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Oviposition site selection in mosquitoes

The role of conspecific larvae, heterospecific larvae and microbes

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Cover: *Aedes aegypti* oviposition
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Oviposition site selection in mosquitoes: The role of conspecific larvae, heterospecific larvae and microbes

Abstract

In mosquitoes, the evaluation and selection of oviposition sites is critical for the growth, development and survival of the offspring. Gravid mosquitoes rely primarily on olfactory cues emanating from breeding water containing, *e.g.*, intra- and interspecific aquatic stages, as well as bacteria for this purpose. In this thesis, I investigated the odour-mediated mechanisms regulating the oviposition preferences of *Aedes aegypti* and *Culex quinquefasciatus* to intra- and interspecific aquatic stages and the commensal *Klebsiella* sp., bacterium. Using multi- and dual-choice oviposition assays, in combination with chemical and electrophysiological assays, volatile organic compounds (VOCs) associated with intraspecific aquatic stages of *Ae. aegypti* were identified and shown to regulate oviposition site choice and egg laying in a stage- and dose-dependent manner **(Paper II)**. Using a similar approach, oviposition site selection by *Ae. aegypti* and *Cx. quinquefasciatus* were shown to be regulated by hydrocarbons emitted by interspecific 4th instar larvae, emphasising that these VOCs are able to regulate niche separation and competitive exclusion between these species **(Paper III)**. The bacteria, *Klebsiella* sp., constitutes a major food resource for *Ae. aegypti* larvae, and gravid mosquitoes are attracted to this resource and the VOCs emitted by the bacteria **(Paper IV)**, which strengthens their commensal interaction. This thesis identified the odour-mediated mechanisms by which mosquitoes detect intra- and interspecific aquatic stages, as well as mosquito-associated bacteria. Furthermore, results of this study provide functional evidence that hydrocarbons, which previously have not been shown to regulate odour-mediated behaviours in mosquitoes, play an important role in regulating oviposition. Besides, adding to our understanding of factors regulating oviposition preference in mosquitoes, this thesis has identified VOCs that may be evaluated under field conditions for their assessment as complementary vector control tools.

Keywords: *Aedes aegypti*, *Culex quinquefasciatus*, egg-laying site selection, volatile organic compounds, intraspecific, interspecific, behaviour, microorganisms, niche separation, competitive exclusion

Dedication

To my parents, first and second family

“Never give up, no matter how hard life gets no matter how much pain you feel. Pain will eventually subside, nothing remains forever, so keep going and don't give up”

Imran Khan

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Zaid Khan**, Rickard Ignell, R. Sharon R. Hill, 2022. Chapter 14: Odour-mediated oviposition-site selection by mosquitoes. In *Sensory Ecology of Disease Vectors* (pp. 373-417). Wageningen Academic Publishers, Wageningen, the Netherlands.

II. **Zaid Khan**, Björn Bohman, Rickard Ignell, Sharon R. Hill, (2023). Odour-mediated oviposition site selection in *Aedes aegypti* depends on aquatic stage and density. *Parasites & Vectors*, *16, 264*

III. **Zaid Khan**, Betelehem Wondwosen, Björn Bohman, Rickard Ignell, Sharon Rose Hill (2023). Hydrocarbons associated with interspecific larvae shape niche segregation and competitive exclusion in *Aedes aegypti* and *Culex quinquefasciatus* (manuscript).

IV. Katherine D. Mosquera*, **Zaid Khan***, Betelehem Wondwosen, Beatrix Alsanius, Sharon R. Hill, Rickard Ignell, Marcelo G. Lorenzo, (2022). Odour-mediated response of gravid *Aedes aegypti* to mosquito-associated symbiotic bacteria. *Acta Tropica*, 237, 106730.

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Paper II is an open access article.
*Equal contribution.

The contribution of Zaid Khan to the papers included in this thesis was as follows:

I. Drafted the initial manuscript and co-wrote the final draft with the co-authors.

II. Designed the study together with the co-authors, performed all the experimental work, data collection and data analyses, with consultation with the co-authors. Drafted the manuscript with the input of the co-authors.

