under laboratory and field conditions. The use of such blends for monitoring and control is discussed. Besides acting as a potential host-habitat cue, cattle urine also serves directly as a source of nutrients. This nitrogenous-rich resource provides host-seeking and gravid mosquitoes with energy for flight, and gravid mosquitoes with nutrients for egg development, and thus sheds light on an important aspect of the nutritional ecology of this species.

1.2. Research Questions

Location and recognition of potential hosts and other resources in nature is challenging for mosquitoes, as these resources usually are patchily distributed, and as such affects the distribution of vectors and their vectorial capacity (Chaves *et al*., 2010; Ferguson *et al*. 2012). Identifying the mechanism(s) by which mosquitoes solve this issue may lead to the development of novel control strategies (Burkett-Cadena *et al*., 2013; Takken and Verhulst, 2013). Recent research has shown that insects make use of habitat cues, volatile chemical cues released over a relatively large area, to increase their chances of locating their resources (Webster and Cardé, 2017). For malaria mosquitoes, such cues have been implicated to be important for the location of blood hosts (Takken and Verhulst, 2013) and oviposition sites (Sumba *et al*., 2004). However, the identity of the bioactive compounds remains largely enigmatic. Building on previous studies by Kweka *et al*. (2011) and Mahande *et al*. (2010), showing that host-seeking and gravid *An. arabiensis* are attracted to cattle urine, this study extends our understanding of how malaria vectors may use host habitat cues to locate their blood hosts. Furthermore, this study reveals that mosquitoes make use of the urine directly to increase energy reserves

and fitness. Thus, this study sheds important light on how malaria mosquitoes locate critical resources for survival, fecundity and development, behaviours which may be targeted in future efforts to control malaria.

1.3. Justification

The recent decline in malaria morbidity and mortality, due to the large scale use of LLINs and IRS, is threatened by increasing vector chemical and behavioural resistance (Benelli and Mehlhorn, 2016; Sougoufara *et al*., 2017). To overcome this issue, and in order to develop novel control strategies, an increased understanding of the biology of the dominant malaria vectors is required. This study is aimed at identifying a mechanism regulating resource location in a major vector of malaria in Sub-Saharan Africa, *An. arabiensis* (Patton). From a fundamental perspective, this study sheds important light on the mechanism on how this and other mosquito species locate hosts for blood feeding. The study also sheds novel light on the nutritional ecology of malaria mosquitoes. From a more applied perspective, this study identifies a blend of volatile chemicals that may be used for monitoring and control of malaria mosquitoes, within a wide range of physiological states.

1.4. Objectives

1.4.1. General Objective

The general objective of this study is to analyse the role of cattle urine as a host-habitat cue and a source of nutrients for malaria mosquitoes, as well as to develop a synthetic odour blend that can be used for odour-mediated control of female *Anopheles arabiensis*.

1.4.2. Specific Objectives

1. To analyse the behavioural response of host-seeking and gravid *Anopheles arabiensis* mosquitoes to the odour of aging cattle urine.
2. To identify blends of bioactive volatile chemical compounds in aging cattle urine that elicit behavioural responses in host-seeking and gravid *An. arabiensis*, as well as in host-seeking *Anopheles coluzzii* and *Anopheles quadriannulatus*, under laboratory conditions.
3. To evaluate the efficacy of attractive blends of synthetic volatile compounds identified under laboratory conditions, in attracting malaria mosquitoes the field.
4. To analyse the effect of imbibing cattle urine, and its main nitrogenous constituent, urea, on flight duration and fecundity as well as egg and larval size, in *An. arabiensis* under laboratory conditions.

Chapter 2 – Literature Review

2.1. Human Malaria – the Parasites, the Vectors and Disease Burden

Mosquitoes undergo holometabolous development in which the life cycle goes through egg and larval stages followed by a pupal stage, before emerging as adults (Becker *et al*., 2010). Female *Anopheles* mosquitoes lay their eggs in different types of aquatic habitats, which range from fresh water habitats to saline and brackish mineral water bodies (Coetzee *et al*., 2013). The breeding habitats of *Anopheles* mosquitoes range from large permanent or semi-permanent breeding sites associated to small and scattered temporary rain pools, with a wide range of biotic and abiotic characteristics (Huang *et al*., 2005; Kibret *et al*., 2015). Anopheline eggs do not resist desiccation, and embryonic development starts immediately within the egg after being laid (Clements, 1999). First instar larvae emerge from the eggs within two to three days after egg laying, depending on the external temperature (Christiansen-Jucht *et al*., 2014). There are four larval instars in their life cycle which takes about two weeks, with the fourth instar larvae transforming into a non-feeding pupal stage, which lasts for three to four days depending on the temperature in the mosquito breeding habitats (Clements, 1999; Rejmankova *et al*., 2013). The newly emerged adult male and female mosquitoes spend hours completing the development of many organs until fully matured, and then feed on sugars from floral and other fermenting plant parts to acquire metabolic energy for flight in search of mates and blood meal host finding by the females (Foster, 1995; Stone *et al*., 2011; Nikbakhtzadeh *et al*., 2014).

The vector competence of mosquitoes is affected by the growth and development of larval stages (Alto and Lonibos, 2013), with larval survival and development, as well as adult fitness and even the next filial generation being highly

dependent on the amount of nutrients available for the larval stages (Merritt *et al*., 1992). Trade-offs between the onset of foraging and mating in both sexes (Blanckenhorn *et al*. 1995), are affected by the teneral reserves in eclosed adults from well-fed or under-nourished larvae (Oliver and Brooke, 2013). In male *Anopheles* mosquitoes, body size resulting from larval resource use, and fitness measures, including longevity, number of mating attempts and mating success are inversely correlated: small males are favoured over larger males during shortage of food, time and energy devotion (Blanckenhorn *et al*., 1995). In addition, access to nutrients by newly emerged adult females of *An. gambiae* affects the balance between survival and immediate reproduction (Stone *et al*., 2011), as well as disease transmission (Shapiro *et al*., 2016).

2.2.2. Emergence and Sugar Feeding in Adult Mosquitoes

Male mosquitoes emerge earlier than females, and for both sexes the onset of sugar feeding on floral nectar, extra-floral nectar and honey dew occur within 24-36 h post-eclosion to ensure survival and reproduction (Foster and Takken, 2004; Stone *et al*., 2011). Besides sugars, floral nectar contains amino acids and non-protein amino acids (Nicolson and Thornburg, 2007; Roy *et al*., 2017), which may prolong the life of mosquitoes by up to 5 % (Vrzal *et al*., 2010), as well as secondary components such as minerals, vitamins, lipids and antioxidants (Nicolson and Thornburg, 2007). While both male and female mosquitoes consume sugar throughout their life cycle, the timing of sugar feeding is affected by environmental factors and sex (Rund *et al*., 2013). Generally, for anautogenous mosquitoes, sugar feeding occurs prior to blood feeding, as well as when females are gravid (Stone and Foster, 2013). In a laboratory set up, *An. gambiae* male mosquitoes were shown to consume sugar twice every night, sustainably over 17 days, whereas for females the frequency of sugar feeding averaged to once

every fourth night, when provided *ad libitum* access to blood and oviposition sites (Gary and Foster, 2006). Moreover, field observations indicate that males consume sugar every 1-2 days, whereas females feed more infrequently on sugar, every 6-9 days (Stone and Foster, 2013). However, a slight interruption in the availability of either blood or oviposition sites increases the sugar feeding tendency to more than once per night until the resource becomes available again (Gary and Foster, 2006).

Sugar (carbohydrates) in the nectar is directly translated to flight energy or, when in excess, can be converted for storage as glycogen in the fat body or flight muscles (Takken and Koenraadt, 2013). The total protein, lipid and glycogen reserves are correlated with body size at eclosion (Briegel *et al*., 2001a), and mosquitoes adjust their flight in relation to the availability of preferred sugar sources, which vary in density and spatial distribution (Kaufmann *et al*., 2013a; Samson *et al*., 2013; Stone and Foster, 2013). This can be explained by the optimal foraging theory, which states that animals strive to gain the most energy for the lowest cost during foraging, so as to maximize their fitness (Straif and Beier, 1996).

2.2.3. Nutritional Requirements for Reproduction in Mosquitoes

Mosquitoes generally begin mating within 24-48 h of adult emergence (Clements, 1992), with mating success being dependent on overall body size, along with other factors, in both males and females (Takken *et al*., 1998; Sawadogo *et al*., 2013). The innate sexual preference of male mosquitoes for large-sized females correlates with greater fecundity (Okanda *et al*., 2002), and larger males are more likely to successfully copulate with and inseminate females (Sawadogo *et al*., 2013). Larval nutritional status affects the size of adult mosquitoes which in many ways affects its fitness (Merritt *et al*., 1992; Vantaux *et al*., 2016). Vitellogenesis in anautogenous mosquitoes is mainly determined by nutrition (Attardo *et al*., 2005). As stated above (2.2.2), sugar feeding

post-emergence enhances the likelihood of mating, improves fecundity (Foster, 1995; Stone *et al*., 2011; Maïga *et al*. 2014), prolongs the life span and increases opportunities for females to feed on blood, which is necessary for an anautogenous female to initiate and maintain egg production (Straif and Beier, 1996; see 2.2.4.).

The main factors associated with the blood meal that affect the reproductive success in mosquitoes are the choice of hosts as blood meal sources, and the frequency of blood feeding. The blood meal imbibed as a result of host selection, differ in the energetic cost of its digestion, rather than the energy gained, which is similar between different hosts (Lyimo and Ferguson, 2009). In general, host preference reflects the ability of mosquitoes to obtain an optimal proportion of amino acids found in the host blood for vitellogenesis (Chang and Judson, 1979; Hansen *et al*., 2004; Attardo *et al*., 2005). Moreover, the frequency of blood feeding in a single gonotrophic cycle benefits successful egg production and increases malaria transmission (Scott and Takken, 2012). In smaller females, lacking sufficient nutrient reserves of protein, glycogen and lipids, two successive blood meals can be required to develop eggs (Takken *et al*., 1998). However, even after the eggs have begun to develop, a subsequent blood meal or sugar meal can provide further nutrients for the eggs, and the energy stores, that can result in larger eggs and egg batches (Manda *et al*., 2007; Bargielowski *et al*., 2012; Kaufmann *et al*., 2013b). Increased blood feeding per each gonotrophic stage, clearly enhance the chance of parasite acquisition by vector mosquitoes and thereby increase the entomological inoculation rate, consequently improving the efficiency of *Plasmodium* transmission by vector mosquitoes (Takken and Lindsay, 2003; Scott and Takken, 2012).

The nutrients obtained from larval and adult feeding, which provide the molecular building blocks for both energy and reproduction, as well as non-energy nutrients like

vitamins, salts, metals and sterols (Rivera-Perez *et al*., 2017), are affected by the frequency of feeding, the size of the mosquito, its age (feeding regime), and the availability of sugar and blood meals (Straif and Beier, 1996; Gary and Foster, 2001; Harrington *et al*., 2001; Braks *et al*., 2006; Damiens *et al*., 2013; Farjana *et al*., 2013). Whether mosquitoes make use of other nutrient resources as part of the regular life cycle, or in the scarcity of other resources, e.g. hosts, is currently unknown. Finding the sources for these energy and non-energy nutrients may be challenging for mosquitoes (Smallegange *et al*., 2011). However, a host-habitat containing abundant access to fresh cattle urine can provide some of these nitrogenous nutrients like urea, amino acids, ammonia and many more similar compounds (Bristow *et al*. 1992; Kilande *et al*., 2016; Miah *et al*., 2017).

2.2.4. Reproductive Energetics of Egg Development in Mosquitoes

Nutrient availability affects the first two stages of egg development in mosquitoes (Raikhel and Dhadialla, 1992; Attardo *et al*., 2005): the previtellogenic stage, which is characterized by fat body maturation to become capable of intense synthesis of yolk protein precursors (YPPs); and the vitellogenic stage, when YPPs are produced by the fat body and stored in the yolk bodies of oocytes (Tufail and Takeda, 2008). During the previtellogenic period, there is cumulative effects of teneral reserves and sugar feeding, which increases both the lipids deposited in the ovary and the transcription of genes integral to oocyte development (Clifton and Noriega, 2012). The consumption of blood initiates a cascade of events in the fat body, which are hormonally regulated, engaging follicles to synthesize yolk proteins (Raikhel *et al*., 2002; Hansen *et al*., 2004; Attardo *et al*., 2005).

In anautogenous female mosquitoes, the concentrations of free amino acids such as leucine, isoleucine, lysine, phenylalanine, threonine, tryptophan, valine, cysteine,

arginine and asparagine, rise in the haemolymph as a result of blood meal digestion and absorption (Harrington *et al*., 2001; Hansen *et al*., 2004; Attardo *et al*., 2005). These free amino acids provide the stimulation to initiate egg development (Uchida *et al*., 2001). In addition, these amino acids are used by the fat body to generate vitellogenin for deposition in the developing oocytes (Clifton and Noriega, 2012; Roy and Raikhel, 2012). For efficient accumulation of yolk proteins, female mosquitoes with low nutritional reserves wait to take a blood meal until after consuming a sugar meal, to regulate reproductive output (Gary and Foster, 2001; Clifton and Noriega, 2011). In extreme cases, when females have very low energy reserves, their oocytes do not develop beyond stage I after the first blood meal, and a subsequent blood meal is required for oocyte maturation (Briegel *et al*., 2001a). Thus, during the vitellogenic stages, both sugar and blood meals have an effect on egg development (Clements and Boocock, 1984; Stone and Foster, 2013). To ensure improved reproductive success, additional nutrient resources are needed to affect egg development. This relies on multiple intrinsic and extrinsic factors, and may not be limited to repeated sugar and blood feeding (Gulia-nuss *et al*., 2015). Other resources in the environment such as a substrate for amino acid synthesis, may be able to provide supplementary nutrients for further oocyte development. Such resources for anautogenous mosquitoes are currently not known.

2.3. Chemical Ecology of Malaria Mosquitoes

Interactions between mosquitoes and their surrounding environment greatly affect their behaviour, physiology and metabolism (Burkett-Cadena *et al*., 2013; Christiansen-Jucht *et al*., 2015; Webster and Cardé, 2017), and are accomplished by highly orchestrated sensory systems (Sutcliffe, 1994; Jacquin-Joly and Merlin, 2004; Majeed, 2013).

Olfactory cues are undoubtedly the most important group of external stimuli affecting mosquito behaviour (Takken and Verhulst, 2013; Haverkamp *et al*., 2018). Under this subtopic, I briefly present the behaviours known to be regulated by chemical cues; sugar seeking, host seeking and oviposition site seeking. In addition, I provide an overview of the anatomy of the olfactory system and the mechanisms underlying odorant reception.

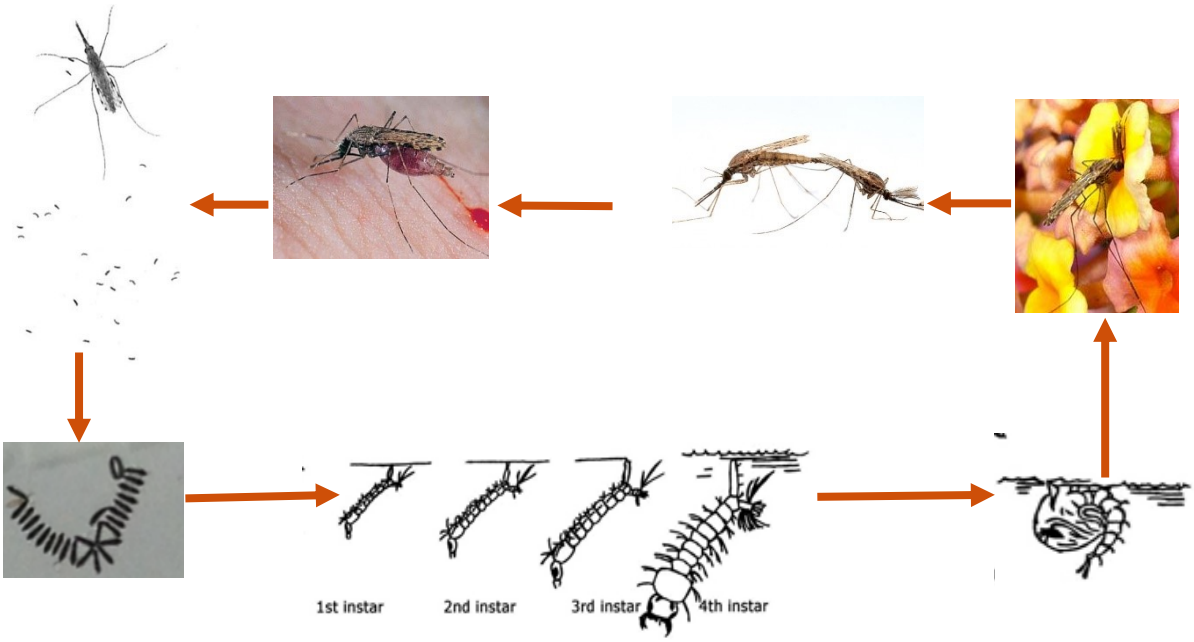

Figure 2.1. Physiological Stages of Anopheles mosquitoes. Sources: Woodbridge Foster, info@wumcd.org, CDC/ James Gathany and Elizabeth Miller

2.3.1. Chemical Ecology of Sugar Seeking

Mosquitoes, including members of the *An. gambiae* species complex, are known to feed on plant sugars (Figure 2.1.) to increase their energy budget, a behaviour which also significantly affects their vectorial capacity (Gary and Foster, 2004; Gu *et al*., 2011; Stone and Foster, 2013). The production of phytochemicals and the behavioural response by the mosquitoes are genetically controlled both at the emitter and receiver level, and regulated by frequent interactions between the mosquitoes and the habitats

(Takken and Dicke, 2006). Recent field and laboratory studies of naïve and conditioned *Anopheles* and *Culex* mosquitoes indicate consistent olfactory behavioural response patterns to natural sugar sources and floral volatile compounds (Jhumur *et al*., 2006; Gouagna *et al*., 2010). Moreover, Gouagna *et al*. (2010) and Manda *et al*. (2007b) reported that some plants are being favoured over others, and that this preference is linked to increased survival and fecundity. This preference seems to be linked to both qualitative and quantitative differences in the volatile profile of host plants (Nyasembe *et al*., 2012). These cues include aldehydes, alcohols, ketones, phenols and terpenes (Lutz *et al*., 2017). Further studies are, however, required to fully understand the role of these volatile cues in host plant recognition and discrimination.

2.3.2. Chemical Ecology of Host Selection and Preference

Host seeking behaviour of female adult mosquitoes is determined by both intrinsic and extrinsic factors (Lyimo *et al*., 2012; Takken and Verhulst, 2013). Host preference studies of mosquitoes show that natural selection often favours a restricted host breadth, a behaviour that evolved and continues to evolve due to the increased fitness obtained by feeding on specific hosts (Cohuet *et al*., 2010; Kitau *et al*., 2012; Majeed *et al*., 2016). This can be exemplified by studies on members of the *An. gambiae* species complex, in which *An. gambiae s.s.* displays a preference for humans, *An. quadriannulatus* displays a preference for cattle, and *An. arabiensis* is more opportunistic in its host choice (Broek and Otter, 1999). This innate preference is predominantly regulated by volatile cues emanating from their respective hosts, including both attractants and repellents (Fernández-grandon *et al*., 2015; Main *et al*., 2016).

Host location and discrimination by mosquitoes includes a series of sequential behaviour. Initial detection of potential hosts is believed to be regulated by host habitat

cues that are released over a large area and indicate a site where more specific host cues are more likely to be found. Emitted by all vertebrates, carbon dioxide is the most characterized of the host habitat cues (Gillies, 1980), and has been shown to activate and gate the attraction of host seeking mosquitoes to host odours over a range of distances (Dekker *et al*., 2005). At closer range, volatiles emanating from the breath or body of potential hosts, play a more significant role (Qui *et al*., 2011; Webster *et al*., 2015). The complexity of host odours, with e.g human emanations containing some 300-1000 volatile compounds (Bernier *et al*. 2000), has made the identification of bioactive compounds enigmatic (Smallegange *et al*., 2003). Recent studies have also shown that host recognition is contextual and dependent on quantitative and qualitative differences in odour blends, as well as the olfactory codes evoked (Qiu *et al*., 2006; Cardé, 2015). To date only few host attractants have been reliably characterized, including carboxylic acids (Smallegange *et al*., 2009) and 1-octen-3-ol (Majeed *et al*., 2016), and further work is need to fully comprehend the complexity underlying host selection in mosquitoes.

2.3.3. Chemical Ecology of Oviposition Site Selection

Ovipositional flight in mosquitoes commences with the completion of egg development (Bentley and Day, 1989). In general, the decision for ovipositional flight is governed by the ability of female mosquitoes to assess potential oviposition sites and make decisions to optimize offspring performance and fitness (Yoshioka *et al*., 2012; Suh *et al*., 2016). In doing so, female mosquitoes appear to use both habitat cues and more specific cues emanating from the potential oviposition sites (Sumba *et al*., 2008; Himeidan *et al*., 2013; Wondwosen *et al*., 2016; 2017; 2018; Asmare *et al*., 2017).

The chemical ecology of malaria mosquitoes is in its infancy, and it is only recently that researchers have identified cues that attract gravid mosquitoes from a

distance. These habitat cues include water vapour (Okal *et al*., 2013), but also volatiles emanating from wild and domesticated grasses (Wondwosen *et al*., 2016; 2017; 2018; Asmare *et al*., 2017), as well as other vegetation (Nikbakhtzadeh *et al*., 2014; Eneh *et al*., 2016). Odours emanating from these grasses are used by female mosquitoes not only for attraction but also discrimination (Asmare *et al*., 2017). Volatiles released by domesticated grasses, mainly including aldehydes and terpenes, have been shown to attract gravid *An. arabiensis* from a distance as well stimulate oviposition (Wondwosen *et al*., 2016). Closer to the oviposition site, additional attractants as well as repellents appear to play a role for the final decision of females to lay their eggs. These volatiles originate from bacteria and other potential nutrient sources (Sumba *et al*., 2004), eggs (Ganesan *et al*., 2006), larvae (Gotifrid *et al*., 2014) and predators (Munga *et al*., 2006; Warburg *et al*. 2011), associated with the water bodies (Huang *et al*., 2006; Himeidan *et al*., 2013: Takken and Knols, 1999). Elucidating the ecological interaction of gravid mosquitoes with their habitat is believed to provide novel avenues for alternative control strategies (Bentley and Day 1989).

2.3.4. Peripheral Olfactory System

Volatile chemical cues are detected by the antennae, as well as by secondary olfactory organs, including the maxillary palp and proboscis in mosquitoes (Zweibel and Takken, 2004). The antenna of female mosquitoes, which is attached to the head by a ring-shaped scape, consists of 13 flagellar segments connected to a rounded pedicel (Schneider, 1963; Mciver, 1978). Similarly, the maxillary palp is divided into 4-5 segments depending on species, of which only a subset are involved in detecting olfactory cues (Mciver and Siemicki, 1975). In contrast to the antenna and maxillary palp, it is only the labella at the tip of the proboscis that serves as an olfactory organ (Syed and Leal, 2007). These olfactory organs are covered by multiporous hairs, known

as sensilla (Sutcliffe, 1994; Kwon *et al*., 2006; Montell and Zwiebel, 2016). These sensilla are categorized based on their structural properties as single-walled (trichoid and capitate peg) or double-walled (coeloconic and grooved peg) sensilla (Sutcliffe, 1994; Steinbrecht, 1997; Ghaninia *et al*., 2007; Guidobaldi *et al*. 2014). Pores in the cuticle of the sensilla serve as an entry point for the odour molecules to gain access to the sensillum lymph (Steinbrecht, 1997). The sensillum lymph contains water-soluble odorant-binding proteins (OBPs) (Bohbot and Vogt, 2005), which binds and transports the volatiles compounds to the chemosensory receptors expressed in the dendritic membrane of the odorant receptor neurons (ORNs) (Leal, 2013). The ORNs express specific sets of chemosensory receptors, including odorant receptors (ORs), ionotropic receptors (IRs) and gustatory receptors (GRs) (Vosshall *et al*., 1999; Iatrou and Biessmann, 2008), which dictates the functional properties of the neurons (Ghaninia *et al*., 2007). The tuning of these chemosensory receptors to natural ligands have allowed for recent progress in our understanding of host selection (Bohbot and Dickens, 2012).

To increase the possibilities of volatiles that can be used as attractants or repellents, additional research into the peripheral olfactory system is required (Carey and Carlson, 2011). The OBPs, ORs and IRs can be used as molecular targets to screen for behaviourally active volatiles (Brito *et al*., 2016). The development of reverse chemical ecology may also be used for the *in silico* selection of ligands to be used for subsequent laboratory and field evaluations (Leal *et al*., 2008; Brito *et al*., 2016). Additionally, the traditional methodologies in chemical ecology, as used in the current study, remain valid and offer the means of identifying volatile cues that can be used to modulate the behaviour of disease vectoring mosquitoes.

2.4. Control Toolbox in the era of Resistance Development

Since ancient times, vector borne diseases control strategies can rely on parasite treatment or vector management strategies where, vector control has remained the best way for controlling the transmission of vector borne diseases, such as malaria (McGraw and ONeill, 2013). Beside pathogen treatment strategies, integrated vector management strategies considers decision-taking from vector control, environment manipulation, biological control strategies of larval and adult mosquitoes and genetic manipulation (Takken and Knols, 2009). Comprehensive vector control strategies are classified as chemical-based and non-chemical-based strategies (Poopathi and Tyagi, 2006). Indoor residual spraying (IRS), Insecticide Treated Nets (ITNs), Long Lasting Insecticide Nets (LLINs), chemical based personal protections, and attract and kill mosquito traps, are considered chemical-based tools. Environmental modifications, such as drainage and other types of breeding habitat sanitation methods, as well screening windows, sterile insect techniques and biological control are categorized under non-chemical based control strategies (Mogi, 2007; McGraw and ONeill, 2013). Of these control methods, odour-based surveillance and management strategies have a strong potential to expand and improve the efficiency of the range of tools in integrated vector management, and is therefore the main focus for this review.

2.4.1. Chemical Control

Vector control predominantly relies on insecticides, a tool that has been used since 1000 BC with inorganic sulphur, arsenic, lead arsenate, cryolite and boric acid (Casida and Quistad, 1998). The introduction of dichloro-diphenyl-trichloroethane (DDT) in 1939, and other organochlorines, as well as organophosphates, including carbamates and pyrethroids, have profoundly enhanced the control of vector borne diseases (Casida and Quistad, 1998; Becker *et al*., 2010; Coetzee and Koekemoer, 2013). Currently, the wide

use of IRS and LLINs, which combine the chemical and barrier control methods, play a major role in reducing the disease transmission on a global scale (Enayati and Hemingway, 2010). The increased coverage of ITNs in Africa has resulted in a 68 % decline of malaria cases between 2000 and 2015 (WHO, 2017a). However, insecticidal resistance, a highly evolving phenomenon in most disease vector mosquitoes (Coleman *et al*., 2017), is a current threat for the success of currently available insecticides (Coleman *et al*., 2017; WHO, 2017b). It is also important to note that ITNs and IRS are indoor-based control methods and are, therefore, largely ineffective against exophilic and exophagic vectors.

In Ethiopia, IRS and LLINs have remained the pillars for malaria prevention and control strategies for more than 40 years (Hamusse *et al*., 2012). In 2016, out of 48 million people at risk of malaria, 62 % of the population had access to ITNs, and more than 15 million house-holds (22 %) were sprayed by IRS (WHO, 2017b). Pyrethroid-treated LLINs and ITNs have been shown to be effective against *An. gambiae* and *An. funestus* (Strode *et al*., 2014), whereas *An. arabiensis* is generally not affected due to its exophilic and exophagic behaviour (Killeen *et al*., 2017). Moreover, *An. arabiensis* is strongly resistant to DDT and pyrethroids in all surveyed sites of the country (Balkew *et al*., 2010; Yewhalaw *et al*., 2011; Alemayehu *et al*., 2017).

2.4.2. Environmental Management

Not intended to replace other vector control strategies, but to be used as a component in IVM, environmental management is defined as activities that reduce the abundance of mosquitoes or as targeted projects of “species sanitation” of vectors of major vector borne diseases, particularly malaria mosquitoes (WHO, 1982; Walker, 2002). The goal of this strategy is to change the vector habitat in order to inhibit the growth of the immature stages or to minimize the contact between vectors and humans. Broadly, the

strategy can be classified as environmental modification, environmental manipulation and enhancement of human habitation (WHO, 1982; Keiser *et al*., 2005). Some of the classical examples of environmental management include swamp alteration, basic sanitary measures, drainage filling and grading, planting barriers and house screening (WHO, 1982). Draining surface water in the landscape, reclamation and filling of the land that harbour large water storage containers and small breeding sites are all included under environmental modification. Environmental modification also includes water level manipulation like flushing and drain clearance, as well as exposing or shading potential breeding sites (Fillinger and Lindsay, 2011). Modifying human surroundings and behaviour greatly assist in reducing human-vector-pathogen contact. Settlement away from breeding sites, mosquito-proofing houses and improved housing designs are some of the recommended human habitation modifications (Keiser *et al*., 2005).

Mosquito ecology and population dynamics, as well as regional disease epidemiology, determines which actions of environmental management will be effective (WHO, 1982; Smith *et al*., 2013). Fewer adverse ecological effects, increased sustainability and wiser use of local resources and knowledge adds the environmental management control strategy as another main pillar in IVM (Utzinger *et al*., 2001). Regular maintenance of the modified environment, and the matching of the ecological requirements in habitat manipulation with traditional house designs and construction methods, as well as with outdoor sleeping habits, will greatly affect the successes of environmental management in general (Walker, 2002; Keiser *et al*., 2005).

2.4.3. Biological Control

The aim of the biological control strategy is to reduce the vector population below threshold level without adverse effects on the ecosystem (Becker *et al*., 2010). Some of the agents used in biological control are vertebrate predators, such as fish, amphibians,

birds, bats, as well as invertebrate predators, including hydra, flatworms, spiders, mites, crustaceans and insects (Lacey, 2007; Mogi, 2007; Poulin, 2012; Benelli *et al*., 2016). Microbial agents have also been deployed in times when exploitation of parasites and predators has become too expensive and/or is not feasible to use in large-scale application (Mogi, 2007; Lacey *et al*., 2015). These agents which includes fungi, protozoa, bacteria and viruses (Becker *et al*. 2010), are released either in small number (inoculation) or in overwhelming number (inundation) into the habitat (Lacey *et al*., 2001; Becker *et al*. 2010).

Examples of biological control strategies that have been successfully implemented include the use of entomopathogenic fungi, including *Beauveria, Metharhizium, Coelomomyces, Culicinomyces, Lagendiu* and *Entomophthora* (Fang *et al*., 2011; Darbro *et al*., 2012); applications of bacterial agents such as *Bacillus thuringiensis* var *israeliensis* (*Bti*), *B. sphaericus* and *Wolbachia* (Tchicaya *et al*., 2009; Hughes *et al*., 2011); the use of predacious copepods from the genus *Mesocyclops* (Kay and Nam, 2005); and larvivorous fishes, including *Poecilia reticulate, Gambusia affinis,* and Tilapia spp. (Kusumawathie *et al*., 2008; Seng *et al*., 2008; Kamareddine, 2012). In order for biological control to be successful, detailed knowledge of the biology of the agents and their interaction with the ecosystem are essential (Benelli *et al*., 2016).

2.4.4. Genetic Manipulation

In an era in which technologies to control vectors with minimal negative environmental impact are required, development of genetic engineering tools for disease vectors has become promising and sought after. Population suppression and population replacement are the ultimate goals in this control strategy (Christophides, 2005; Leftwich *et al*., 2016). This can be accomplished by manipulating reproduction in both males and females, and by blocking pathogen transmission.

The mass release of sterile male mosquitoes that will mate with indigenous females and produce non-fertile offspring is a form of the common sterile insect technique (SIT) (Gilles *et al*., 2014; Oliva *et al*., 2014). Another form of SIT is the induction of cytoplasmic incompatibility through the use of bacteria of the genus *Wolbachia* (Lees *et al*., 2015). Here, *Wolbachia*-infected males provide uninfected females with sperm, resulting in the failure of embryonic development (Alphey *et al*., 2013). Furthermore, by using recently developed genetic engineering technology, such as the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein9 (Cas9)-mediated gene-drive system (Gantz *et al.* 2015), it is feasible to suppress *An. gambiae* populations to levels that do not support malaria transmission, by editing the genes responsible for recessive female sterility phenotype (Hammond *et al*., 2016). Using genetic engineering technology to block malaria parasite transmission, through the introduction of foreign genes that express anti-parasitic changes in the midgut of the mosquito also reported by Wang and Jacobs-lorena, (2013).

Overall, the genetic control strategy is endowed with advantages like being species-specific and utilizing the natural biology of the vector species (Coleman and Alphey, 2004). Besides these benefits, others such as cost-effectiveness, affordability, acceptability, equity, accessibility and sustainability contribute to the success of the usage of genetic control strategies (Coleman and Alphey, 2004; Alphey *et al*., 2013). Therefore, the implementation potential of this method, if made to influence the chance of contact between mosquito, pathogen and the host, a greater leap in the development of a novel control strategy can be achieved (McMeniman, 2016).

2.4.5. Odour Mediation for Development of Push-Pull Strategies

Semiochemical cues influence a repertoire of behaviours, including sugar seeking, host seeking and oviposition site selection (Zwiebel and Takken, 2004; Nyasembe and Torto,

2014). While recent research has allowed for an increased understanding of the chemical ecology of malaria mosquitoes, much of this work is confined to laboratory and semi-field settings (Majeed *et al*., 2016; Mweresa *et al*., 2016). However, recent research has provided promising evidence that odour baits for trapping indoor as well as outdoor *Anopheles* mosquito species has great potential for use in future IVM strategies (Menger *et al*., 2015; Homan *et al*., 2014). An eminent example of this, is the solar-powered human odour-baited mosquito trapping system setup on Rusinga Island in Lake Victoria, Western Kenya. This system has proven that odour baits may be used to reduce the malaria prevalence by suppressing the population of malaria mosquitoes (Homan *et al*., 2016). The simultaneous use of both attractive and repellent volatiles may enhance the system and strengthen existing malaria prevention strategies (Menger *et al*., 2015). The use of natural repellents, such as non-host volatiles, for this purpose is both interesting and important as it may reduce the risk of resistance among mosquitoes (Gikonyo *et al*., 2002; Chauhan *et al*., 2012; Jaleta *et al*., 2016). The advantages of using odour-baited trapping strategies include reproducibility, objectivity and relatively low cost, as compared to other standard traps. Moreover, their use in outdoor settings strengthen their functionality (Okumu *et al*., 2010; Mukabana *et al*., 2012; Menger *et al*., 2015).

Chapter 3 - Identification of Host Habitat Cues for Malaria Mosquitoes - Behavioural and Electrophysiological Responses of Host-seeking and Gravid *Anopheles gambiae sensu lato* to Cattle Urine Volatiles

3.1. Introduction

Mosquito survival depends on the ability of the insects to locate suitable hosts and oviposition sites. Understanding the mechanisms underlying this process is a prerequisite for an increased exploitation of the ecology and evolution of mosquito-host interactions, as well as for the development of novel tools in vector control strategies (Cardé and Willis, 2008; Barreaux *et al*., 2017; Webster and Cardé, 2017). Natural selection favours a restricted range of hosts and oviposition sites, However, the identification and discrimination of range of hosts based on the sensitivity to generic and host specific volatiles determine plasticity in preferring available and suitable hosts (Bowen, 1991; Majeed *et al*, 2016; 2017; Montell and Zwiebel, 2016). The olfactory system of mosquitoes is adept at both qualitative and quantitative detection, as well as at the discrimination of ecologically relevant volatile cues, including those emanating from hosts and oviposition sites. The physical properties of odour plumes (Murlis *et al*., 1992) and the decreasing concentration of bioactive compounds at longer distances (Cardé and Willis, 2008), however, makes finding patchily distributed resources in nature difficult. To overcome this, mosquitoes may use ´habitat odour´ cues, which tend to be released at higher concentrations (Webster and Cardé, 2016), to locate the area in which resources can be located. Such cues often lack in specificity, but provide insects a way to subsequently encounter more specific host and oviposition site cues (Dekker *et al*., 2005; Beyaert and Hilker, 2014; Meiners, 2015).

Urine, produced in large quantities by host and non-host animals alike, has previously been shown to act as a source of host habitat cues, and attract a range of hematophagous insect species (Bursell *et al*., 1988; Okech and Hassanali, 1990; Mihok and Mulye, 2010; Kweka *et al*., 2011). Average volume of urine voided by cattle within 48 h indicated that 1600 ml of urine with range of 850 to 2850 ml found to cover 1651 cm^2 and with consideration of the 5 cm edge of the patch, the total area covered by urination can reach up to 2159 cm^2 (Doak, 1952). Moreover, in Ethiopian context where cattle were kept in an open shed overnight. However, at early evening and morning, cattle were observed to stay in the human inhabitation (yard) with for milking and common practice which makes difficult to demarcate specific habitat for human and cattle. The level of attraction is, however, dependent on the origin (Okech and Hassanali, 1990) and age of the urine (Mahande *et al*., 2010). Aged urine from buffalo, cattle, horse, sheep, waterbuck, bush pig and warthog has been demonstrated to attract host-seeking tsetse, horseflies, tabanids and culicine mosquitoes (Owaga *et al*., 1988; Den Otter, 1991; Madubunyi *et al*., 1996; Mihok and Mulye, 2010; Kweka *et al*., 2011; Baldacchino *et al*., 2013), whereas fresh cattle urine has been shown to attract both host-seeking and gravid anopheline mosquitoes, under laboratory and field conditions (Kweka *et al*., 2011; Mahande *et al*., 2010). Microbial decomposition of the urine is responsible for the observed attraction of tsetse to aged buffalo urine, by affecting the chemical composition of released volatiles (Murry and Adams, 1988; Okech and Hassanali, 1990). Chemical analyses of cattle and buffalo urine volatiles indicate a significant change in the odour profile with age. Most noticeable is the gradual formation of phenolics, including 3- and 4-methyl-, ethyl- and propyl phenols (Bursell *et al*., 1988; Murry and Adams, 1988; Owaga *et al*., 1988), metabolized by select microorganisms (Okech and Hassanali, 1990; Martin, 1982). Of these, 3- and 4-

methylphenol, as well as 3-ethyl- and propyl phenol, have consistently been shown to elicit physiological responses across haematophagous insects (Den Otter, 1991; Mihok and Mulye, 2010; Kweka *et al*., 2011; Baldacchino *et al*., 2013). Other constituents in cattle urine found to elicit physiological responses in haematophagous insects include naphthalene, (*Z*)-3-hexen-1-ol, 1-octen-3-ol, undecane, 2-heptanone, sulcatone and linalool (Birkett *et al*., 2004).

In this chapter, the behavioural response of host-seeking and gravid *Anopheles arabiensis,* a major vector of malaria in sub-Saharan Africa, to headspace volatiles collected from fresh and aged cattle urine, is analysed. Combined gas chromatographic and electroantennographic analyses, combined with chemical analysis, is used to identify the bioactive compounds in the aging urine. From this analysis, synthetic blends based on the natural ratio of bioactive compounds were developed and tested for their ability to attract host-seeking and gravid *An. arabiensis* under laboratory conditions. Comparative behavioural analysis is made with the closely related species, *Anopheles coluzzii* and *Anopheles quadriannulatus.* Based on these results, the importance of cattle urine as host habitat cue is discussed.

3.2. Materials and Methods

3.2.1. Mosquito Rearing

Anopheles arabiensis (Dongola strain), *Anopheles coluzzii* (Suakoko strain) and *Anopheles quadriannulatus* (SANQUA) were maintained at 25±2 °C, 65 ± 5 % RH and at a 12:12 h light: dark cycle. Larvae were reared in plastic trays (20 cm × 18 cm × 7 cm), filled with distilled water, and fed on Tetramin® fish food (Tetrawerke, Melle Germany). Pupae were collected in 30 ml cups (Nolato Hertila, Sweden) and transferred to Bugdorm cages (30 cm × 30 cm ×30 cm; MegaView Science, Taiwan) for the adults

to emerge. Adults were provided with 10 % sucrose solution *ad libitum*. For experiments with host-seeking mosquitoes, 4-day post-emergence females were used. To prepare gravid mosquitoes for behavioural and electrophysiological bioassays, 4-day post-emergence females were provided de-fibrinated sheep blood (Håtunalab, Bro, Sweden) using a membrane feeding system (Hemotek Discovery Workshops, Accrington, UK). Engorged females were subsequently transferred to a separate cage and provided 10 % sucrose solution *ad libitum* until used for the experiments three days post-blood feeding. For behavioural analyses, host-seeking and gravid mosquitoes were transferred to the experimental room, 4-6 h prior to the experiments, and provided distilled water *ad libitum*. Then, 2-3 h before the onset of the experiments, individual mosquitoes were placed in separate release cages (Fig. 3.1A).