III. Designed the study together with the co-authors, established and performed all of the experiments, data collection and statistical analyses for the behavioural assays. Analysed the physiological and chemical data produced. Drafted the manuscript with the input of the co-authors.

IV. Designed the part on mosquito oviposition behaviour with synthetic blend together with the co-authors. Performed all experimental workflow and data analysis for this part. Contributed to the final paper by providing feedback.

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Abbreviations

ANOVA	Analysis of variance
CFU	Colony forming unit
GC-EAD	Gas chromatography-electroantennogram detection
GC-FID	Gas chromatography-flame ionization detection
GC-MS	Gas chromatography-mass spectrometer
IR	Ionotropic receptors
OBPs	Odorant binding proteins
OR	Odorant receptor
Orco	Odorant receptor co-receptor
OSNs	Olfactory sensory neurons
SPE	Solid phase extraction
SPME	Solid phase microextraction
TSB	Tryptic soy broth
VOCs	Volatile organic compounds
WHO	World health organisation

1. General introduction

Of the 3586 known mosquito species in the world, approximately 200 are considered important vectors of human diseases (Yee *et al*., 2022). According to the World Health Organisation (WHO), 80% of people worldwide, especially in tropical and subtropical regions, are at risk of contracting one or more arboviral disease (WHO, 2017, 2022). Arboviral diseases are classified as neglected tropical diseases by the WHO (WHO, 2020). One example is dengue, which infects more than 3.9 billion people in over 129 countries each year (Bhatt *et al*., 2013; WHO, 2022). Another disease is West Nile fever, which is endemic in Europe, Africa, East Asia and North America, and which caused an estimated 126,000 cases of infection in Europe during its last outbreak (Andreadis, 2012; McMullen *et al*., 2011; Nasci & Mutebi, 2019; Paz & Semenza, 2013). These diseases are increasing in intensity and expanding in their geographic distribution due to factors that include insecticide resistance and their adaptation to lay eggs in man-made breeding sites in urban and peri-urban areas (David *et al*., 2018; Lima *et al*., 2011; Karunaratne *et al*., 2018; Talipouo *et al*., 2021; Thanispong *et al*., 2008).

Oviposition is an important component of vectorial capacity, as this behaviour is intimately correlated with mosquito reproduction, and thus population size (Liang *et al*., 2015). For this reason, an effective control strategy for vector management and surveillance by targeting gravid mosquitoes is required to reduce arboviral transmission (Wooding *et al*., 2022). One such strategy is to target the odour-mediated behaviour of mosquitoes (Takken & Knols, 1999). Mosquito species are attracted and stimulated to lay eggs in response to volatile organic compounds (VOCs) emitted from potential breeding sites in a habitat (Khan *et al*., 2022). However, until now, only a limited number of behaviourally active VOCs have been identified that significantly influence oviposition of gravid

mosquitoes (Afify & Galizia, 2015; Khan *et al*., 2022, 2023; Mwingira *et al*., 2020a; Wooding *et al*., 2020).

When female mosquitoes leave the habitat where they have taken a blood meal and rested, females must make decisions over multiple spatial levels by orienting themselves, as well as searching for and accepting potential oviposition sites in a habitat (Alcalay *et al*., 2019; Hopkins, 2022). Deciding where to lay eggs is an important factor in regulating offspring growth and development, as immature aquatic stages are unable to move to a suitable breeding site when conditions become adverse (Silberbush *et al*., 2019; Vonesh & Blaustein, 2010). The selection of a breeding site by gravid mosquitoes relies mainly on biotic, *i.e.*, intra- and interspecific competitors, predators and food resources, mainly microbiota, as well as abiotic factors, *i.e.,* physical and chemical factors, and their interaction (Alfonzo *et al*., 2005; Blaustein & Chase, 2007; Blaustein *et al*., 2004; Dhileepan, 1997; Do Nascimento *et al*., 2022; Khan *et al*., 2022; Mutero *et al*., 2004; Xia, 2021a). To do so, gravid mosquitoes use multiple cues, such as vision and taste, but primarily rely on odour cues emanating from the oviposition site (Afify & Galizia, 2015; Day, 2016; Dhileepan, 1997; Khan *et al*., 2022, 2023; Mwingira *et al*., 2020a; Wooding *et al*., 2020). This thesis provides an up-to-date overview of the odour mediated cues associated with the biotic factors regulating oviposition site selection in mosquitoes, with a focus on the sympatric species *Aedes aegypti* and *Culex quinquefasciatus.*

2. Background

2.1 Mating

Mating is a critical for reproduction in order to increase fitness and maintain species populations (Charlwood & Jones, 1980; Takken *et al*., 2004). Male mosquitoes emerge earlier than females and become sexually mature within 18-24 h, while it takes 1-3 days for females (Clements, 1999). After adult emergence, mosquitoes rest and then fly in search of flower nectaries to replenish their nutrient reserves to gain energy for flight (Foster, 1995) and mating (Oliva *et al*., 2011). *Aedes aegypti* copulate within 2-3 days after adult emergence (Degner & Harrington, 2016). Copulation between pairs occurs in a position in which the genitalia interlock, during which time the semen is transferred in less than 1 min (Degner & Harrington, 2016). Females of *Ae. aegypti* and *Anopheles* spp. have been shown to mate in swarms of flying males, often consisting of a few to many thousands of mosquitoes (Oliva *et al*., 2014; Wang *et al*., 2021, 2023).

Mosquitoes use both auditory (Feugere *et al*., 2021, 2022) and may or may not use chemical cues to attract both sexes to mate in a swarm (Fawaz *et al*., 2014; Mozuraitis *et al*., 2020; Poda *et al*., 2022; Wang *et al*., 2021, 2023). Both sexes are sensitive to auditory cues and swarm in the air to mate (Feugere *et al*., 2021; Su *et al*., 2018). Auditory cues, which are generated by the beating of male wings, are used by females to enter into the swarm (Feugere *et al*., 2021;2022; Su *et al*., 2018). Other researchers have suggested that swarm formation in some mosquito species is mediated by chemical cues. For example, in *Ae. aegypti* aggregation pheromones have been suggested to attract both sexes to swarm and mate (Fawaz *et al*., 2014).

In addition, the chemical cues have been proposed in *Anopheles* mosquitoes *e.g.*, *Anopheles gambiae* and *Anopheles coluzzii* exhibit swarming behaviour at specific times of the day (Mozuraitis *et al*., 2020; Wang *et al*., 2021, 2023). This behaviour is circadic and regulated by clock genes, entrained by light and temperature, which in turn regulate the expression of the desaturase gene *desat1* regulating the production of heptacosane and induce mating activity (Wang *et al*., 2021). After mating the female becomes refractory to further mating and, shortly thereafter, the search for a host to obtain a blood meal to continue to develop the fertilised eggs in the ovaries (Chapman, 2009; Lima-Camara *et al*., 2014).

2.2 Host seeking and blood feeding

Most mosquito species are anautogenous, *i.e.,* require a blood meal from a vertebrate host to obtain protein for egg development and maturation (Clements 1999; Reeves *et al*., 2018; Tempelis, 1975; Verhulst *et al*., 2018). After adult emergence, female mosquitoes require 1-3 days for the mouthparts and other organs to develop in order to take a blood meal (Alto *et al*., 2003; Armstrong & West 1965; Clements, 199). Therefore, the blood-feeding activity of mosquitoes increases with the age of the female, correlating with the tendency of female mosquitoes to search for hosts (Davis, 1984; McCann *et al*., 2009; Omondi *et al*., 2019).