3.2.2. Headspace Volatile Collections from Fresh and Aged Cattle Urine

Headspace volatiles from fresh (1 h after urine sampling), 24 h, 72 h and 168 h aged urine were collected from samples collected from Zebu cattle, Arsi race, in the Meki district of central Ethiopia. For convenience and availability, the urine sample collections were carried out early in the morning while the cattle were still in the shed. In total, samples were collected from 10 individuals in separate clean PVC plastic buckets (Fig. 3.1.). Then, 100-200 ml of the samples were transferred into separate polyamide roasting bags (Toppits Cofresco, Frischhalteprodukte GmbH & Co,, Minden, Germany), placed inside a 3 l Polyvinylchloride plastic bucket container, kept at room temperature. To collect the volatiles from fresh and aged urine, a closed loop headspace system was used, by circulating an activated charcoal-filtered airstream (100 ml min^{-1}) through the polyamide bag onto an adsorbent column, using a diaphragm vacuum pump (KNF Neuberger, Freiburg, Germany), for 2.5 h. As a control, headspace collection from an empty polyamide bag, was performed. The adsorbent column was

made of Teflon tubing (5.5 cm × 3 mm i.d.) holding 35 mg Porapak Q (50/80 mesh; Waters Associates, Milford, MA, USA) between glass wool plugs (Fig. 3.1.). The columns were rinsed with 1 ml re-distilled n-hexane (Merck, Darmstadt, Germany) and 1 ml pentane before use. Adsorbed volatiles were eluted with 400 µl pentane (99.0 % pure solvent GC grade, Sigma Aldrich). Headspace collections were pooled and then stored at -20 °C until used for behavioural, physiological and chemical analyses.

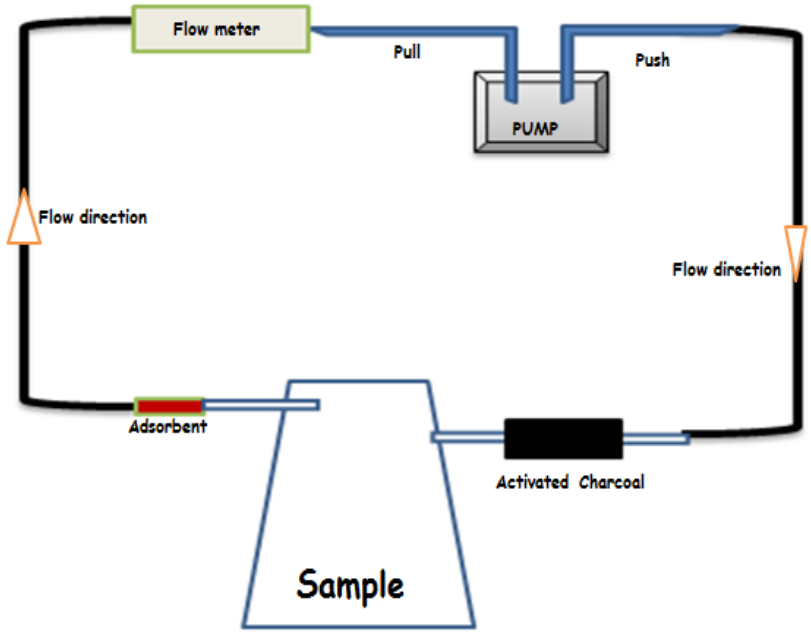

Figure 3.1. A closed loop headspace odour collection set up. A urine sample placed inside a cooking bag and kept in bucket connected with Teflon tubes circulating stream of air through the headspace using the pump. An adsorbent trapping the volatiles and an activated charcoal connected at both ends.

3.2.3. Behavioural Responses of Host-seeking and Gravid *Anopheles arabiensis* to Headspace Extracts of Fresh and Aged Cattle Urine Odour

Behavioural responses of host-seeking and gravid *An. arabiensis* mosquitoes to the headspace volatile extracts collected from fresh, 24 h, 72 h and 168 h aged urine were analysed using a straight glass tube olfactometer (Majeed *et al*., 2014). The experiments were conducted during the peak host-seeking activity period, 19:00 to 21:00, of *An. arabiensis* (Majeed *et al*., 2014). The glass olfactometer (80 cm × 9.5 cm i.d.) was illuminated from above at 3 ± 1 lx. A charcoal-filtered and humidified air stream (25±2 °C, 65±2 % relative humidity) flowed through the bioassay at 30 cm s^{-1} (Fig. 3.2E). The air passed through a series of stainless-steel mesh screens to generate a laminar flow and a homogenous plume structure (Fig. 3.2E). Dental wicks (4 cm × 1 cm; L:D; DAB Dental AB, Upplands Väsby, Sweden) were used as stimulus dispensers. Initial preliminary bioassays, designed to identify the optimal concentration of the extracts to be used in subsequent experiments, revealed a significant attraction of host-seeking mosquitoes (n = 12-34; $F = 8.008$, $df = 13$, $p < 0.001$) to a 1:10 dilution (10 µl; approximately 3 min equivalents) of the headspace volatile extracts, collected from fresh ($p = 0.023$) and 24 h aged urine ($p < 0.001$), whereas no significant difference in attraction was observed to extracts from 72 h ($p = 0.087$) and 168 h ($p = 0.65$) aged urine headspace volatiles, compared to a pentane control. Lower concentrations (1:100 and 1:1000) of the extracts elicited no significant attraction ($p > 0.05$) in host-seeking mosquitoes. Thus, for the main experiments, 10 µl of all extracts at a 1:10 dilution was used, with an equivalent amount of pentane used as a control. Both treatment and control dispensers were suspended from a 5 cm wire coil at the upwind end of the olfactometer (Fig. 3.2D) and replaced every 5 min. The release

cage (Fig. 3.2A), containing individual host-seeking or gravid mosquitoes, was placed at the down-wind end of the olfactometer, and allowed 1 min to acclimatize before the butterfly valve of the cage was opened for their release. Attraction to either treatment or control was analysed as the proportion of mosquitoes that made source contact within 5 min after release. Each headspace volatile extract and control was replicated at least 30 times, and to avoid any day effect, the same number of treatments and controls were tested each experimental day.

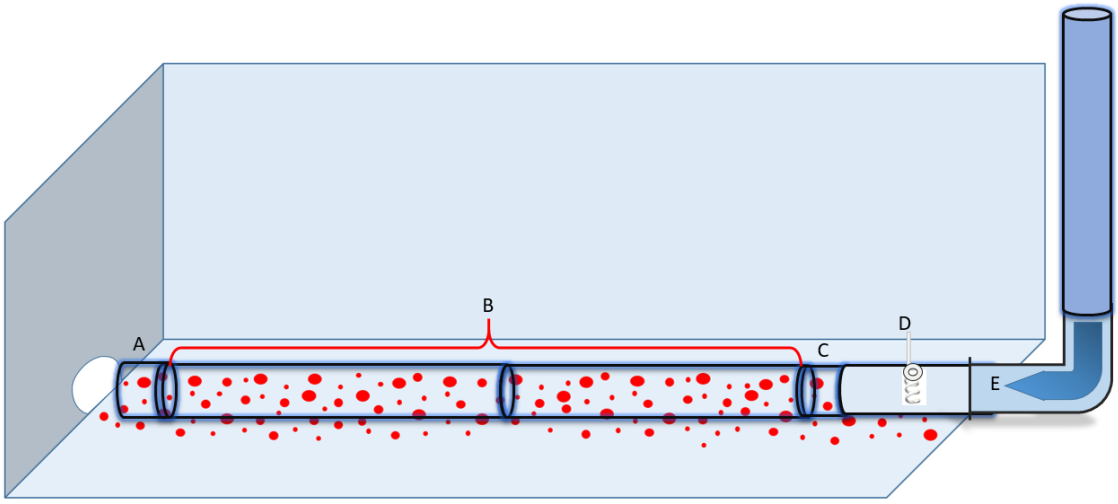

Figure 3.2. Glass tube olfactometer design. A. release cage, B. flight tube, C. trapping cage, D. odour delivery opening, E. air flow entry.

3.2.4. Combined Gas Chromatography and Electroantennographic Detection Analysis

Combined gas chromatography and electroantennographic detection (GC-EAD) analyses of host-seeking and gravid *An. arabiensis* were performed, as previously described (Wondwosen *et al*., 2016). In short, insects were anaesthetized on ice for 1-2 min, and then the head was excised. After cutting the distal tip of the first flagellomere with micro-scissors, the antenna was inserted into a recording glass electrode filled with Beadle–Ephrussi Ringer (140 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2 \cdot 2H_2O$). A similar

electrode serving as the reference was inserted into the head capsule through the occipital foramen. The recording electrode was connected to a pre-amplifier probe (10×) connected to a high impedance DC amplifier interface box (IDAC-2; Ockenfels-Syntech GmbH, Kirchgarten, Germany).

An Agilent Technologies 6890 GC (Santa Clara, CA, USA) equipped with an HP-5 coated fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies), was used to separate the headspace volatile extracts of fresh and aged urine. Hydrogen was used as the mobile phase at an average linear flow rate of 45 cm s^{-1}. Each sample (2 µl) was injected in splitless mode, for 30 s, at an injector temperature of 225 °C. The GC oven temperature was programmed from 35 °C (3 min hold) at 10 °C min^{-1} to 300 °C (10 min hold). At the GC effluent, 4 psi of nitrogen was added and split 1:1 in a Gerstel 3D/2 low dead volume four-way cross (Gerstel, Mülheim, Germany) between the flame ionization detector and the EAD. The GC effluent capillary for the EAD passed through a Gerstel ODP-2 transfer line, that tracked the GC oven temperature plus 5°C, into a glass tube (30 cm × 8 mm), where it was mixed with charcoal-filtered, humidified air (1.5 l min^{-1}). The antenna was placed 0.5 cm from the outlet of this tube. Each individual mosquito accounted for a single replicate, and at least three replicates were performed for each age of the urine samples, for both host-seeking and gravid mosquitoes.

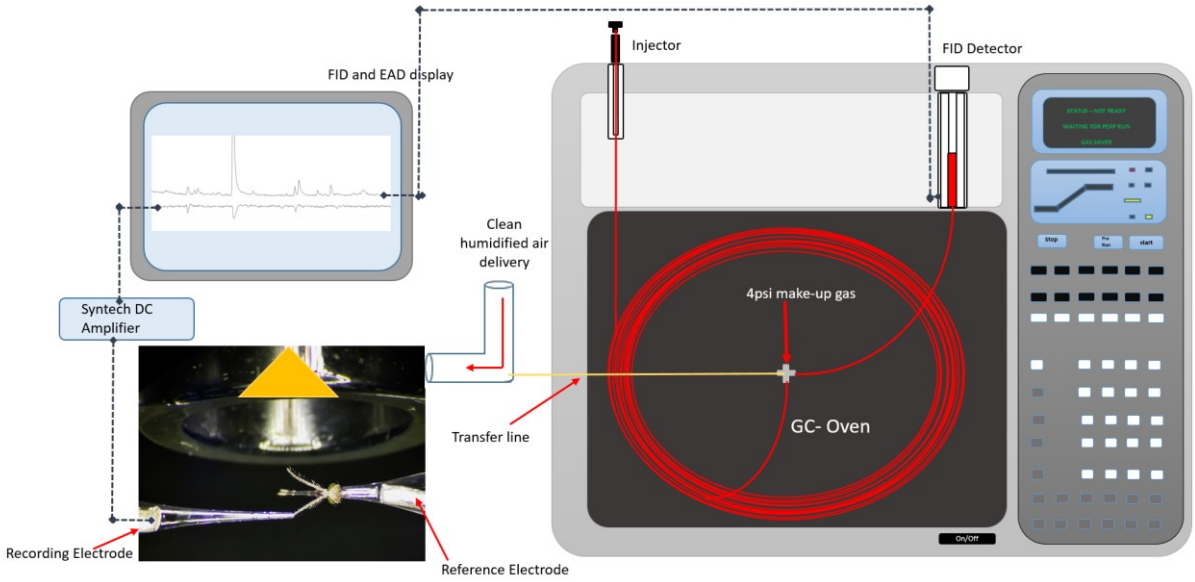

Figure 3.3. Diagrammatic representation of the combined gas chromatography (GC) and electroantennographic detection (EAD) setup. The headspace sample is injected onto the GC column in which the complex odour extract is fractionated and the flow split between the flame ionization detector (FID) and the transfer line. The transfer line passes the fractionated odorants over the antenna, via a glass tube receiving clean humidified air. The FID chromatogram and the physiological responses are displayed on a computer screen. (modified from Ghaninia *et al*., 2008).

3.2.5. Chemical Identification of Bioactive Compounds

Bioactive compounds in the headspace collections of fresh and aged cattle urine, eliciting an antennal response in the GC-EAD analyses, were identified using a combined GC- and mass spectrometer (GC–MS; 6890 GC and 5975 MS; Agilent Technologies), operated in the electron impact ionization mode at 70 eV. The GC was equipped with an HP-5MS UI coated fused silica capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness), and helium was used as the mobile phase at an average linear flow rate of 35 cm s^{-1}. Two µl of sample were injected using

the same injector settings and oven temperatures as for the GC-EAD analysis. Compounds were identified according to their Kovát's indices (retention times) and mass spectra, in comparison with custom made (Alnarp chemical list) and NIST14 libraries (Agilent). The Kovát´s retention indices (RI) were calculated according to:

$$RI\boldsymbol{a} = 100N + 100n\ \frac{t_{Ra} - t_{RN}}{(t_{R(N+n)} - t_{RN})}$$

where ***a*** = compound ***a***

t_R = retention time

$t_{R\boldsymbol{a}}$ = retention time for the compound ***a***

N = is the number of Carbon in shorter hydrocarbon; before compound ***a***

n = is the number of Carbon more in the longer hydrocarbon; before compound ***a***

N+n = is the number of Carbon in longer hydrocarbon; before compound ***a***,

For determination of RI, a homologous series of straight-chain n-alkanes (C_8-C_{20}) and (C_{21}-C_{40}) Kováts mix 2 µl ng^{-1} in hexane was used, and the values were compared with published values with similar chromatographic column and experimental conditions. Identified compounds were confirmed by the injection of authentic standards (Table 3.1). Quantification of identified compounds were performed by comparison of their peak areas with heptyl acetate (10 ng, 99.8 % chemical purity, Aldrich).

3.2.6. Behavioural Analyses of Host-seeking and Gravid Malaria Mosquitoes towards Synthetic Odour Blends

To assess the efficacy of synthetic odour blends, composed of the bioactive compounds identified in fresh and aged urine, to attract host-seeking and gravid *An. arabiensis*, the

same olfactometer and protocol was used as described above (see section 3.2.5.). The synthetic blends mimicked the composition and ratio of compounds in the pooled headspace volatile extracts of fresh, 24 h, 48 h, 72 h and 168 h aged urine (Fig. 3.4). These blends were tested in preliminary dose-response bioassays using the synthetic blend of the 24 h aged urine odour. This revealed that 10 µl of a 1:100 dilution of the full synthetic blends, at a release rate of approximately 140 ng h^{-1}, giving one minute equivalent per minute, attracted host-seeking and gravid mosquitoes at a level similar to that of the natural extracts. As the natural extract of 24 h aged urine, along with its synthetic blend, elicited the highest level of attraction in host-seeking *An. arabiensis*, subtractive bioassays with both host-seeking and gravid mosquitoes were conducted to determine the relative activity of the identified components. For this purpose, single compounds were removed from the full blend to create subtractive blends to be tested against the full blend.

Table 3.1. Synthetic compounds used for electrophysiological and behavioural analyses

	Purity (%)	Supplier	Kovát´s (Retention) Indices (RI)	CAS No.
phenol	99.5	Sigma-Aldrich	977-979	108-95-2
2-cyclohexen-1-one	96	VWR Int.	1024	930-68-7
m-cresol	97	Sigma-Aldrich	1076	108-39-4
p-cresol	99	Sigma-Aldrich	1075	106-44-5
4-ethyl phenol	99	Sigma-Aldrich	1168-1170	123-07-9
3-nonen-2-one	95	Sigma-Aldrich	1096	18402-83-0
decanal	98	Sigma-Aldrich	1206	112-31-2
2-ethyl-1-hexenol	99	Sigma-Aldrich	1030	104-76-7
linalool	97	Sigma-Aldrich	1104	78-70-6
S-(-)-limonene	95	Sigma-Aldrich	1035	5989-54-8

Finally, a comparative analysis was performed to assess the attraction of closely related species within the *Anopheles gambiae s.l.* complex, including *An. arabiensis*, *An. coluzzii* and *An. quadriannulatus*, to the synthetic odour blend of 24 h aged urine. For this purpose, a Y-tube olfactometer (Emami *et al.*, 2017) was used, with conditioning of the insects done as described above (see section 3.2.3). The synthetic blend and the pentane control was applied as described above (see section 3.2.6.), and then inserted in either arm of the olfactometer. The position of the treatment and control was altered in between each trial to reduce positional bias. A preference index (PI) was calculated as:

$$PI = \left(\frac{(N_T - N_C)}{(N_T + N_C)}\right) \times 100$$

where, N_T is the number of mosquitoes flying to the treatment arm and N_C is the number of mosquitoes flying to the control arm. Positive values indicate that mosquitoes prefer to enter the arm containing the synthetic blend whereas negative values indicate a preference to enter the control arm (Wondwosen *et al*., 2016).

3.2.7. Data Analysis

Responses of host-seeking and gravid *An. arabiensis* to the headspace collections and their respective synthetic blends were analysed using a general linear model (GLM) followed by a post hoc Tukey HSD test. Behavioural responses to the subtractive blends were compared to that of the 24 h synthetic blend, using a GLM followed by Dunnett´s post hoc analyses at a 95 % significance level. Individual flight responses were recorded as the dependent variable, and the headspace volatiles, synthetic and subtractive blends were taken as factors responsible for the observed behaviour. The behavioural responses of the three *Anopheles* species were analysed using a nominal logistic regression

followed by a Likelihood Ratio Test comparing between species and doses (JMP® Pro 14.0.0 SAS Institute Inc., Cary, NC, USA.

3.3. Results

3.3.1. Behavioural Responses of Host-Seeking and Gravid *Anopheles arabiensis* to Headspace Volatile Extracts of Fresh and Aged Cattle Urine

The overall attraction of host-seeking, but not of gravid, *An. arabiensis* to the headspace volatile extracts of cattle urine, as assessed in a glass tube olfactometer, was significantly affected by the age of the urine ($F = 4.237$, $df = 4$, $p = 0.003$) and ($F = 2.379$, $df = 4$, $p = 0.054$) (Fig. 3.4), respectively. Host-seeking mosquitoes showed a significant attraction to the headspace volatile extract of 24 h aged urine as compared to the extracts of 72 h ($p = 0.026$), 168 h ($p = 0.05$) and pentane as a control ($p = 0.002$) (Fig. 3.4A). No significant difference in attraction was observed between the headspace volatile extracts of fresh ($p = 0.47$) and 24 h aged urine (Fig. 3.4A). In contrast, gravid mosquitoes only demonstrated a significant attraction to the headspace volatile extract of 72 h aged urine, as compared to the control ($p = 0.022$) (Fig. 3.4B). No significant difference in attraction of gravid *An. arabiensis*, was found between the headspace volatile extracts of the differently aged urine samples ($p > 0.05$) (Fig. 3.4B).