Most anautogenous mosquitoes are generalists that may feed opportunistically on a distinct range of hosts, which can include, *e.g.,* humans, livestock, birds and amphibians (Edman, 1968; Reeves *et al*., 2018; Tempelis, 1975; Verhulst *et al*., 2018). In mosquitoes, generalist behaviour may evolve, when the probability of finding a particular host species is low, or the fitness benefits of feeding on a limited number of available hosts in a habitat are low (Lyimo & Ferguson, 2009). In contrast, some mosquito species are specialists in terms of host preference, such as *Ae. aegypti*, which prefers to take a blood meal from humans (Bernier *et al*., 2002; Geier *et al*., 2007; Takken & Verhulst, 2013). The reason for such a preference of mosquitoes could be that host species are abundant and provide an additional fitness advantage. For example, the isoleucine content of human blood has been associated with an increased egg production, fitness and energy reserves in *Ae. aegypti* (Harrington, 2001; Harrison *et al*., 2021). The high

preference of *Ae. aegypti* for humans as hosts may also be due to the fact that this species is able to find favourable locations near human dwellings to benefit from the man-made water containers in which female deposits eggs (Rose *et al*., 2020). Females that have taken a blood meal are inhibited to seek hosts by two endogenous mechanisms, firstly by distention-induced inhibition resulting from the activation of abdominal receptors following the engorgement of a blood meal, and secondly by oocyte-induced inhibition, as a consequence of egg development (Brown *et al*., 1994; Duvall *et al*., 2019; Klowden & Lea 1979a, 1979b). The female mosquito becomes refractory to host odour until after oviposition (Edman *et al*., 1975; Klowden & Briegel, 1994; Klowden & Lea, 1978, 1979b; Takken *et al*., 2001). The onset of oviposition begins 2-3 days after the complete digestion of a blood meal (Klowden & Blackmer, 1987).

2.3 Oviposition site selection

Egg-laying site selection is an important stage in the life cycle of mosquitoes and a critical event for the growth, development and survival of the next generation, as well as for maintaining population dynamics (Bentley & Day, 1989; Day, 2016; Khan *et al*., 2022). The decision of where to lay their eggs is crucial for female fitness, as immature aquatic stages are unable to switch breeding sites if the situation becomes adverse. According to life history theory, female mosquitoes must choose a site that increases fitness by promoting offspring growth and development, as well as reducing the mortality rate (Silberbush *et al*., 2019; Vonesh & Blaustein, 2010). As a result, the choice of oviposition site location is dependent on the availability of nutrients for the larvae and the degree of competition and predation at the breeding site.

When searching for a potential oviposition site, female mosquitoes may travel long distances, and is required to make decisions across multiple spatial scales (Hopkins, 2022). For this, mosquitoes may use both long-range (<10 m; Carde, 2015) and short-range volatile cues emanating from potential breeding sites (Afify & Galizia, 2015; Day, 2016; Khan *et al*., 2022). Gravid mosquitoes have been demonstrated to respond to both water vapour and VOCs when selecting an oviposition site (Afify & Galizia 2015; Khan *et al*., 2022, 2023; Lindh *et al*., 2015; Mwingira *et al*., 2020a; Okal *et al*., 2013;

Wondwosen *et al*., 2016, 2017, 2018; Wooding *et al*., 2020). The quality of these cues varies widely and differentially affects the oviposition site selection of mosquitoes (Afify & Galizia 2015; Khan *et al*., 2022).

Gravid females primarily use short- medium- and long-range odour signals emanating from the breeding sites to select an oviposition site (Afify & Galizia, 2015; Carde, 2015; Day, 2016). For example, odours emanating from conspecific and heterospecific aquatic stages of mosquitoes, as well as the aquatic stage conditioned water, attract and stimulate mosquitoes to lay eggs (Allan & Kline, 1998; Ganesan *et al*., 2006; Gonzalez *et al*., 2014; Khan *et al*., 2023; Laurence & Pickett 1982, 1985; Mendki *et al*., 2000; Ong & Jaal, 2015; Seenivasagan *et al*., 2009; Shragai *et al*., 2019; Wachira *et al*., 2010; Zahiri & Rau, 1998; Zahiri *et al*., 1997). Moreover, water vapour is a strong pre-egg-laying attractant to potential breeding sites, signalling gravid females to locate water sites over short- to medium-ranges of 15-20 cm and 60 cm distances, respectively (Carde, 2015; Okal *et al*., 2013; Raji *et al*., 2019). Once mosquitoes come into contact with the substrate for laying eggs, females detect short-range humidity levels, and may also use gustatory cues to assess the water quality, to make the ultimate yes-or-no decision of ovipositing eggs (Laursen *et al.*, 2023).

Different terms are often used to describe the response of gravid mosquitoes to VOCs encountered in various oviposition assays. An “oviposition attractant" is a volatile chemical that elicits gravid mosquitoes to fly towards the target source used for oviposition, while a "stimulant" induces egg laying after the female has landed on the substrate, using contact stimuli. A “repellent” can be described as a volatile chemical compound that induces mosquitoes to avoid or move away from the oviposition site, and a “deterrent” induces an avoidance of oviposition by gravid mosquitoes using contact chemoreception (Afify & Galizia, 2015; Day, 2016; Khan *et al*., 2022). I will use this terminology throughout the thesis.

The oviposition site and the surrounding environment are complex, and a gravid mosquito needs to detect and discriminate among relevant VOCs in a noisy chemical background in the environment (Afify & Galizia 2015; Khan *et al*., 2022; Mwingira *et al*., 2020a). This thesis discusses the identity and role of behaviourally relevant VOCs emitted from breeding sites by

conspecific and heterospecific aquatic immature stages of mosquitoes, as well as mosquito-associated microorganisms, in regulating oviposition site choice in two important disease vectors, the yellow fever mosquito *Ae. aegypti* and the southern house mosquito *Cx. quinquefasciatus*.

3. Factors affecting oviposition site selection

Gravid mosquitoes are required to evaluate both abiotic and biotic factors when selecting a site for oviposition in a habitat (Day, 2016; Xia, 2021a; Xia *et al*., 2021b), as these factors may have a significant effect on the development and survival of their offspring (Christophers, 1960; Dom, 2019; Lounibos, 1981). Various mosquito species may occupy a wide variety of breeding sites depending on the niche requirements of the individual species (Gimnig *et al*., 2001; Lounibos, 1981; Xia *et al*., 2021b), emphasising that different species may have evolved different preferences and strategies to cope with various physical and chemical environments, as well as the potential limitations set by some biotic factors. In this section, I provide an overview of the factors regulating the choice of gravid mosquitoes where to lay their eggs.

3.1 Abiotic factors

The chemical and physical factors in the environment, which impact living organisms in terms of growth, reproduction and survival, are known as abiotic factors. Abiotic factors, such as the moisture in and around the breeding habitat as well as the temperature, may significantly affect the abundance of aquatic immature stages (Custódio *et al*., 2019; Do Nascimento *et al*., 2022; Talaga *et al*., 2020; Yang *et al*., 2014). These two abiotic factors can be subdivided to include *e.g.*, humidity, salinity and rainfall, as well as temperature and shade, which are presented in this section in relation to how these influence the oviposition site choice and egg-laying response of gravid mosquitoes (Do Nascimento *et al.*, 2022; Wong *et al*., 2011).

3.1.1 Humidity and Salinity

Gravid mosquitoes are able to detect the humidity associated with a breeding site and the chemical composition of the water, including salinity (Canyon *et al.,* 1999; Matthews *et al*., 2019; Ramasamy & Surendran, 2012; Saifur *et al*., 2010; Xia, 2021a). Gravid mosquitoes prefer to lay eggs in areas with high humidity to ensure that water is available in the breeding sites throughout the development of the aquatic stages (Canyon *et al.,* 1999; Saifur *et al*., 2010). High humidity provides an environment that encourages egg laying, while also inducing mosquitoes to lay more eggs in a short period of time (Canyon *et al*., 1999; Madeira *et al*., 2002; Okal *et al*., 2013; Saifur *et al*., 2010). In addition, moisture supports egg hatching, larval development and pupae to emerge as adults (Canyon *et al*., 1999; Madeira *et al*., 2002; Okal *et al*., 2013; Raji *et al*., 2019; Saifur *et al*., 2010). In contrast, low humidity has a negative effect on oviposition (Canyon *et al.,* 1999; Saifur *et al*., 2010). Gravid mosquitoes retain eggs for a few days under low humidity conditions, to protect the eggs from the stress of dehydration (Canyon *et al*., 1999; Saifur *et al*., 2010). After egg-laying, *Ae. aegypti, Ae. albopictus* and *Ae. vexans* eggs require sufficient levels of humidity for 24-48 h, but after this the eggs can tolerate the stress of low relative humidity (42%) (Canyon *et al*., 1999; Christophers, 1960; Sota & Mogi, 1992). After 48 h, the outer shell of the eggs, otherwise known as the chorion, becomes hard and egg hatching can be delayed for up to 6-8 months (Canyon *et al*., 1999; De Almeida Costa *et al*., 2010; Saifur *et al*., 2010; Wang *et al*., 2016). An increase in evaporation from a breeding site decreases the water volume, which accidentally will increase the salinity level, which also affects the oviposition site choice of mosquitoes (Ramasamy & Surendran, 2012).