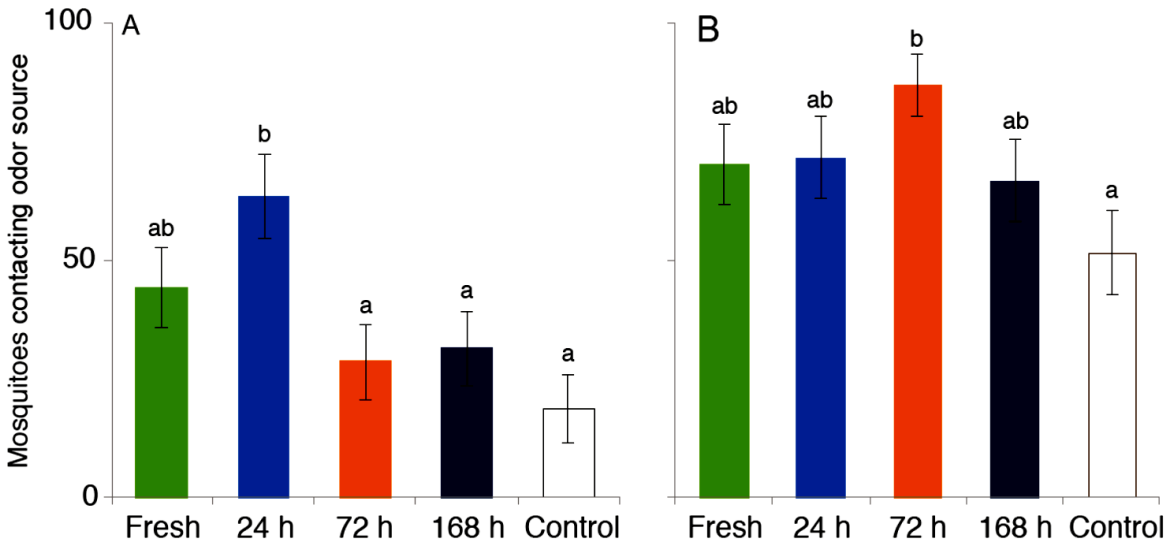

Figure 3.4. Behavioural responses of host-seeking (A) and gravid (B) *Anopheles arabiensis* to the headspace volatile extracts of fresh and aged cattle urine. Different lowercase letters indicate significant differences, as revealed through Tukey's post hoc analysis ($p < 0.05$). Error bars indicate the standard error of the mean.

3.3.2. Qualitative and Quantitative Changes in Bioactive Compounds

For host-seeking mosquitoes, the GC-EAD and GC-MS analyses identified eight, six, three and three bioactive compounds in the headspace volatile extracts of fresh, 24 h, 72 h and 168 h aged cattle urine, respectively (Fig. 3.5). While gravid mosquitoes displayed a similar response profile, they did not respond to 2-cyclohexen-1-one and (*S*)-(-)-limonene found in the headspace volatile extracts of fresh urine, when presented at an ecologically relevant concentration (data not shown). The injection of a five times stronger synthetic blend onto the GC, however, elicited a physiological response to these compounds (data not shown).

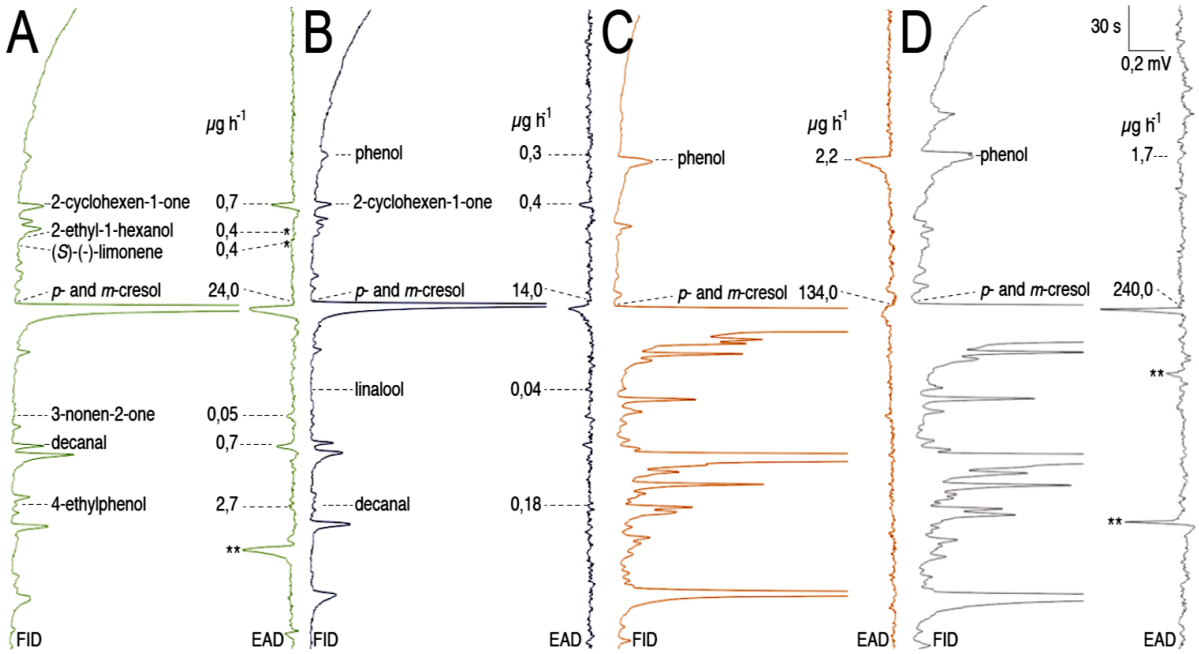

Figure 3.5. Antennal responses of host-seeking *Anopheles arabiensis* to fractioned headspace extracts from fresh (A), 24 h (B), 72 h (C) and 168 h (D) aged cattle urine. Electroantennographic detection (EAD) traces show voltage changes in response to the bioactive compounds in the headspace eluting from the gas chromatograph and detected by the flame ionization detector (FID). The identity and release rate (µg h^{-1}) of the bioactive compounds are shown. A single asterisk (*) indicates consistent low amplitude responses. A double asterisk (**) indicates irreproducible responses.

Despite the observed difference in the number of bioactive compounds, all were present in the headspace volatile extracts of fresh and aged urine (Fig. 3.6). Overall, the volatile release rate of these compounds in the headspace collections increased from 30 µg h^{-1} in fresh urine sample to 242 µg h^{-1} in 168 h aged urine sample, mainly due to the increase of 3- and 4-methylphenol, as well as phenol, 4-ethylphenol (Fig. 3.6). In contrast, the release rate of 2-cyclohexen-1-one, (*S*)-(-)-limonene, decanal, and 2-ethyl-

1-hexenol decreased with an increasing age of the urine samples (Fig. 3.6), which correlates with the decrease physiological response to these compounds.

Figure 3.6. Heat plot showing the change in release rate of compounds found to be GC-EAD active in at least one of headspace extracts from fresh, 24 h, 72 h or 168 h aged urine. The quantification of compounds is based on the manual integration of the area of each chromatogram.

3.3.3. Behavioural Responses to Synthetic and Subtractive Urine Odour Blends

Synthetic blends containing the bioactive compounds identified in the headspace volatile extracts of fresh and aged urine, approximating their natural ratio (Fig. 3.5), elicited attraction in host-seeking ($F = 4.319$, $df = 4$, $p = 0.003$; Fig. 3.7A) but not in gravid mosquitoes ($F = 1.388$, $df = 4$, $p = 0.239$; Fig. 3.7B). In addition, a multiple comparison of the means revealed a significant attraction of host-seeking mosquitoes to

the synthetic blend of 24 h aged urine, as compared to the pentane control (p = 0.003) (Fig. 3.7A).

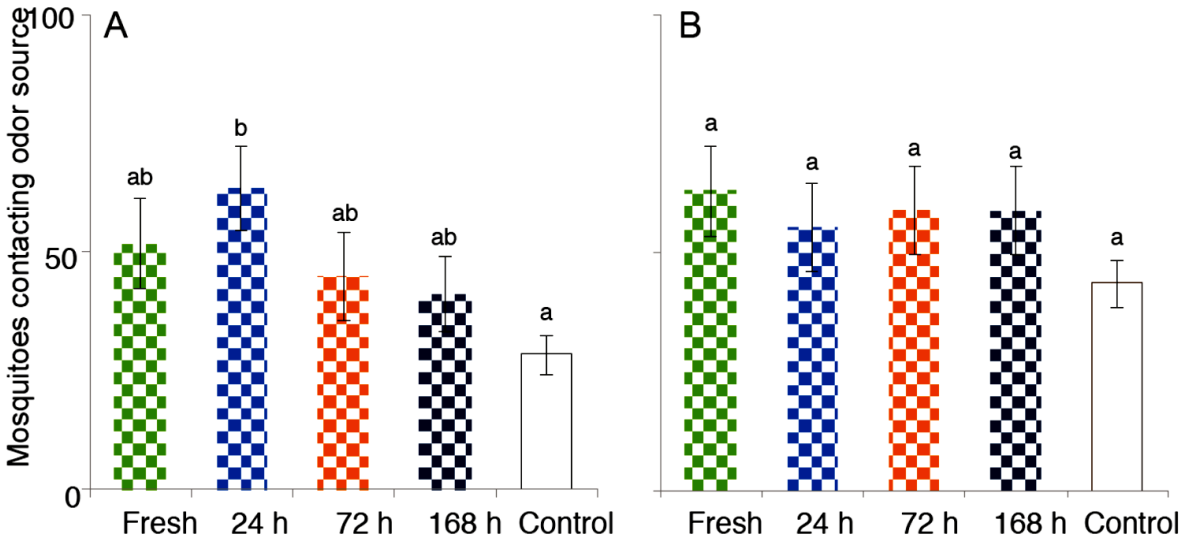

Figure 3.7. Behavioural responses of host-seeking (A) and gravid (B) *Anopheles arabiensis* to the synthetic blends of fresh and aged cattle urine odour. Different lowercase letters indicate significant differences, as revealed by a Tukey HSD post hoc analysis ($p < 0.05$). Error bars indicate the standard error of the mean.

To further assess the role of each individual component in the synthetic blend of 24 h aged urine, six subtractive blends, from which individual compounds removed, were evaluated against the full blend in a Y-tube assay. For host-seeking mosquitoes, subtraction of individual compounds from the full blend had a significant effect on the behavioural response ($F = 3.876$, $df = 6$, $p = 0.001$; Fig. 3.8), with all subtractive blends being less attractive than the full blend. In contrast, the removal of individual compounds from the full synthetic blend did not affect the behavioural response of gravid mosquitoes ($F = 1.794$, $df = 6$, $p = 0.102$; Fig. 3.9).

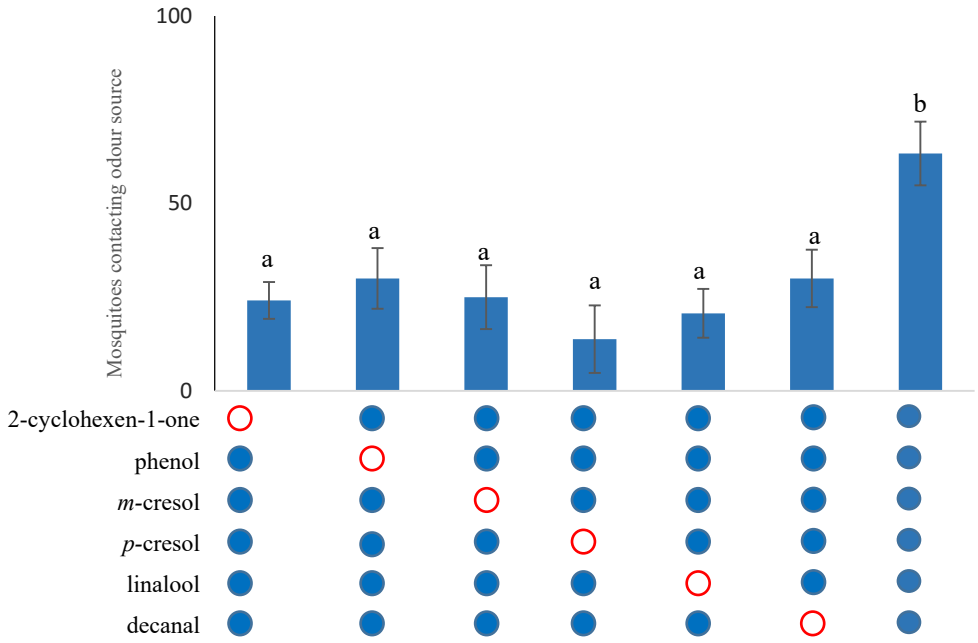

Figure 3.8. Behavioural responses of host-seeking *Anopheles arabiensis* to subtractive blends of volatile compounds from 24 h aged cattle urine. Attraction was significantly reduced compared to that of the full blend (far right) by a general linear model followed by Dunnet´s post hoc analysis ($p < 0.05$). Error bars represent the standard error of mean.

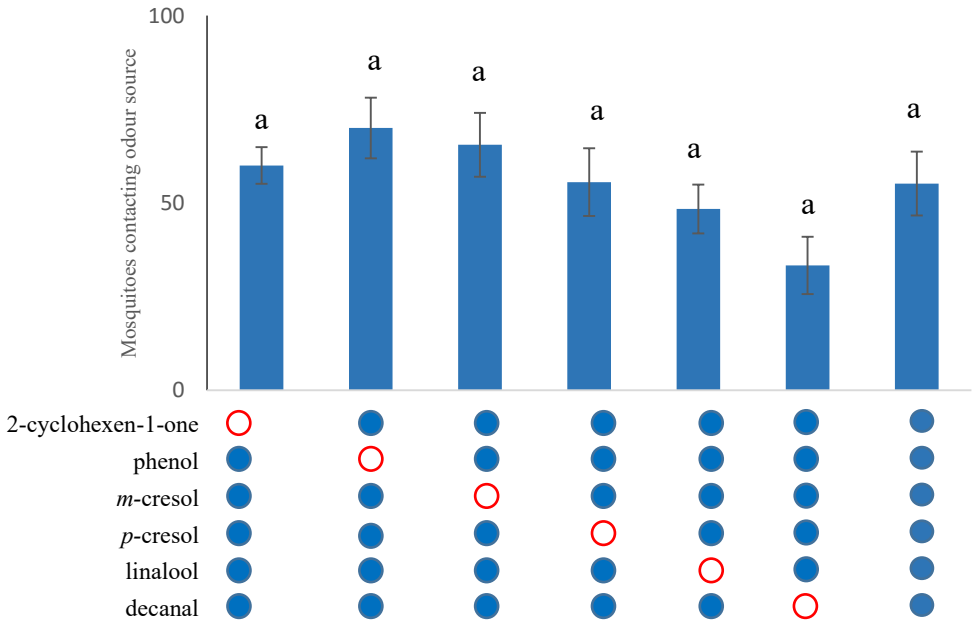

Figure 3.9. Behavioural responses of gravid *Anopheles arabiensis* to subtractive blends of volatile compounds from 24 h aged cattle urine. Attraction was not significantly affected by the removal of the compounds compared to that of the full blend (far right) as shown using a general linear model followed by Dunnett´s post hoc analysis ($p < 0.05$). Error bars represent the standard error of mean.

3.3.4. Comparative Behavioural Analysis of *Anopheles gambiae Sensu lato* Mosquitoes

The efficacy of the synthetic blend to attract *An. gambiae, An. quadriannulatus* and *An. arabiensis* was compared using a Y-tube olfactometer, indicating significant differences in the dose-dependent responses. The behavioural responses of the three species differed significantly at a 10^{-3} dilution of the blend ($\chi^2 = 7.12$, df = 2, $p = 0.028$) (Fig. 3.10) with *An. quadriannulatus* > *An. coluzzii* (odds ratio = 0.177, $p = 0.041$) > *An. arabiensis* (odds ratio = 6.99, $p = 0.022$). In addition, the behavioural response of *An. quadriannulatus* was significantly different from that of *An. arabiensis* at a 10^{-1} dilution of the synthetic blend (odds ratio = 0.286, $p = 0.046$).

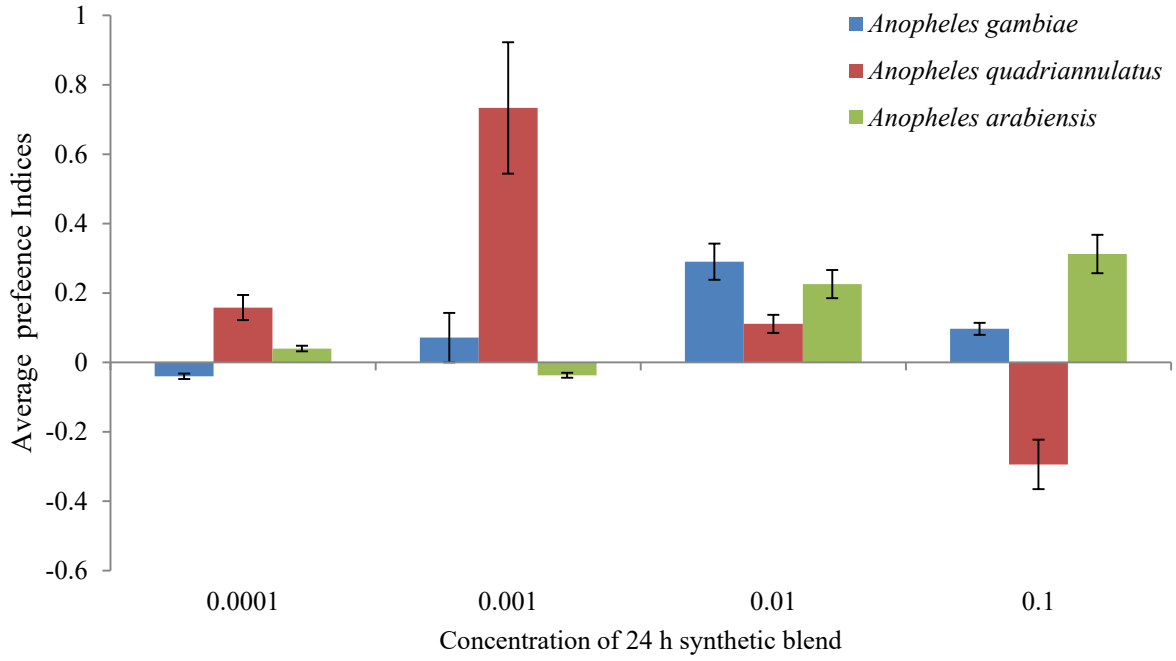

Figure 3.10. Behavioural responses of host-seeking *Anopheles gambiae, Anopheles arabiensis* and *Anopheles quadriannulatus* to four decadic dilutions of the synthetic odour blend of 24 h aged cattle urine. A likelihood ratio test was employed to test for statistical differences, using nominal logistic regression. Error bars represent standard error of mean.

3.4. Discussion

Survival and reproduction of mosquitoes rely on the successful location of potential hosts, which often are patchily distributed (Cardé, 2015). Host habitat cues, such as urine, have been shown to offer effective host-characteristic cues that indicate where potential hosts or oviposition sites may be located (Bursell *et al*., 1988; Okech and Hassanali, 1990; Mihok and Mulye, 2010; Kweka *et al*., 2011). While previous studies on *An. arabiensis* have shown that host-seeking (Kweka *et al*., 2009) and gravid (Mahande *et al*., 2010) mosquitoes are attracted to cattle urine, the mechanisms regulating this interaction was unknown. Host-seeking mosquitoes, including not only

An. arabiensis but also the closely related *An. quadriannulatus,* are attracted to cattle urine deposited within 24 h (Broek and Otter, 1999; this study), are correlated with qualitative and quantitative changes in the volatile chemical composition of aging cattle urine, and are confirmed behaviourally using synthetic odour blends. This is different from that which has been demonstrated for tsetse flies and tabanids, which are attracted to aged urine, and volatiles associated with it, predominantly *m*- and *p*-cresol, as well as 3-propylphenol (Okech and Hassanali, 1990; Mihok and Mulye, 2010). The identification of host habitat cues that attract host-seeking malaria mosquitoes provides an important input for future mosquito control measures.

Locating both hosts for blood feeding and sites for oviposition is essential for mosquito survival and reproduction (Takken and Verhulst, 2013; Sumba *et al*., 2004). To overcome this challenge, mosquitoes and other hematophagous insects appear to use host habitat cues, such as urine and faecal odour (Qui *et al*., 2011; Cooperband *et al*., 2008), which often are released in large quantities, as shown here, making them more easily detectable over longer distances. Previous studies by (Mahande *et al.* 2010), demonstrated that host-seeking *An. arabiensis* are differentially attracted to fresh cattle urine, which is in line with our observations. This behavioural response appears to be contextual, as host-seeking *An. arabiensis* only respond to cues that indicate that a potential host has been present with 24 h, and thus has the potential of strengthening the spatial association with the host. This is unlike that observed for other hematophagous insects, including tsetse and tabanid flies, which are attracted to volatiles emanating from aged urine (Madubunyi *et al*., 1996; Baldacchino *et al*., 2013), suggesting different strategies for host location among these insects. The general lack of response of gravid mosquitoes to volatiles of fresh and aging cattle urine, with the exception of that to the headspace volatile extract of 72 h aged urine, is intriguing as it contradicts that observed

by (Kweka *et al*., 2011). Plausible explanations for this discrepancy may be that the collections of headspace volatiles failed to capture all bioactive compounds or that it was not feasible to identify these in the GC-EAD and GC-MS analyses. More likely is that the overall high behavioural activity of gravid mosquitoes made it difficult to assess attraction using a straight glass tube assay; a subsequent field evaluation, however, clearly demonstrate that gravid mosquitoes are attracted to the synthetic blend of 24 h aged urine (Chapter 4).