The salinity level in breeding sites can determine the type of mosquito species that uses the site for oviposition (Balasubramanian & Nikhil, 2015; Matthews *et al*., 2019; Ramasamy & Surendran, 2012). For example, the salt-tolerant mosquitoes, *Culex sitiens*, and *Culex tritaeniorhynchus*, are adapted to tolerate a high salinity level (Balasubramanian & Nikhil, 2015; Ramasamy & Surendran, 2012), while *Ae. aegypti,* and *Ae. albopictus* select oviposition sites with low salinity, *i.e.,* fresh water, and avoid saline water breeding sites (Gunathilaka *et al*., 2018). For *Ae. aegypti* and *Ochlerotatus taeniorhynchus*, an increase in salinity decreases larval growth rate, development and lengthens the time to reach to the pupal stage, which is not

the case for the species adapted to high salinity levels (Albers *et al*., 2011; Clark *et al*., 2004). The underlying reason for this is that larvae that are not adapted to high salinity levels have to expend a significant amount of energy for ionoregulation to reach to the adult stage, resulting in small adults with low fecundity (Clark *et al*., 2004; Ramasamy *et al*., 2011). Gravid mosquitoes use different mechanisms to detect the humidity and saline levels at and around a breeding site.

Mosquitoes detect the moisture level through humidity-sensing sensilla located on the last segment of the antenna (Laursen *et al*., 2023), whereas the salinity level in a breeding site is assessed by sensory neurons on the legs and proboscis after the mosquito makes contact with the water during oviposition site evaluation (Matthews *et al*., 2019). *Aedes aegypti* and *An. gambiae* express a conserved ionotropic receptor, *Ir93a*, that is critical for the detection of humidity as gravid females search for a breeding site (Laursen *et al*., 2023). The *AaIr93a* allows gravid *Ae. aegypti* to locate potential breeding sites by detecting water vapor plumes emanating from these, but is not needed for egg laying once the female arrives at the oviposition site (Laursen *et al*., 2023). Mutant females, in which the function of *AaIr93a* has been knocked out, fail in their search for an oviposition site (Laursen *et al*., 2023). The cation channel *ppk301*, which is expressed in legs (tarsi) and proboscis, and is activated by both fresh and saline water, however, regulates the decision of egg deposition upon contact with water (Matthews *et al*., 2019). The data presented and discussed above reveal that oviposition involves multiple sensory pathways. How these and other pathways, including olfactory and gustatory, interact require further studies.

3.1.2 Rainfall

Rainfall impacts oviposition site choice and the number of eggs laid per gravid female by limiting the flight activity and dispersion of mosquitoes when searching for suitable breeding sites (Dhimal *et al*., 2015). In the event of heavy rainfall, existing aquatic immature stages may be flushed out of the breeding sites, which affects the local population abundance of *e.g.*, container breeding mosquito species, such as *Ae. aegypti* (Paaijmans *et al.*, 2007; Paul *et al*., 2018). In addition, strong rainfall may change the concentration of nutrients, *e.g.*, ammonia, at the breeding sites due to the physical disturbance of the water, which might affect the oviposition

preference of gravid mosquitoes (Chaves & Kitron, 2011; Nguyen *et al*., 2014). More limited rainfall, on the other hand, generates new breeding sites or refills existing ones, which increases the available choices for gravid mosquitoes (Dhimal *et al*., 2014). For example, a high abundance of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* have been reported during the post-monsoon rainy season, suggesting that light rainfall is attributing to the peak abundance of these species (Dhimal *et al*., 2014). Thus, oviposition is strongly associated with rainfall-induced regimes shift (Nguyen *et al*., 2014), and results in fitness benefits for the next generation of mosquitoes (Chaves & Kitron, 2011).