Mechanistically, the observed behavioural responses of host-seeking *An. arabiensis* to fresh and aging cattle urine is correlated with qualitative and quantitative changes in the volatile chemical composition of the urine. The composition and overall release rate of bioactive compounds change with the aging of the urine (this study), predominantly due to bacterial activity (Murry and Adams, 1988; Okech and Hassanali, 1990). The GC-EAD and GC-MS analyses identified nine and six bioactive compounds in the headspace volatile extracts of fresh and 24 h aged cattle urine, respectively, revealing a partial turnover of bioactive compounds. While several of these compounds have previously been shown to be associated with host (Qui *et al*., 2011) and oviposition site odour (Wondwosen *et al*., 2016; 2017; Eneh *et al*., 2016), and to elicit physiological responses across mosquito species, only a subset has been identified in urine odour (Mihok and Mulye, 2010; Baldacchino *et al*., 2013). When presented in the natural ratio and at the approximate release rate, these compounds elicit behavioural responses in host-seeking *An. arabiensis* reflecting that observed to the headspace volatile extracts of fresh and 24 h aged cattle urine. Importantly, subtractive bioassays reveal that the full component blends are required to elicit the behavioural response of host-seeking mosquitoes, which is in line with previous studies on *An. arabiensis* showing that blend perception is critical for behavioural responses (Meiners, 2015; Majeed *et al*., 2016). In

contrast to fresh and 24 h aged urine, the GC-EAD and GC-MS analyses consistently revealed phenol, *m*- and *p*-cresol to be the bioactive compounds in 72 h and 168 h aged urine. Previous chemical analyses have also identified these compounds, along with 3-propylphenol, to be the major constituents of cattle (Madubunyi *et al*., 1996) and buffalo (Den Otter, 1991) urine, derived from the bacterial breakdown of tyrosine (Murry and Adams, 1988). The lack of behavioural responses to blends of these compounds (this study; Lindh*et al*., 2008; Huang *et al*., 2006) may thus be linked to an aversion to potentially harmful bacteria, as demonstrated for *Drosophila melanogaster* (Mansourian *et al*., 2016). Future studies are needed to assess this association.

An increased understanding of the behavioural ecology of malaria mosquitoes have been deemed critical (WHO, 2017b) for the sustainable control of the disease situation. The use of odour-baited traps (Homan *et al*. 2016), has been shown to be an effective way to supplement current IVM strategies, however additional research is needed to increase the efficacy of existing lures, not least develop lures that target physiological states other than host-seeking mosquitoes. The host habitat cues identified in the current study provide such a tool (see also Chapter 4). Additional research into the functional role of urine is also required to fully comprehend its role in the ecology of malaria mosquitoes. For example, data presented in Chapter 5 of this thesis, reveal that cattle urine may be used both as a cue for locating potential host, as well as a cue to locate nutrients for flight energy and reproduction.

Chapter 4- Field Evaluation of Synthetic Cattle Urine Odour as ´Host Habitat´ Lure to Attract Malaria Mosquitoes in the Central Rift Valley of Ethiopia

4.1. Introduction

Current control strategies developed to manage malaria in sub-Saharan Africa are challenged by the increased physiological and behavioural resistance of the vectors that transmit the disease, the *Anopheles* mosquitoes (Hemingway *et al*., 2004; Benelli, 2015; Benelli and Beier 2017; Ranson *et al*., 2011; Sougoufara *et al*., 2017; Zalucki and Furlong, 2017; Ranson and Lissenden, 2016). Overall, current literature suggest that behavioural resistance and species changes in response to long lasting insecticide treated nets and indoor residual spraying may be a lead cause for residual malaria and the stagnation of progress in malaria control (Durnez and Coosemans, 2011; Kitau *et al*., 2012; Gatton *et al*., 2013; Cibulskis *et al*., 2016). To overcome these issues, an increased understanding of the behaviour and ecology of malaria mosquitoes is required (Killeen *et al*., 2017; Brady *et al*., 2016). For this reason, emphasis has been put on research unveiling the mechanisms underlying host selection, a key behaviour directly impacting the malaria parasite transmission cycle (Bowen, 1991; Takken and Verhulst, 2013).

Host selection is composed of a series of behaviours that are sequentially expressed and rely heavily on olfactory cues (Lutz *et al*., 2017), some of which have been used with some success to control malaria mosquitoes, and reduce malaria prevalence (Mweresa *et al*., 2016). While the great majority of studies have focused on identifying cues emanating directly from potential blood hosts (Takken and Verhulst, 2013; Majeed *et al*., 2016), recent studies have indicated that mosquitoes, and other hematophagous insects, use ‘habitat cues’ to locate a place where more specific host

cues may be more easily found (Webster and Carde, 2017). Urine, produced in large quantities by host and non-host animals alike, attract a range of hematophagous insect species, including malaria mosquitoes (Belete *et al.,* 2004; Mahande *et al.,* 2010; Baldacchino *et al.,* 2013; Nordéus *et al.,* 2014). Fresh, and to a lesser extent aging cattle urine, has previously been shown to be efficient for sampling one of the major vectors of malaria in sub-Saharan Africa, *Anopheles arabiensis* (Kweka *et al.,* 2010; 2011). Subsequent behavioural analyses along with combined gas chromatography and electroantennodetection analyses allowed for the identification of synthetic odour blends that differentially attracted host-seeking and gravid *An. arabiensis* under laboratory conditions (Chapter 3). One blend in particular, that of 24 h aged cattle urine, demonstrated great potential as a malaria mosquito attractant.

In this study, the efficacy of the synthetic odour blend composed of the six bioactive compounds identified in the headspace volatile extract of 24 h aged cattle urine (Chapter 3) is tested under field conditions in Ethiopia. Predominantly host-seeking and blood fed mosquitoes, of the genus *Anopheles* and *Culex* are shown to be attracted to traps baited with this lure. The potential of using the synthetic odour blend for monitoring and control purposes is discussed.

4.2. Materials and Methods

4.2.1. Experimental Site

The study was conducted in a malaria endemic village nearby the town of Meki in the Dugda district of East Shewa, in the Oromia region of Ethiopia. The village (Fig. 4.1) is positioned on the northern shore of Lake Ziway at 8°11′08′′N, 38°81′70′′E, at an altitude of 1636 m above sea level, with an average rainfall and temperature of 150 mm and 28 °C, respectively. The main rainy season in the area is from June to mid-

September, during which time the mosquito populations reaching their maximum. The study was conducted between mid-August to mid-September before the annual indoor residual spraying commenced. The housing, commonly called ´Mana Chitaa´, are thatched houses made from mud-walls, with a thatched roof and eaves in between for ventilation. Livestock rearing, including cattle, sheep, goats and poultry, is very common. Most of the houses have sheds for cattle, goats and sheep. Five pairs of houses (20 -50 m apart from each other) located in the periphery of the village were selected for the study (Fig. 4.1). The criteria used to select the houses were: no animals were allowed to be kept inside the houses, no cooking (smoking fire wood or charcoal) was allowed indoors (at least during the trial period), and houses with maximum of two inhabitants, sleeping under a non-insecticide treated bed nets.

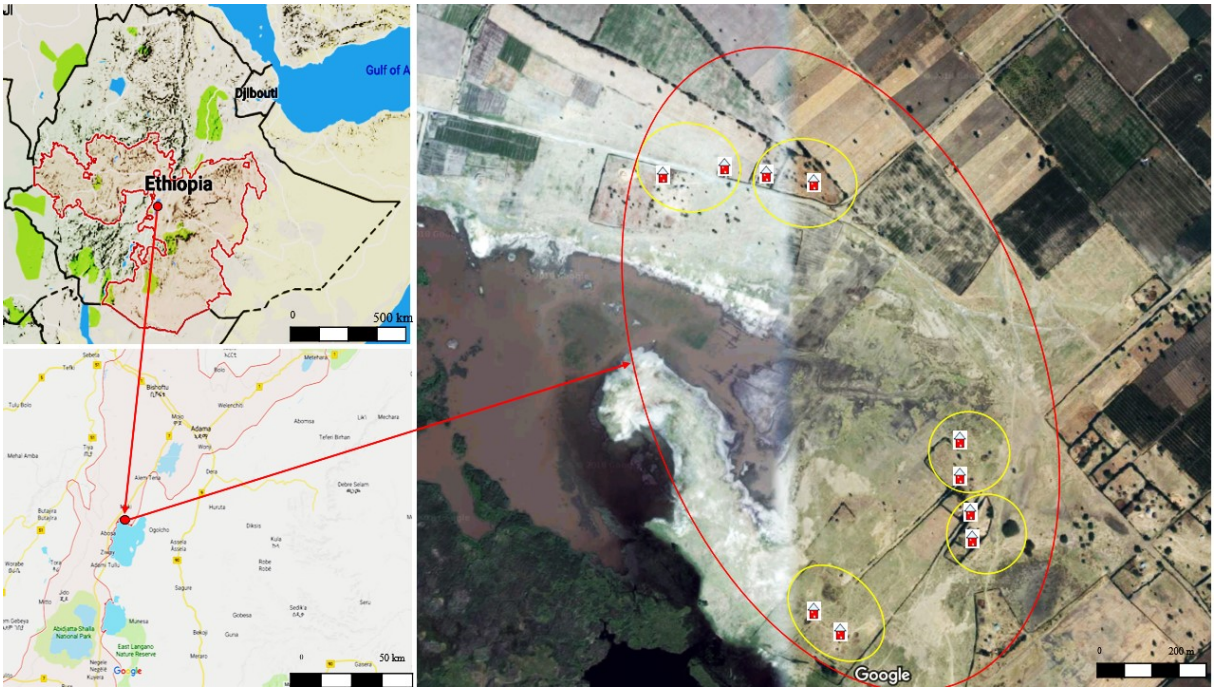

Figure 4.1. Map of the study site showing the five pairs of houses in the village selected for the study, located on the proximity of Lake Ziway nearby the town of Meki. (Source Imagery© DigitalGLobe, Map data© 2018 Google Ethiopia).

4.2.2. Synthetic blend preparation and CDC light trap set up

The synthetic blend was dissolved in heptane (97.0 % solvent GC grade, Sigma Aldrich). The blend contained the bioactive compounds identified in the headspace volatile extracts of 24 h aged cow urine, approximating their natural ratio (7:9:156:156:1:4; 2-cyclohexen-1-one: phenol: *p*-cresol: *m*-cresol: decanal: linalool) (Chapter 1). This blend was released at 140 ng h^{-1} using wick dispensers consisting of a 2 ml vial with a hole in the center (1.7 mm i.d.) of the cap through which a Teflon tube (1.6 mm i.d. × 3 cm) containing a cotton wick protruded (Wondwosen *et al*., 2016). The wick dispensers allow for the release of all compounds in constant proportions throughout the 12 h experiment, giving one minute equivalent per minute. Heptane was used as a control. The vials were hung on the side of CDC (Center for Disease Control and Prevention) light traps to ensure that insects were attracted and captured (Jaleta *et al*., 2016). The traps were suspended 0.8 – 1 m above the ground next to the foot side of a bed with a volunteer sleeping under an untreated bed net, and turned on at 18:00 and turned off the following morning at 06:30, and then enumerated and identified to species as described below. The experimental design followed a 2 × 2 Latin square design, in which the treatments and control blends were assigned to paired houses at the first night and rotated between paired houses the next experimental night. This procedure was replicated 10 times. In addition, to estimate the activity of mosquitoes in the selected houses, CDC traps, without synthetic blend dispensers, were set to operate during the same hours of the day, at the beginning, mid and end of the field trials for 5 nights.

4.2.3. Identification of Mosquitoes

Mosquitoes were sorted by sex and physiological states as unfed, fed, semi-gravid and gravid (WHO, 1975). Subsequently, these mosquitoes were identified morphologically

to species (Verrone, 1962; Gillies and Coetzee, 1987) and placed in 1.5 ml Eppendorf tube with dry silica gel. Five per cent of the mosquitoes that were morphologically identified as *An. gambiae* s.l. were subsequently screened using polymerase chain reaction (PCR) analysis at Swedish University of Agricultural Sciences, Disease Vector group, Alnarp by Dr. Elsa Quellary to identify the specimens to the species level (Wilkins *et al*., 2006).

4.2.4. Ethical Approval

Ethical approval was obtained from the Institutional Research Ethics Review Board, College of Natural Sciences, (CNS-IRB), Addis Ababa University (IRB/022/2016), according to the guidelines set out by the World Medical Association Declaration of Helsinki. Consent from each household head was obtained with assistance of health extension workers. The whole process was endorsed by the local administration at district and ´Kebele´ level.

4.2.5. Data Analysis

To assess the effect of treatment to that of the control, the trap captures of the paired houses were analysed using a nominal logistic fit model, in which attraction was the dependent variable and treatment (synthetic blend vs. control) the fixed effect (JMP® 14.0.0. SAS Institute Inc., Cary NC, USA). Here, we report the χ^2 and p-value from the Likelihood Ratio Test.

4.3. Result

A total of 4861 mosquitoes were collected and identified during the study of which 45.7 % were *An. gambiae sensu lato,* 18.9 % were *An. pharoensis* and 35.4 % were *Culex spp. Anopheles arabiensis* was the only member of the *An. gambiae* species complex to be identified, following PCR analyses of 261 (5 %) female mosquitoes. On

average, 320 mosquitoes were caught for each of the 10 trapping nights, during which the traps baited with the synthetic blend caught more mosquitoes than the paired traps without the blend ($\chi^2_{(0, 3196)} = 170.0$, $p < 0.0001$). During each of the five nights in which the non-baited traps were set to operate at the beginning, middle and end of the trial, an average of 333 mosquitoes were caught, with similar numbers caught in each paired trap, demonstrating that there was no bias between houses ($\chi^2_{(0,1665)} = -9 \times 10^{-13}$, $p > 0.05$). The number of host seeking ($\chi^2_{(0, 2107)} = 138.7$, $p < 0.0001$), recently blood fed ($\chi^2_{(0, 650)} = 32.2$, $p < 0.0001$) and gravid ($\chi^2_{(0, 228)} = 6.27$, $p = 0.0123$) mosquitoes were significantly higher in traps containing the synthetic blend compares to the control traps. This was also reflected in the total number of mosquitoes caught: host seeking > blood fed > gravid > semi-gravid > males.

The three species were differentially attracted to the traps containing the synthetic blend. A significantly higher number of host seeking ($\chi^2_{(1, 1345)} = 71.7$, $p < 0.0001$), blood fed ($\chi^2_{(1, 517)} = 16.7$, $p < 0.0001$) and gravid ($\chi^2_{(1, 180)} = 6.11$, $p = 0.0134$) *An. arabiensis* were caught in traps releasing the synthetic blend (Fig. 4.2 A), whereas no difference in the number of *An. pharoensis*, at different physiological states, was found (Fig. 4.2 B). For the *Culex* spp., only the number of host seeking was found to be significantly higher in the traps baited with the synthetic blend ($\chi^2_{(1, 1319)} = 12.6$, $p = 0.0004$; Fig. 4.2. C), compared to the control trap.

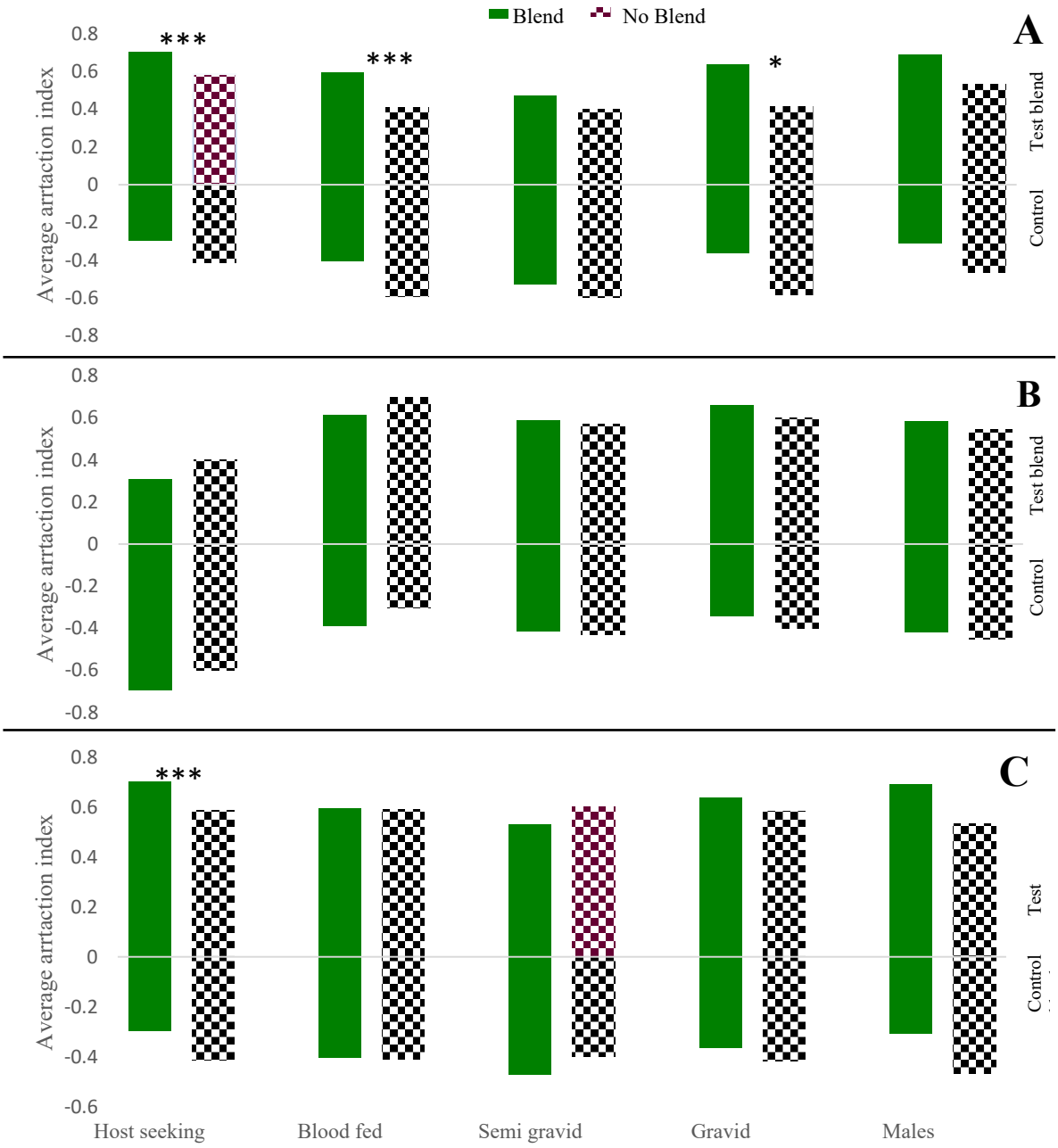

Figure 4.2. Captures of male and different physiological states of female *Anopheles arabiensis* (A), *Anopheles pharoensis* (B) and Culex spp. (C) in traps baited with a synthetic odour of 24 h aged cattle urine. Solid bars represent the average attraction indices of mosquitoes caught in paired odour baited and control traps (N = 10), whereas hatched bars represent the preference indices of mosquitoes

caught in paired control traps (N = 5). Asterisks denote the level of statistical significance (*P = 0.01 and ***P < 0.0001).

4.4. Discussion

Bovine urine has been demonstrated to act as host-habitat cues for a range of hematophagous insects, including malaria mosquitoes (Webster and Cardé, 2017). This study shows that a range of physiological states of *An. arabiensis*, as well as host-seeking *Culex* spp., are attracted and trapped by a synthetic blend of volatile compounds identified in 24 h aged cattle urine (Chapter 3). As such, it confirms and extends previous studies by Mahande *et al*. (2010) and Kweka *et al*. (2011), showing that *An. arabiensis* and culicine mosquitoes are attracted to the odour of fresh cattle urine. As such, this odour blend offers an alternative tool for surveillance and control of mosquitoes.