3.1.3 Temperature

Temperature is an important factor affecting egg laying and egg production of mosquitoes (de Almeida Costa *et al*., 2010; Yang *et al*., 2009). Most mosquito species, *e.g*., *Ae. aegypti*, *Ae. albopictus*, and *Cx. pipiens, Cx. quinquefasciatus* and *Cx. restuans* as well as *An. gambiae* prefer an average temperature range between 20-30°C for oviposition (Christiansen-Jucht *et al*., 2015; Ciota *et al.*, 2014; Do Nascimento *et al*., 2022; Mayne, 1926; Neto & Navarro-Silva, 2004; Santos *et al*., 2020). Gravid *Aedes* species, *e.g.*, *Ae. aegypti* and *Ae. albopictus*, oviposit more eggs in a temperature range of 25-28 °C. When the temperature falls outside the optimal range, mosquitoes lay significantly fewer eggs (Ciota *et al.*, 2014). At lower temperatures, female *Ae. aegypti* may also delay oviposition or may not be able to produce mature eggs (Yang *et al*., 2009). In contrast, an increase in temperature shortens the development time of the offspring, resulting in small adults with reduced fitness (Alto & Juliano, 2001; Christiansen-Jucht *et al*., 2015; Mayne, 1926). Moreover, female mosquitoes use the energy gained from the blood meal for survival rather than for egg production under adverse conditions (Alto & Juliano, 2001; De Almeida Costa *et al*., 2010; Nayar, 1972).

3.1.4 Shade

Gravid mosquitoes select breeding sites in a habitat on the basis of shade and light intensity (Cardo *et al*., 2018; Strickman, 1982; Vezzani & Albicócco, 2009). Many female culicine mosquitoes are stimulated to lay more eggs in a shaded breeding site (Strickman, 1982). For example, *Ae. aegypti* and *Aedes vexans* select and colonise breeding sites in zones that provide shade

for most of the day, which tend to be cooler and more humid (Cardo *et al*., 2018; Strickman, 1982; Xia, 2021a). The shaded breeding sites are often present in the vicinity of trees, which protect the mosquito aquatic immature stages from extreme temperatures, as well as decrease water evaporation from the breeding sites (Cardo *et al*., 2018). This preference of mosquitoes for shaded breeding sites can also provide secondary benefits in terms of increased nitrogen enrichment from detritus in the breeding sites (Nguyen *et al*., 2014). Not all culicine mosquitoes, however, prefer to lay eggs in shaded areas. For example, high densities of *Culex pipiens* larvae are found in containers exposed to direct sunlight, which hold warmer water than those in the shade, and in which the immature stages grow and develop at a faster rate with a reduced larval mortality (Carrieri *et al.,* 2003).

3.2 Biotic factors

Biotic factors are the living organisms, such as plants, animals and microorganisms, in the environment, that may influence the selection of an oviposition site by gravid mosquitoes (Blaustein, 1999; Diaz-Nieto *et al*., 2016; Khan *et al*., 2023; Merritt *et al*., 1992; Zahiri *et al*., 1997). In this section, I provide an overview of the major biotic factors associated with a breeding site and how these, and the associated cues, affect the behaviour of a female mosquito searching for eggs-laying sites.

3.2.1 Oviposition cues associated with conspecific and heterospecific aquatic stages

In the aquatic environment, the aquatic stages are restricted in their movement and thus the development and survival of the offspring relies on the correct decision of female mosquitoes (Bentley & Day, 1989; Day, 2016; Kohandani *et al*., 2017; Xia *et al*., 2021). For this, female mosquitoes are required to evaluate the level of intra- and interspecific competition (Allan & Kline, 1998; Gonzalez *et al*., 2015; Khan *et al*., 2023; Schoelitsz *et al*., 2020; Suh *et al*., 2016; Zahiri *et al*., 1997). This subsection addresses our current understanding of the sources and chemical nature of VOCs identified from the aquatic stages of mosquitoes.