Landscapes are heterogeneous, and searching insects need to make choices at increasingly refining spatial scales, a process that can be broken down to habitat finding, resource finding, resource recognition, and resource acceptance (Sumba *et al*. 2008; Sanford and Tomberlin 2011; Stone *et al*., 2011; Webster *et al*., 2015). Habitat cues, such as cattle urine, which often are released in large quantities (Webster and Cardé, 2017; Chapter 3), have been demonstrated to be essential for a range of insect species to locate potential hosts that often are patchily distributed and mobile (Burkett-Cadena, McLure, *et al*., 2013). Results presented in this study demonstrate that both host-seeking and blood fed *An. arabiensis*, as well as host-seeking *Culex* spp., are attracted to the synthetic odour of 24 aged cattle urine. In general, this supports the findings of Mahande *et al*. (2010) and Kweka *et al*. (2011). However, Mahande *et al*. (2010) reported a higher number of semi-gravid and gravid *An. arabiensis* caught in baited resting boxes. The

observed discrepancy between this study and that of Mahande *et al*. (2010), is likely due to the trap type used. Combined, these studies clearly indicate that host-seeking *An. arabiensis* and culicine mosquitoes may use the odour of fresh cattle urine, but not that of aged urine (Chapter 3), as a true indicator for host availability. In contrast, the role of cattle urine for blood-fed *An. arabiensis* remains elusive. Although, Mahande *et al*. (2010) and Kweka *et al*. (2011) observed an increase in attraction and oviposition choice of *An. arabiensis* in response to fresh urine, mosquitoes were deterred by the odour of aged urine, suggesting that cattle urine may serve a different role in semi-gravid and gravid mosquitoes. As an alternative, blood fed *An. arabiensis* may use cattle urine as a nutrient source as shown in Chapter 5 of this thesis.

The synthetic odour of cattle urine used in this study differs substantially from that used to attract other hematophagous insects in terms of identity and ratio of compounds, as well as release rate (ng h^{-1}; this study). Tsetse flies, tabanids, biting midges and mosquitoes have all been shown to be attracted to synthetic blends containing various combinations of 3- and 4-methylphenol, as well as 3-propylphenol, often released at a very high rate (mg h^{-1}). This discrepancy is intriguing, suggesting that hematophagous insects may have developed different strategies to locate potential hosts, which may be related to differences in flight behaviour, especially of the fast flying tsetse flies and tabanids compared to the comparatively slow flight of mosquitoes (Brady and Griffiths, 1993; Spitzen *et al*., 2013). Alternatively, it may related to difference in the ecology of the hematophagous insects, in which for example some species of biting midges have been shown to oviposit in dunghills (Ninio *et al*., 2011).

At times when existing control strategies are challenged, it is important to develop alternative sampling, surveillance, and control tools (Benelli, 2015; Benelli and

Beier, 2017). Building on efforts, aimed at increasing the understanding of the ecology of malaria mosquitoes, this study provides direct evidence that a synthetic odour blend, based on volatile compounds identified in 24 h aged urine (Chapter 3), can be used to attract both host seeking and blood fed *An. arabiensis*. The study also provides further evidence that cattle urine is used as a host habitat cue, and/or a cue to locate nutrient sources (Chapter 5). The odour blend provides many benefits over that of currently available lures, most importantly the lack of need of carbon dioxide and the fact that it attracts multiple physiological states. Further evaluation of the blend, identifying the formulation and optimal release rate for field use, as well as the trap type to be used in combination with this lure are recommended.

Chapter 5- Reproductive and Flight Performance of Gravid and Host Seeking *Anopheles arabiensis* (Patton) Fed on Cattle Urine and Urea

5.1. Introduction

Environmental cues detected by malaria mosquitoes can act as reliable signals for locating resources or an environmental feature that increases the likelihood of locating a resource (Webster and Cardé, 2017). Fresh cattle urine acts as an indirect signal of the presence of hosts (Mahande *et al*., 2010; Chapters 3 and 4) and oviposition sites (Kweka *et al*., 2011), both of which contribute to the survival and reproductive fitness of female *Anopheles arabiensis*, one of the primary vectors of malaria in sub-Saharan Africa (Sinka *et al*., 2012). This study investigates the potential of cattle urine as a fitness-enhancing resources in flight and reproduction of adult female mosquitoes under laboratory conditions.

Sufficient energy reserves, which accumulate as a result of larval (Arrese and Soulages, 2010) and adult feeding (Briegel *et al*., 2001b; Kaufmann *et al*. 2013a), are required for the successful exploitation of resources by sustained flight of the adult female (Clements, 1955; Kaufmann and Briegel, 2004). As sugar is rapidly digested, it can be used directly for flight (Nayar and Sauerman, 1973; Briegel *et al*., 2001b), or, like blood, it may be converted and stored as energy reserves (Van Handel, 1965; Clements, 1992; Foster, 1995; Naksathit *et al*., 1999a). As energetic resources, sugar and blood are interchangeable, however, the digestion of blood is metabolically less efficient than that of sugar (Van Handel, 1965; Clements, 1992; Foster, 1995). To complete egg development in anautogenous mosquitoes, the protein from at least one blood meal is required, with supplemental sugar and blood feeding shown to increase fecundity, particularly in females with low energy reserves or those which have initially

fed on sub-optimal host blood (Rowley and Graham, 1968a; Nayar and Sauermann, 1975; Magnarelli, 1978; Straif and Beier, 1996; Manda *et al*., 2007; Bargielowski *et al*., 2012; Kaufmann *et al*., 2013b). Urine is both an attractive (Kweka *et al*., 2011; Chapters 3 and 4) and nitrogen-rich (Bannink *et al*., 1999; Dijkstra *et al*., 2013; Kilande *et al*., 2016; Miah *et al*., 2017) resource that is commonly found in the habitats of both host-seeking and gravid *An. arabiensis*, and has the potential to supplement the flight-sustaining energy reserves of host-seeking females and provide additional nutrients supporting egg development in gravid mosquitoes and their subsequent larval development.

Anopheles mosquitoes can fly up to 10 km within 22 h, often composed of short bouts of flying less than one hour (Kaufmann and Briegel, 2004). Such sustained flight is fuelled from carbohydrate and lipid reserves derived from teneral sources, as well as sugar and blood meals (Kaufmann and Briegel, 2004; Kaufmann, Reim, *et al*., 2013). While sugars are converted into triglycerides and glycogen within 24 h of consumption more efficiently than blood, blood is converted into lipids faster than sugar (Naksathit *et al*., 1999; Ziegler and Ibrahim, 2001; Kaufmann and Briegel, 2004). While up to one third of the calories from the protein-rich blood meal is used by anophelines to produce yolk protein and lipids for oogenesis, the residual can be used to enhance maternal protein and lipid stores, impacting the energy metabolism and survival of the female, or be catabolised and excreted (Briegel, 1990; Kaufmann and Briegel, 2004; Nayar and Sauermann, 1975). As one-two days of starvation can lead to the depletion of more than 50 % of all protein, lipid and carbohydrate reserves in anopheline females (Briegel 1990), these reserves need to be regularly replaced from available resources. The availability of sugar meals to the anopheline female during the gonotrophic cycle can provide such a resource, leading to increased survival and fecundity (Gary and Foster,

2001). Interestingly, female *An. gambiae* may increase the frequency of taking blood meals to compensate the lack of sugar meals without suppressing reproductive fitness (Foster, 1995; Gary and Foster, 2001; Ramasamy *et al*., 2000). Such supplementary feeding practices demonstrate that *An. gambiae* females can make use of multiple substrates to satisfy energetic, survival and reproductive needs.

Fresh cattle urine contains urea, which makes up 50-95 % of the total nitrogen. Other forms of nitrogen include allantoin, hypoxanthine and xanthine, creatinine, creatine, hippuric acid, amino acids, and ammonia. As cattle urine ages, microbes make use of these resources, particularly hydrolysing urea to ammonia, and generally reducing the complexity of nitrogen-containing compounds (Kilande *et al*., 2016; Miah *et al*., 2017). With the rapid increase in ammonia correlating with the decline in organic nitrogen (Kilande *et al*., 2016), alkalophilic microbes, many of which produce compounds toxic to mosquitoes, thrive. This suggests that cattle urine aged less than 24 h contains more nitrogen resources that are available for use in energy production and reproduction by the anopheline mosquito, with fewer microbes to challenge the female's immune system. The urea hydrolysis indicated by Doak (1952), revealed that at 23° C the whole urea contained in urine completely hydrolysed within 96 h, where 40 % hydrolysed within 24 h and 70 % within 48 h. However, there is scarcity for the reports indicating the hydrolysis of other nitrogenous constituents of urine.

Table 5.1. Ranges of concentrations of urine N containing compounds in cattle urine expressed in g l^{-1} from 10 individual animals (Source: Bristow *et al*., 1992)

Urine constituents	**Amount (g l^{-1})**
Urea	8.4 -41.13
Allantoin	0.77 -3.4
Uric acid	0.15 – 0.53
Creatinine	0.54 -1.75
Creatine	0.37 -1.58
Hippuric acid	5.96 – 8.93
Hypoxanthine	0.07 -0.23
Free amino Acids	0.15 -1.58
Ammonia	0.03 – 1.3

The attraction of female malaria mosquitoes to cattle urine aged less than a few days both in the laboratory and field (Chapter 3 and 4), together with their ability to use nitrogen sources to build carbohydrate and lipid reserves that enhance female fitness, suggests that nitrogen-rich urine could be used as a resource during the gonotrophic cycle of female anopheline mosquitoes. This chapter investigates the use of cattle urine, as well as the principal nitrogenous component urea, as a possible nutrient resource for *An. arabiensis*. In addition, cattle urine and urea are evaluated for their potential to enhance both sustained flight in adult females and the development of eggs and larvae. As the composition of cattle urine changes by fermentation over time, multiple ages of urine and concentrations of urea are analysed. The implications of cattle urine as an additional nutrient resource in the gonotrophic cycle of malaria mosquitoes, and its potential impact on vectorial capacity, are discussed.

5.2. Materials and Methods

5.2.1. Experimental Insect Rearing

A colony of *An. arabiensis*, Dongola strain, were maintained using a standard insect rearing procedure of 12 h:12 h light and dark cycle in a climate-controlled mosquito rearing chamber kept at 25 ± 2 °C, 65 ± 5 % RH. The rearing protocols for feeding and maintenance followed the same procedure as has been explained previously in Chapter 3. Prior to the feeding, flight performance and reproductive benefits bioassays, female mosquitoes were provided with 10 % sucrose solution *ad libitum* for 4 days, and then starved with *ad libitum* access to only distilled water for an assay-specific period described below.

5.2.2. Quantification of Urine and Urea meals taken by *Anopheles arabiensis* (Patton)

Two-choice feeding assays were used to quantify the consumption of urine and urea by *An. arabiensis* adult females, as described by Ignell *et al.* (2010), with some modifications. Prior to these bioassays, mosquitoes were starved for 24 h with only access to distilled water. Preliminary studies of gravid mosquitoes were analysed on number of eggs laid by surviving female *An. arabiensis* (Patton) fed on 10 % urine, 10 % urine plus 10 % sucrose, 1 % urine plus 10 % sucrose and 1 % urine alone. The results indicated that early ages of urine at 1 % have trends of increased egg laying female mosquitoes where the decision was made to use 1 % urine alone for further studies. All figures for these are attached in the Appendices B supplementary data. Then, a test solution of either 1 % urine (fresh, 24 h, 72 h and 168 h post excretion) or various concentrations of urea (2.69 µM, 26.9 µM, 134 µM, 269 µM, 1.34 mM, 2.69 mM, 13.5 mM, 26.9 mM, 135 mM or 269 mM), containing a known concentration of blue dye

(1 mg ml^{-1} Xylene cyanole FF; CAS number 2650-17-1; Sigma-Aldrich), was provided in a 4 × 8 matrix of 250 µl microfuge tubes (PCR-0208-C Axygen Scientific, Sweden) interspersed with a control solution of undyed distilled water. The concentration of urine used in this and the following experiments was determined by pilot experiments considering optimal survival and fecundity exhibited by gravid mosquitoes (Supplementary B. Figs.1-4). The microfuge tubes were filled to elicit a convex surface above the rim (approximately 300 µl) and alternating between tubes containing the dyed diet and the distilled water. While it has been determined that mosquitoes do not prefer the colour of xylene cyanol over that of other dyes (Ignell *et al*., 2010), it has not been determined whether the colour of xylene cyanol is preferred over that of clear water. To avoid the influence of the colour of the dye, 10 mosquitoes were placed, together with diet solutions, in complete darkness for 48 h consecutively in experimental enclosures (12 cm diameter, 6 cm height; Semadeni, Switzerland) at 25 ± 2 °C and 65 ± 5 % RH. Following exposure to the diets, the mosquitoes were placed at -20 °C until needed. To release the diet imbibed prior to quantification, mosquitoes were individually placed in 1.5 ml Eppendorf tubes with 230 µl of distilled water, and the tissues were disrupted using a disposable pestle and cordless motor (47747-370; VWR International, Sweden), and then centrifuged at 10,000 rpm for 10 min. To be able to determine the volume of dye imbibed, and thus to quantify the diet, a standard curve was prepared by a serial dilution (1:2 ratio) from 0.2 µl to 2.4 µl of 1 mg ml^{-1} xylene cyanol in the above-mentioned 1 % aged urine solutions and concentrations of urea. The supernatants (200 µl) and standards were orderly transferred into 96 wells microplate and the absorbance (λ620 nm) determined using a microplate reader spectrophotometer (SPECTROStar® Nano, BMG Lab Tech, Germany). Then, a linear regression was

produced from optical density values of known dye concentrations and used as a standard curve to estimate the volume of diet imbibed by each mosquito.

Two sets of feeding bioassays were carried out, one for flight performance and the other for reproductive benefits bioassays. For flight performance experiments, blood meal seeking female *An. arabiensis* on their 4th day were provided with a choice between 1 % fresh, 24 h, 72 h, and 168 h aged cattle urine, 10 % sucrose and distilled water. For reproductive benefits bioassays, blood feeding followed the same procedure as indicated in Chapter 3. Then, fully engorged females were offered to imbibe urea (CAS:57-13-6; Sigma) at 2.69 µM, 26.9 µM, 134 µM, 269 µM, 1.34 mM, 2.69 mM, 13.5 mM, 26.9 mM, 135 mM or 269 mM concentrations, diluted in distilled water. The highest concentration is equivalent to that previously reported in cattle urine (269 mM) (Bristow *et al*. 1992). Both urine and urea diets were mixed with blue dye (1 mg ml^{-1} Xylene cyanole FF).

5.2.3. Flight Performance Assessment of *Anopheles arabiensis* (Patton) fed on Cattle Urine and Urea

A custom-made mosquito flight-mill, based on Attisano *et al*. (2015), was designed in three main parts: a flight-mill, a data logger and a computer (Fig. 5.1.). The flight-mill was made from 5 mm thick clear acrylic panels (10 cm W, 10 cm L and 10 cm H) arranged into a box lacking front and back walls; a pair of neodymium magnets, held in parallel to each other, 9 cm apart, at the centre of the upper and lower walls of the box; a pivot assembly made from a worn-out gas chromatography column with the vertical tube (0.25 mm i.d; 7.5 cm L) glued to insect pins at both ends and suspended between the magnets; and a horizontal tube (0.25 mm i.d; 6.5 cm L) divided in half into a mosquito tethering arm and a photo interrupter arm, and glued in the centre

perpendicular to the vertical tube. The pivot assembly was held in between the magnets without being attached to either side creating a near frictionless motion, minimizing the weight to drag for the mosquito. For mosquito tethering, an insect pin was affixed to one arm of the horizontal tube and a small piece of aluminium foil to the other, to create both balance and an interruption signal for the sensor during revolution (Fig. 5.1.). A photo interrupter sensor recorded the flight activity each time the foil passed through an infrared beam. The data logger was customized to accommodate four flight-mill relays and to transfer the data to a computer for data interpretation, display and storage.

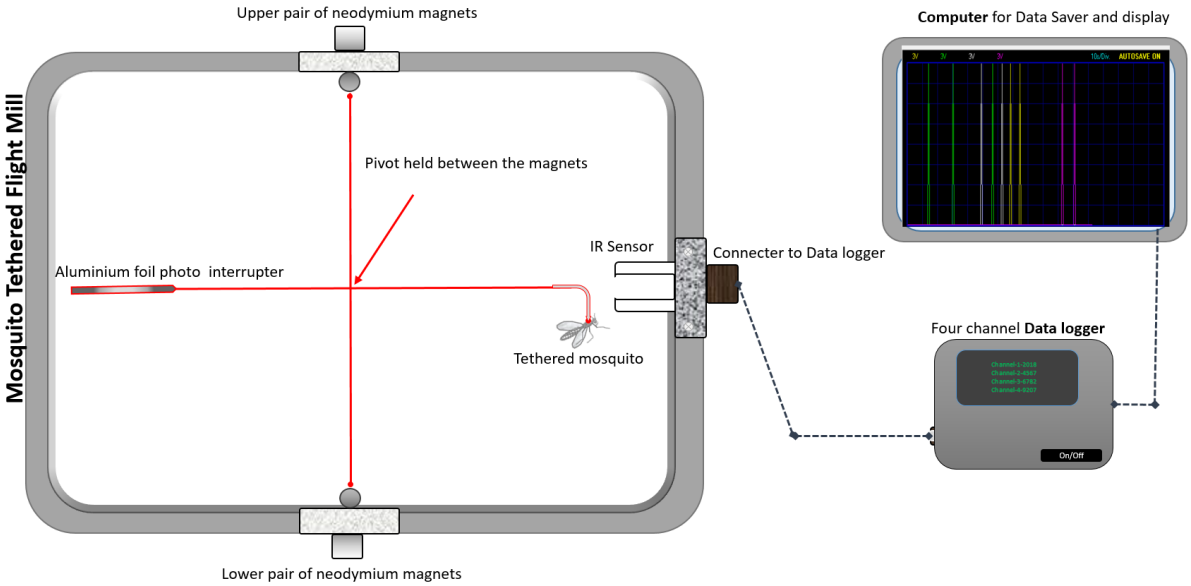

Figure 5.1. Schematic diagram of mosquito tethered flight-mill. The flight mill consists three main parts, a boxed in enclosure composed of acrylic containing a pivot axis, held between two pairs of neodymium magnets, and a horizontal rod, which holds a piece of aluminium foil at one end that triggers the infrared (IR) sensor

and a tethered mosquito at the other end. The signal from the IR sensor feeds into a data logger for display, storage and further analysis.

Host seeking female mosquitoes (4 dpe) were starved for 4-6 h with only access to distilled water. Prior to tethering, starved females, were provided an access for 30 min to diets containing either 10 % sucrose, water, urine (1 %; either fresh, 24 h, 72 h, or 168 h post-excretion), or urea (either 0.135 mM, 1.35 mM, 13. 5 mM or 135 mM). Fully fed female mosquitoes were individually anaesthetized on ice for 2-3 min and glued onto an insect pin by bee wax (Joel Svenssons Vaxfabrik AB, Munka Ljungby, Sweden) on their mesothorax and tethered onto the arm of the horizontal tube of the flight-mill. Each flight revolution was logged and displayed using the PC-Lab 2000^{TM} software (v4.01; Velleman, Belgium). Finally, the distance flown by each mosquito, as recorded from the distance measured from single revolution (20.41 cm) times number of total revolutions were translated in to total distance flown by each mosquitoes within a given time. An arbitrary categorization used to classify the flight of mosquitoes based on no fight, 100 m, 500 m, 1000 m and above 1000 m of the tethered flights. The flight mill was placed in a climate conditioned room (12 h: 12 h, light: dark, 25 ± 2 °C, 65 ± 5 % RH). Flight performance assays were conducted for a duration of 24 h without provision of any nutrient or water.

5.2.4. Experimental Procedures for Reproductive Performance of *Anopheles arabiensis* Patton Fed on Cattle Urine and Urea

Ten female *An. arabiensis* (4 dpe) were placed in Bugdorm cages (30 cm x 30 cm x 30 cm) after blood feeding, and then provided with experimental diets of urine (1 % of fresh, 24, 72 h or 168 h aged), urea (2.69 µM, 26.9 µM, 134 µM, 269 µM, 1.34 mM, 2.69 mM, 13.5 mM, 26.9 mM, 135 mM or 269 mM), or control diets (10 % sucrose or

water) for 48 h. Each diet regime was replicated 20 times. After the diets were removed, oviposition sites (clear plastic cups with 20 ml water) were provided for 48 h, replacing the cups every 24 h. The eggs that were laid were counted manually and recorded per surviving female for each experimental cage. Egg size (length in µm) was determine from subsamples of the eggs ($n \geq 200$ per diet) by placing the eggs on a microscope slide under a coverslip and photographing each egg individually using a Dialux-20 microscope (DM1000; Leitz Wetzlar, Germany) equipped with a Leica camera (DFC 320 R2; Leica Microsystem Ltd, Germany) and LAS software (v4; Leica Microsystem Ltd, Germany). The remaining eggs were maintained in a climate controlled chamber under standard rearing conditions for 24 h and a subsample of 1st instar larvae ($n \geq 230$ per diet) were then measured (length in µm as an approximation for overall size) following the same microscopy protocol as above.

5.2.5. Data Analysis

The assessments of the diet imbibed, eggs laid, and egg and larval sizes were analysed using a univariate analysis of variance (ANOVA) followed by a Dunnett's *post hoc* pair wise comparison between the treatments and the control (water), with the probabilities reported as multiple comparison adjusted p-values (*P*). All assessments of the proportion of females among different treatments in the feeding and flight performance assays were carried out using binominal logistic regression, followed by a Dunnett's *post hoc* pair wise comparison between the treatments and the control (water). All statistics were calculated using JMP Pro (v13.0.0, SAS Institute Inc., Cary, NC, 1989-2007).

5.3. Results

5.3.1. Quantification of Urine and Urea Imbibed by Blood Fed *Anopheles arabiensis*

Anopheles arabiensis females fed on cattle urine aged from fresh to 7 days (Fig. 5.2A) and on all concentrations of urea tested (2.70 µM to 270 mM; Fig. 5.2B) within 48 h post-blood meal. The highest proportion of females fed on fresh and 24 h aged urine ($\chi^2 = 23.3$, df = 3, $P < 0.0001$), with females imbibing the highest volume of 24 h aged urine ($F = 6.65$, $df_n = 3$, $df_d = 204$, $P = 0.0003$; Fig. 5.2A). Females imbibed the highest volume of urea (0.44-0.65 µl) over a wide range of concentrations (135 µM to 135 mM; Fig. 5.2B). The proportion of females feeding, however, demonstrated a bimodal distribution, with the lowest proportion feeding on 2.70 µM, 1.35 mM and 2.70 mM, with the highest proportion of females feeding on 270 µM, 135 mM, and 270 mM urea ($F = 9.11$, $df_n = 8$, $df_d = 211$, $P < 0.0001$; Fig. 5.2B).

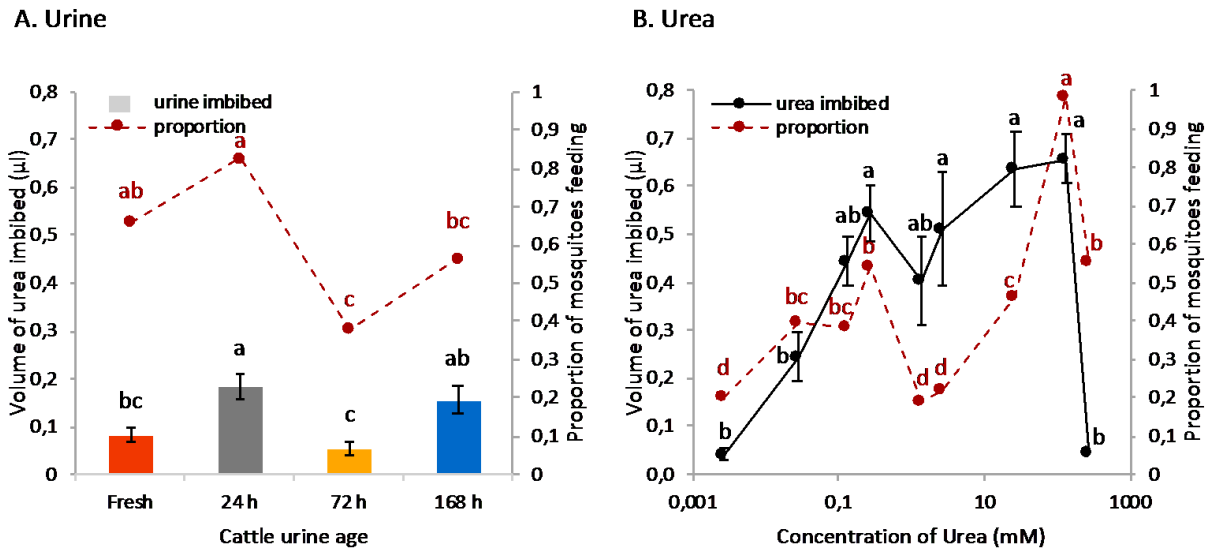

Figure 5.2. Amount and proportion of urine and urea imbibed by female *Anopheles arabiensis* (Patton) within 48 h of a blood meal. **A.** cattle urine (bars) incubated for various times post-excretion, and **B.** urea (black solid line) of various concentrations, with differing proportions (red dashed lines). Ages (cattle urine) and doses (urea) with the same letters in each group are not significantly different, whereas, all pair-wise combinations of data with different letters are significantly different for volume imbibed (black) and proportion (red; $P < 0.05$). Error bars represent the standard error of the mean.

5.3.2. Flight Performance of *Anopheles arabiensis* (Patton) Fed on Urine and Urea

Urine and urea feeding by 4 dpe host seeking females did not result in a similar flight performance as those fed on sugar or on water alone (Fig. 5.3). The proportion of females that flew <100 m was similar between those fed on fresh urine and those fed on sugar ($P = 0.27$), but these differed from those that fed on water and on aged urine ($P \leq 0.027$). Overall, the urine fed females either flew <100 m, or did not fly, with the exception of a few females fed on fresh urine that flew up to 1 km (Fig. 5.3A). The

proportion of females that fed on urea at concentrations from 1.35 mM and above, and flew <100 m, was similar to that of females fed on sugar and water. However, unlike sugar- and water-fed host-seeking females, no urea-fed females flew longer than 100 m ($\chi^2 = 19.24$, $df = 5$, $P = 0.0017$; Fig. 5.3B).

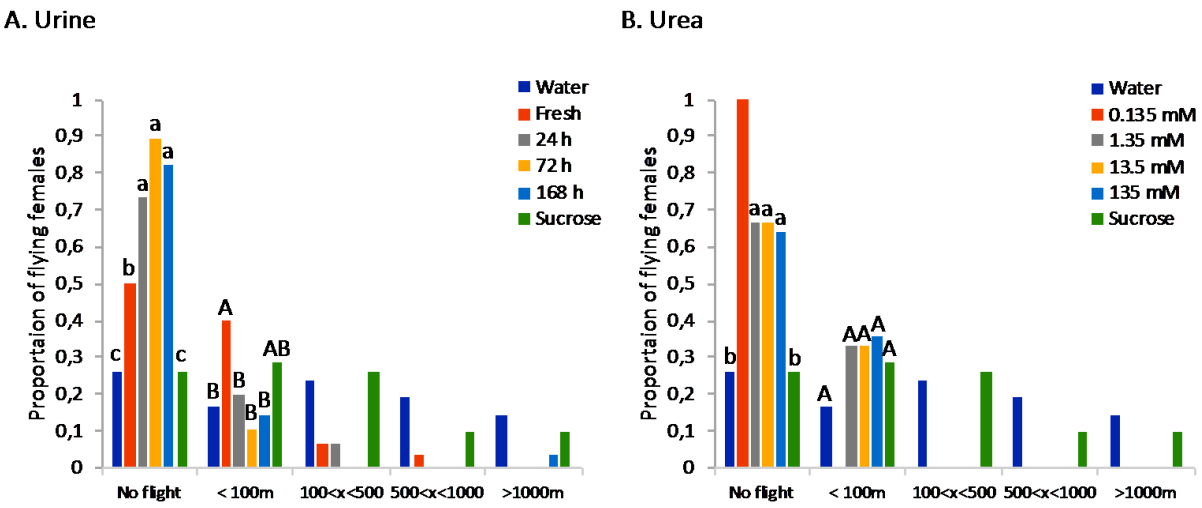

Figure 5.3. Proportions of host-seeking females (4 days post-emergence) fed on **A.** cattle urine and **B.** urea, with water- and sugar-fed controls, that fly different distances on a tread mill. Ages (cattle urine) and doses (urea) with the same letters in each group are not significantly different, whereas, all pair-wise combinations of data with different letters are significantly different ($P < 0.05$).

5.3.3. Reproductive Performances of *Anopheles arabiensis* Patton Fed on Urine and Urea

Female *An. arabiensis* fed on aged urine post-blood meal did not significantly differ in the number of eggs laid from those that fed on either water or 10 % sucrose ($F = 1.137$, $df_n = 5$, $df_d = 119$, $P < 0.345$; Fig. 5.4A) at 0.05 α level. Moreover, the proportion of urine-fed egg laying mosquitoes did not differ from the controls ($\chi^2 = 4.005$, $df = 5$, $P = 0.54$; Fig. 5.4A). The number of eggs laid by female *An. arabiensis* was influenced

by feeding on urea within 48 h after a blood meal ($F = 7.24$, $df_n = 11$, $df_d = 474$, $P < 0.0001$; Fig. 5.4B). The number of eggs laid by females that fed on moderate concentrations of urea (0.135-13.5 mM) increased, similar to that observed for females fed on 10 % sucrose, compared to those with access to only water (Fig. 5.4B black). The proportion of females that laid eggs also increased when fed on a moderate concentration of urea (0.135-0.270 mM), compared with water (Fig. 5.4B red). At the highest concentration of urea tested (269 mM), the proportion of mosquitoes laying eggs was lower than that for all other concentrations of urea.

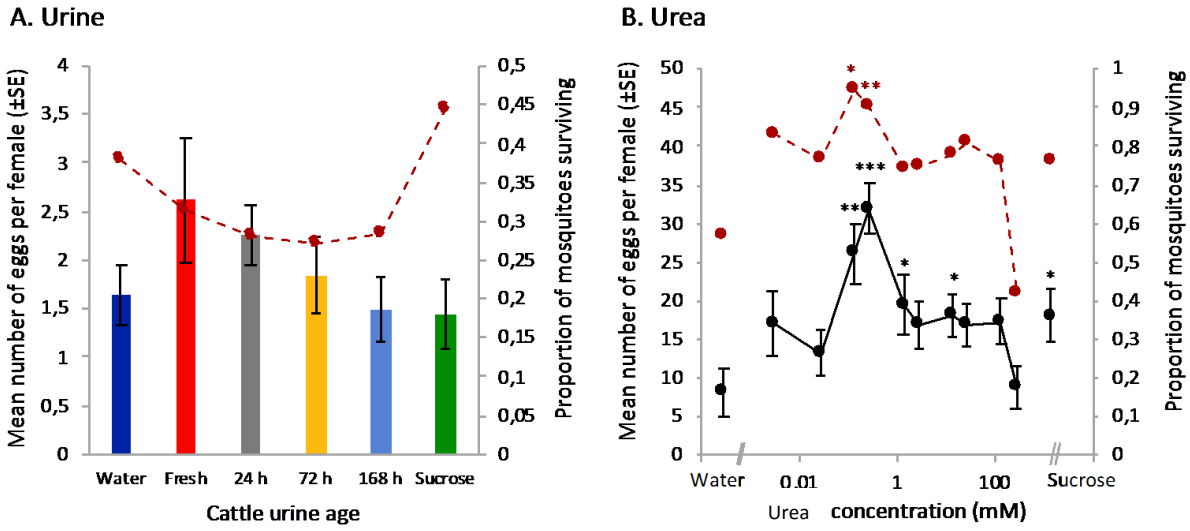

Figure 5.4. Number of eggs laid by females fed on **A.** cattle urine (bars) and **B.** urea (black solid line) from differing proportions of females laying eggs (red dashed line). Ages (cattle urine) and doses (urea) with the same letters in each group are not significantly different, whereas, all pair-wise combinations of data with different letters are significantly different for number of eggs laid (black) and proportion of females laying eggs (red; $P < 0.05$).

Interestingly, female mosquitoes that fed on urine laid eggs with different sizes depending on the age of urine ($F = 12.85$, $df_n = 5$, $df_d = 209$, $P < 0.0001$; Fig. 5.5A).

Blood fed mosquitoes, which were subsequently fed on urine 24 h post-excretion, laid longer eggs (P = 0.0001) than those laid by females that had access to distilled water alone. These eggs had a similar length to those of sugar-fed females (Fig. 5.5A). In contrast, shorter eggs were laid when 168 h post-excretion urine was provided (P = 0.023; Fig. 5.5A). Moreover, sugar-fed and urea-fed female *An. arabiensis* laid longer eggs than those with access to water ($F = 42.72$, $df_n = 11$, $df_d = 4668$, $P < 0.0001$) for all treatments excepting the lowest concentration of urea tested (Fig. 5.5B). Indeed, the concentrations of urea that resulted in the highest numbers of eggs laid, corresponds with those which resulted in the longest eggs, with the exception of the 270 mM urea which resulted in the fewest eggs, but among the longest (Figs 5.4B and 5.5B).

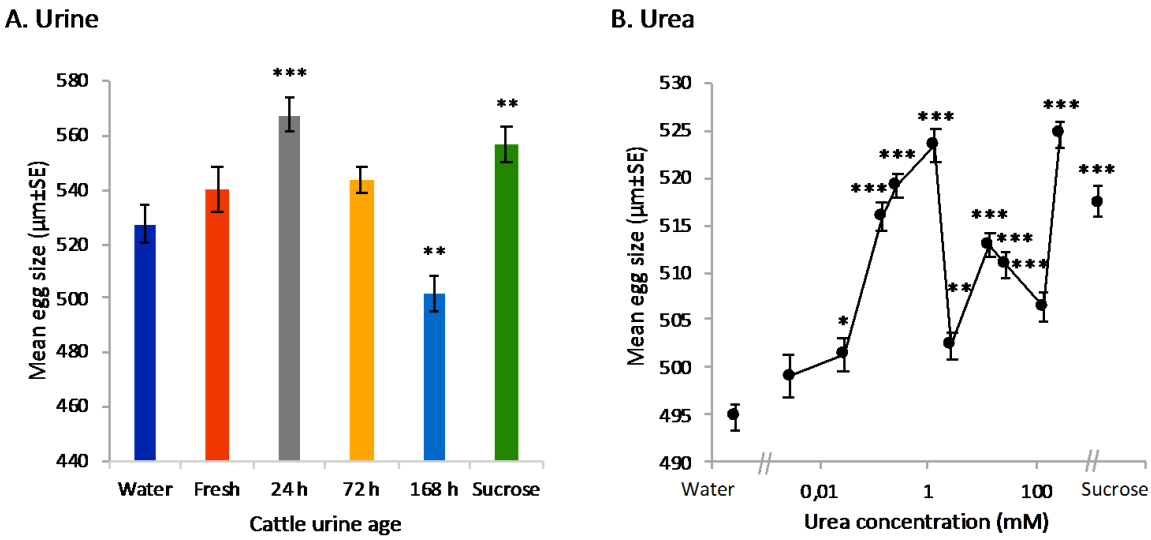

Figure 5.5. Size of the eggs laid by females fed on **A.** cattle urine (bars) and **B.** urea (black solid line). All pair-wise combinations of data with those from water-fed females are significantly different egg sizes when marked with asterisks (* $P <$ 0.05; ** $P < 0.01$; *** $P < 0.001$).

Furthermore, 1st instar larvae that emerged from these eggs, siblings to those which were assessed for length above, revealed that larval sizes followed a similar

pattern to that of the eggs from females fed on urine ($F = 7.86$, $df_n = 5$, $df_d = 187$, $P < 0.0001$, Fig. 5.6A), and those fed on urea ($F = 39.32$, $df_n = 11$, $df_d = 3639$, $P < 0.0001$; Fig. 5.6B). The longer eggs that were laid in response to feeding on 24 h aged urine, hatched into longer 1st instar larvae. Whereas the eggs laid in response to 72 h aged urine meals were not significantly larger in size than those from the water controls, the 1st instar larvae were longer ($P \leq 0.0001$). While the eggs from females fed on 168 h aged urine meals were smaller in size than those from the water-fed controls, this did not appear to influence the length of 1st instar larvae (Fig. 5.6A). While the larger eggs appeared to result in larger 1st instar larvae at concentrations of urea at and above 13.5 mM, the size of the larvae was more variable at concentrations between 0.135 mM and 2.70 mM (Fig. 5.6B).

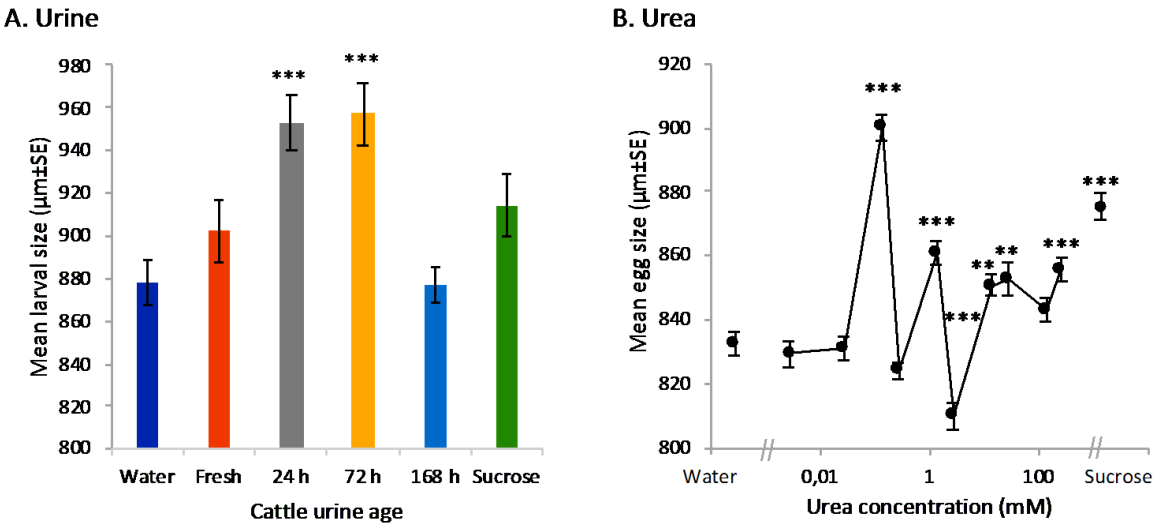

Figure 5.6. Larval size hatched from eggs laid by females *Anopheles arabiensis* (Patton) fed on **A.** cattle urine (bars) and **B.** urea (black solid line). All pair-wise

combinations of data with those from water-fed females are significantly different larval sizes when marked with asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

5.4. Discussion

Female *An. arabiensis* are attracted to cattle urine that has been deposited within the last 24 h (Mahande *et al*., 2010; Kweka *et al*., 2011; Chapters 3 and 4) and are able to use nitrogen-rich resources for both energetic and reproductive processes (Foster, 1995; Naksathit *et al*., 1999a; Briegel *et al*., 2001a; 2001b; Kaufmann *et al*., 2013a; 2013b). This suggests that cattle urine may act as both a host habitat cue and a resource for the mosquito. Both host seeking and gravid mosquitoes will imbibe cattle urine and urea at concentrations approximating those of fresh to 48 h post-deposition urine from common livestock animals (Bristow *et al*., 1992; Thomas *et al*., 1988). Post-ingestion, few to no host-seeking females flew longer distances (>100 m) in contrast with the water-fed and sugar-fed mosquitoes, indicating that these mosquitoes are likely not using the nitrogen-rich urine or urea as an energetic resource. Gravid mosquitoes, however, laid bigger eggs, which developed into bigger larvae, after imbibing on the cattle urine 24 h post-deposition, and urea equivalent concentrations thereof, indicating that females make use of these nitrogen-rich resources for reproduction. These findings suggest that cattle urine is an abundant potential nutrient resource for malaria mosquitoes, which may indeed affect the vectorial capacity of *An. arabiensis*.

Malaria mosquitoes feeding on urine might be a supplement to the nitrogen-rich blood feeding, in an otherwise sugar-rich adult diet, and might correlate with the preference of feeding on varied quality and quantity of carbohydrates and proteins sources (Ignell *et al*., 2010) to balance their C:N intake and metabolism (Arrese and Soulages, 2010; Hood-Nowotny *et al*., 2012). Cattle urine constitutes an abundant

nitrogen resource in the environment of a malaria mosquito. Recently deposited cattle urine contains 100-700 mM urea, 60-95 % of its total nitrogen content, and very little ammonia (Bristow *et al*., 1992). Once deposited on soil, the urea in livestock urine is rapidly degraded into ammonia by microbial action over 24-48 h (Thomas *et al*., 1988), making recently deposited urine a source of available nitrogen resources for use by the mosquito. Malaria mosquitoes have the ability to use to the nitrogen derived from urea and ammonia as an energetic resource to fuel sustained flight (Scaraffia and Wells, 2003; Scaraffia *et al*., 2010). Host-seeking mosquitoes invest energy in flights in search for preferred, or at least available, hosts within the habitat (Killeen *et al*., 2001; Sumba *et al*., 2004; Burkett-Cadena *et al*., 2013; Majeed *et al*., 2016). Urea and ammonia are converted by *Ae. aegypti* midguts into glutamine and alanine, both of which can then be transported to and used by the fat body (Scaraffia *et al*., 2010). It is important to note that, while proline is a product of ammonia detoxification in the fat body, the midgut does not make use of the same metabolic pathway to fix the nitrogen from ammonia into amino acids, and it is the amino acid proline that is the amino acid used as energy during sustained flight in *Ae. aegypti* (Scaraffia and Wells, 2003). The lack of proline biosynthesis by the midgut likely underpins the finding here, that host-seeking *An. arabiensis* did not appear to use the imbibed urine or urea to increase the energy invested in sustained flight. In fact, fewer females engaged in extended flight (>100 m) compared to those which were either starved or sugar fed. This might be interpreted as females that imbibe cattle urine remain in the local habitat, thereby increasing the chance of encountering a suitable host (Cardé, 2015). In a broader context, this suggests that imbibing substances from the host habitat influences the behaviour of host seeking mosquitoes to use the strategy of ´sit and wait´(Webster *et al*., 2015).

The nitrogen-rich urine and urea meals, though not providing additional energy to sustained flight, appeared to supply the nutrients needed to lay more eggs, in response to urea meals, and larger eggs, in response to both recently deposited urine and urea meals, resulting in larger first instar larvae. Up to one third of post-blood meal nitrogen is used for the synthesis of yolk protein and lipids in malaria mosquitoes, while another 15 % is deposited as extra-ovarian protein and lipids stores (Briegel, 1990). Subsequent post-blood meal sugar and blood meals have been shown to further increase these egg and maternal protein and lipid stores, which proves increasingly important to females in suboptimal habitats, or with a history of suboptimal diets (Kloweden *et al*, 1988; Stone *et al*., 2011; Smykal and Raikhel, 2015). This study demonstrates that blood fed *An. arabiensis* can use the nutrients in cattle urine, likely the large urea fraction, in a similar fashion, supplementing the yolk proteins and lipids in their eggs. The accumulated nutrient reserves in the eggs of their offspring likely to lead to an increased fitness (France and Judson, 1979; Foster, 1995; Clifton and Noriega, 2012), providing energy stores during the larval stages and the teneral adults, for locomotion/flight, as well as stores available for reproduction (Arrese and Soulages, 2010; Kaufmann *et al*. 2013a).

This study has described cattle urine, abundant in the malaria mosquito's habitat, as a nitrogen-rich nutrient resource available to female *An. arabiensis*, and revealed that imbibing urine, or its predominant nitrogen component urea, altered flight and enhanced reproductive performances of these mosquitoes. Future studies investigating further habitat-related sources for energy or reproduction enhancing substrates will provide an opportunity to discover more habitat factors that affect the survival and reproductive fitness malaria mosquitoes, and ultimately discover novel control strategies (Webster and Cardé, 2017).

Chapter 6 – Conclusions and Recommendations

6.1. Conclusions

The survival and reproduction of malaria mosquitoes greatly depend on their ability to locate and exploit various resources (Takken and Verhulst, 2013; Sumba *et al*., 2004). In doing so, the mosquitoes are challenged due to the patchy and spatiotemporal distribution of resources in the heterogeneous landscape (Cardé, 2015). To overcome such challenges, searching mosquitoes need to make choices at increasingly refining spatial scales, a process that can be broken down to habitat finding, resource finding, resource recognition, and resource acceptance in which they locate their resources (Sumba *et al*., 2008; Sanford and Tomberlin, 2011; Stone *et al*., 2011; Webster *et al*., 2015). The olfactory system plays an important role for most of these behaviours (Zwiebel and Takken, 2004; Ignell and Hansson, 2004). This study expanded on previous findings by Kweka *et al*. (2011) and Mahande *et al*. (2010), revealing that *An. arabiensis* may use cattle urine as a host-habitat cue to locate potential hosts and oviposition sites. Moreover, this study further explored the direct use of cattle urine as a source of nitrogen, and how this affects flight duration and reproductive rate, as well as egg and larval sizes.

Fresh and aged cattle urine was found to differentially attract host-seeking and gravid *An. arabiensis.* The mechanism underlying this behavioural response was found to be due to a change in the detected chemical volatile profile of aging urine, as revealed through GC-EAD and GC-MS analyses. Behavioural analysis further revealed that blends of bioactive compounds, at their natural ratios and release rates, was able to reproduce the behavioural response to cattle urine. The complexity of bioactive compounds in the odour blends emanating from the cattle urine decreased with age. The

presence of large amounts of phenolic derivatives, including 3- and 4-methyl phenol, caused by the microbial metabolism of tyrosine in the urine (Murry and Adams, 1988), likely explain the lack of behavioural response to aged urine. In contrast, both host-seeking and gravid *An. arabiensis* were found to be attracted to blends of bioactive compounds identified in fresh and 24 h aged urine, under both laboratory and field conditions. This observation contrasts with that found for other hematophagous insects, which have been shown to be attracted to the odour of aged urine (Den Otter, 1991; Madubunyi *et al*., 1996), and emphasises that malaria mosquitoes may use fresh cattle urine as a reliable signal for the presence of blood hosts. The results from the field evaluation of the synthetic blend of 24 h aged cattle urine are interesting, and indicate the potential of this odour to be used in future monitoring and control of *An. arabiensis*, as well as other mosquito species.

Female *An. arabiensis* also make use of cattle urine as a nutrient source for energy and reproduction. Cattle urine is rich in nitrogenous compounds, and feeding on urea has similar effects on energetic flight and reproduction as feeding on urine. These effects were dependent on the age of the urine, and the concentration of urea. A possible explanation for the observed consumption on urine and urea is that mosquitoes use this resource to supplement their diet in the absence of other types of nitrogenous compounds or sugar (Chang and Judson, 1979; Nepi *et al*., 2012). However, evidence presented in this study indicates that mosquitoes may not use urine and urea directly as fuel energy, rather alter their expenditure of their glycogen reserves, in which they metabolise glycogen to proline. Proline, is also a by-product of the detoxification of ammonia, which is another nitrogenous compound found in urine, and has been shown to be used as an energy resource for sustained flight in *Ae. aegypti* (Scaraffia and Wells, 2003; Scaraffia *et al*., 2010).

In summary, the identification of a host-habitat substance that attract host-seeking and gravid malaria mosquitoes provides important information about the ecology of these insects, and identifies a target that can be used for future mosquito control measures.

6.2. Recommendations

- ✓ While the current study has allowed for the identification of a volatile blend, which attracts mosquitoes at various physiological stages, this blend may be further improved by the addition of other volatiles present in urine that failed to be detected by available methods.
- ✓ Further field evaluation of current and future synthetic blends of cattle urine volatiles is required to assess optimal concentration and formulation of the blend, as well as to identify the proper trapping device.
- ✓ Semi-field and field studies are required to further verify the role of cattle urine as a host habitat cue.
- ✓ Further analysis of urine constituents other than urea is needed to assess how these affect survival and reproduction.

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Appendices

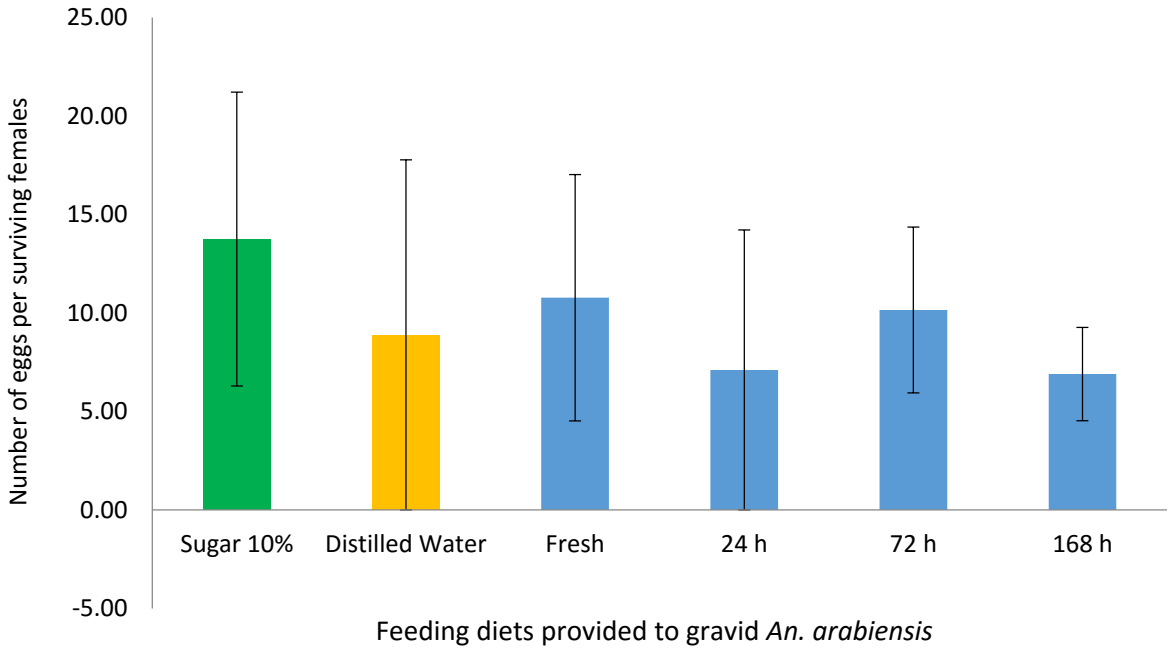
Appendix A. Glossary

The following definitions and concepts for the following terms were are used in this dissertation

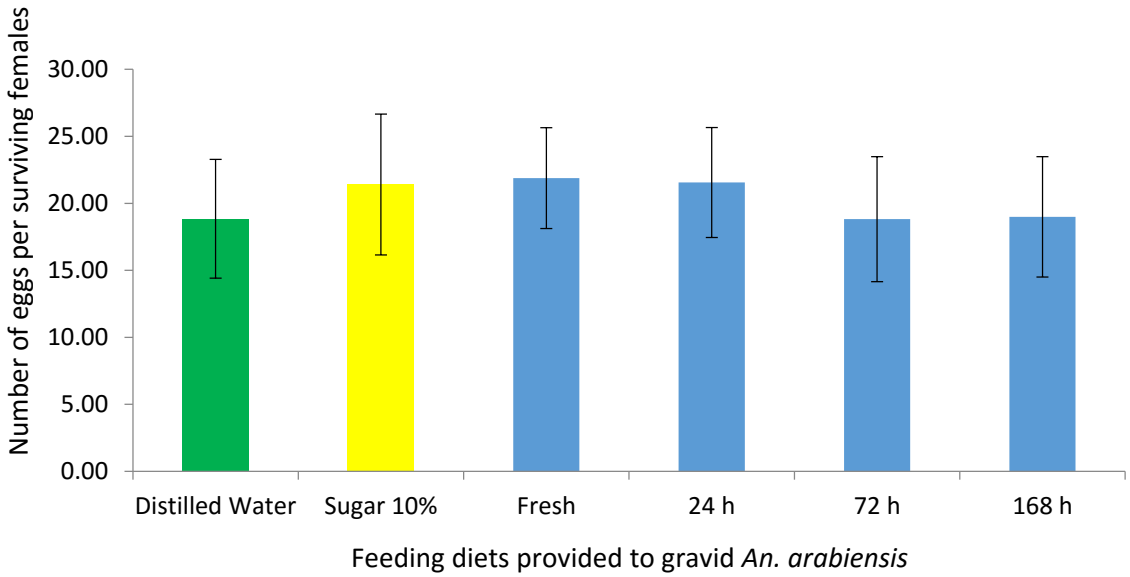
- Habitat defined as any environment that can potentially harbour adult and immature stages of mosquitoes. Based on the availability of the resources e.g. nectar and extra-floral nectaries, blood hosts and oviposition sites; distribution and abundance of malaria mosquitoes found to be determined. For instance, 90 % of anopheline adults were found in houses within 300 meters from the nearest larval habitats (Minakawa *et al*., 2002). Furthermore, to some extent, habitat influences the distribution of hosts (Burkett-Cadena *et al.,* 2013).
- Hosts refers to organisms that can provide resources e.g. flowering plants and mammals that provide malaria mosquitoes with carbohydrate or proteinaceous nutrients.
- Host- habitat can be taken as an environment where hosts exist and can be considered in the presence and absence of hosts
- Host cues include body emanations, breath, excretory substances releasing odorous volatiles, body appearances and coloration used for visual identifications, body heat and other related features of hosts.
- Habitat cues in addition to cues displayed by hosts, any stimuli that can be used by mosquitoes to identify and discriminate hosts in the habitat originating from living or non-living matter. And in this study the collective volatile emanations from hosts and non-hosts in a potential foraging patch is taken as habitat cues (Webster and Cardé, 2016).

- Host-habitat cue with vague demarcation between host cues and habitat cues; host-habitat cues include cues that can represent and used to distinguish the function of the stimuli, source of the volatile cues, quantities emitted, detectability, specificity and behaviours elicited (Webster and Cardé, 2016).
- Mosquito habitat can be demarcated from the behavioural exhibition perspective e.g. aquatic larval feeding habitat, adult mosquito sugar feeding spaces, mating environments, host searching and feeding localities, blood digesting associated with resting sites, oviposition sites that can secure the survival and reproduction of malaria mosquitoes.
- Fitness for the ease of this study, defined as the quantity and quality viable offspring produced and their comparative phenotypic appearances of *An. gambiae* s.l. e.g. survival of adult mosquitoes, number of eggs, sizes of eggs and 1st instar larvae.

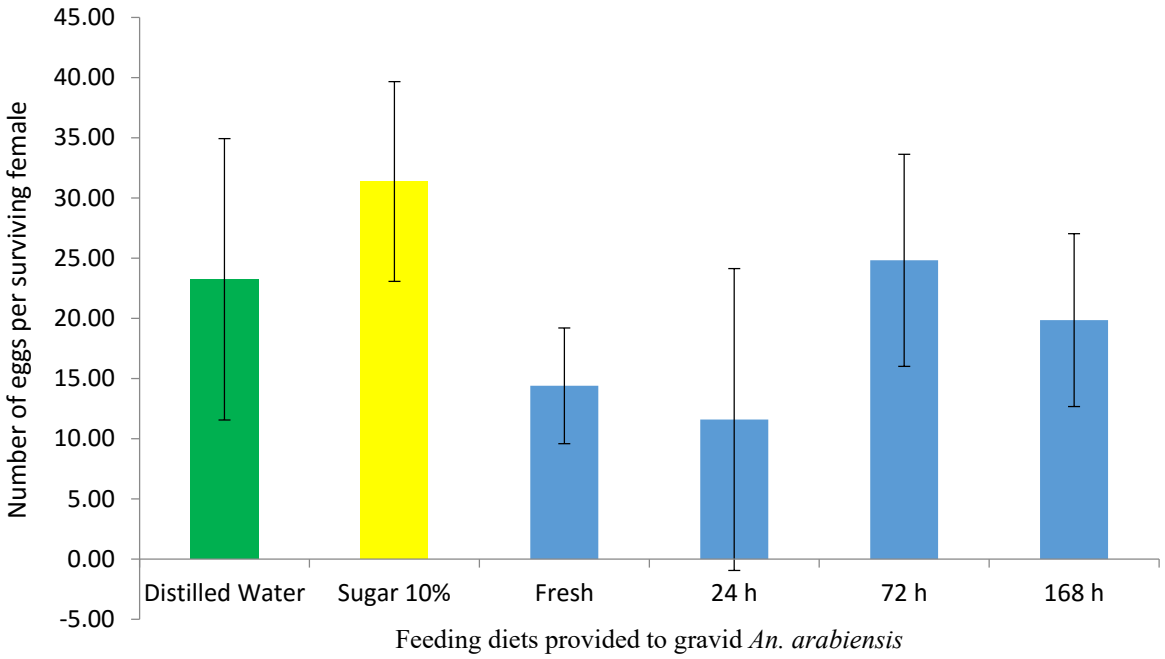
Appendix B. Supplementary Data



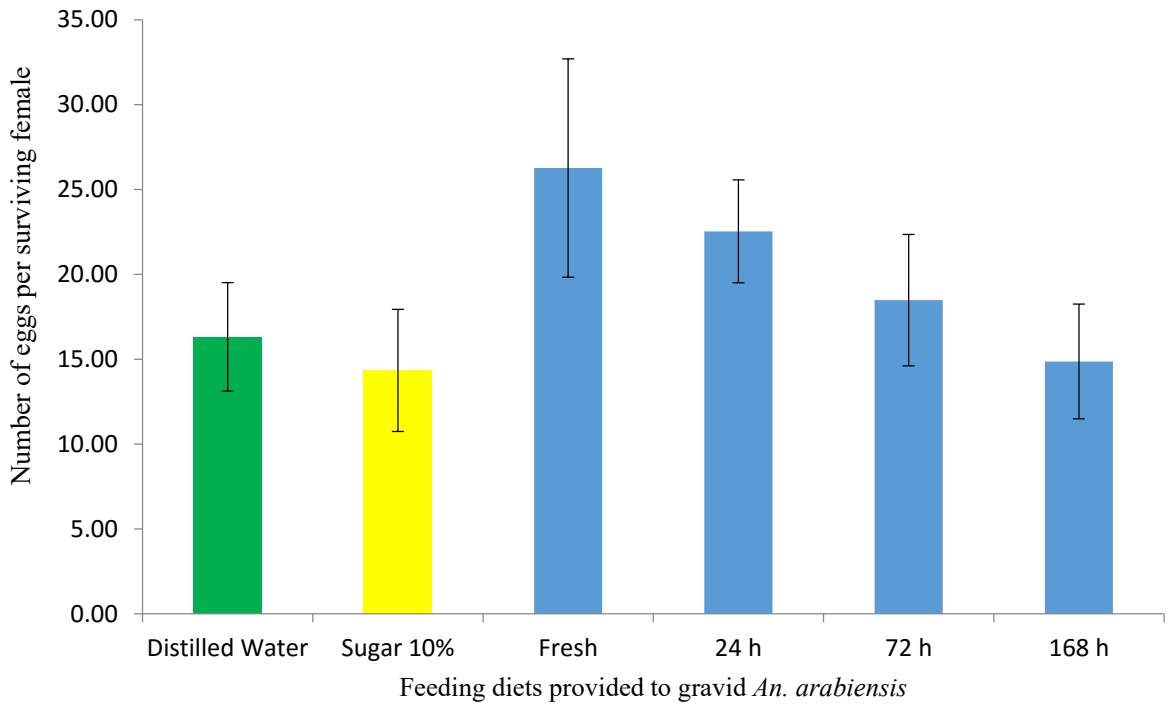
Supplementary Figure 1. Mean ± SEM number of eggs laid by surviving female *An. arabiensis* fed on fresh and aging 10 % cattle urine diluted in distilled water. Eggs were counted 72 h post-feeding on meal diets provided. For each diet, the survival and number of eggs laid were assessed for 10 gravid females in parallel, and replicated 10 times.



Supplementary Figure 2. Mean ± SEM number of eggs laid by surviving female *An. arabiensis* fed on fresh and aging 1 % cattle urine mixed with 10 % sucrose in a 1:1 ratio diluted in distilled water. Eggs were counted 72 h post-feeding on meal diets provided. For each diet, the survival and number of eggs laid were assessed for 10 gravid females in parallel, and replicated 20 times.



Supplementary Figure 3. Mean ± SEM number of eggs laid by surviving female *An. arabiensis* fed on fresh and aging 1 % cattle urine mixed with 10 % sucrose in a 1:1 ratio diluted in distilled water. Eggs were counted 24 h post-feeding on meal diets provided. For each diet, the survival and number of eggs laid were assessed for 10 gravid females in parallel, and replicated 10 times.



Supplementary Figure 4. Mean ± SEM number of eggs laid by surviving female *An. arabiensis* fed on fresh and aging 1 % cattle urine alone diluted in distilled water. Eggs were counted 24 h post-feeding on meal diets provided. For each diet, the survival and number of eggs laid were assessed for 10 gravid females in parallel, and replicated 20 times.

Declaration

I, the undersigned, declare that this doctoral thesis is my original work and has not been presented in any other University, College or Institution, seeking for a similar degree or other purposes. All source of materials used for the thesis have been duly acknowledged.

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Date: September 21, 2018

This doctoral dissertation has been submitted for examination with my approval as a university advisor.

Dr. Habte Tekie

Signature _____

Date _____