3.2.1.1 Cues associated with eggs

Mosquito species are able to detect and respond differentially to cues associated with eggs (Allan & Kline, 1998; Ganesan *et al*., 2006; Khan *et al*., 2023; Laurence & Pickett, 1982, 1985; Ong & Jaal, 2015; Roberts, 2021; Wang *et al*., 2019). Behavioural analyses have demonstrated that culicine mosquitoes respond to VOCs associated with conspecifics and heterospecific eggs in a density-dependent manner (Allan & Kline, 1998; Dhileepan, 1997; Khan *et al*., 2023; Nakamura, 1978, Onyabe & Roitberg, 1997; Roberts, 2021; Wachira *et al*., 2010; Williams *et al*., 2008). In contrast, anopheline mosquitoes avoid oviposition sites containing eggs of conspecifics (Sumba *et al*., 2008), through a yet undescribed mechanism, but some species prefer oviposition sites with heterospecific eggs (Wachira *et al*., 2010). The reason for this could be that *Anopheles* do not prefer the conspecific in order to avoid competition, the larvae use the same food source, but prefer the heterospecific that indicate a productive oviposition site and use the strategy of niche partitioning to avoid competition.

Culicine mosquitoes can modulate their oviposition and are influenced by the density of pre-existing eggs (Allan & Kline, 1998; Apostol *et al*., 1994; Chadee *et al*., 1990; Khan *et al*., 2023; Nakamura, 1978; Wachira *et al*., 2010; Wasserberg *et al*., 2014; Williams *et al*., 2008). Culicine mosquitoes use both pheromones and other VOCs when assessing oviposition sites containing intraspecific aquatic stages (Allan & Kline, 1998; Bruno & Laurence, 1979; Ganesan *et al*., 2006; Khan *et al*., 2023; Laurence & Pickett, 1982, 1985). The major component of the mosquito oviposition pheromone (MOP), erythro-6-acetoxy-5-hexadecanolide, has been identified from the maternally-deposited apical droplet of *Cx. quinquefasciatus* eggs (Bruno & Laurence, 1979; Laurence & Pickett, 1982, 1985). The MOP alone is capable of attracting and stimulating egg laying in *Cx. quinquefasciatus,* and other *Culex* species, including *Cx. pipiens molestus* and *Culex tarsalis*, to oviposition sites (Bruno & Laurence, 1979; Fytrou *et al*., 2022; Hwang *et al*., 1987). However, when comparing the lowest effective dose of MOP, the attraction to the pheromone was 100 times higher in *Cx. quinquefasciatus* than *Cx. tarsalis* (Hwang *et al*., 1987), indicating a species-specific response to this pheromone, and that additional pheromone components are likely required to elicit a response in *Culex* species. Thus, future behavioural analysis is required to assess the role of the other active fractions obtained

from the apical droplets of *Culex* eggs, including methyl esters, fatty acids and fatty acid esters (Starratt & Osgood, 1972).

So far, no specific oviposition pheromone has been identified to be associated with eggs in the genus *Aedes*. However, a number of VOCs have been identified to be associated with *Ae. aegypti* eggs, which regulate oviposition site selection and egg laying in a dose-response manner (Boullis *et al*., 2021; Ganesan *et al*., 2006; Hwang *et al*., 1982; Khan *et al*., 2023; Ong & Jaal, 2015). Available data indicate that, *Ae. aegypti* respond to a wide range of chemical classes, including long-chain aliphatic acids and methyl esters, straight short-chain aldehydes and alkenes, as well as monoterpenes, associated with eggs and egg-conditioned water from conspecifics (Boullis *et al*., 2021; Ganesan *et al*., 2006; Hwang *et al*., 1982; Khan *et al*., 2022, 2023; Ong & Jaal, 2015). Compared to *Aedes*, *Culex* species have shown an oviposition preference to more limited classes of chemical compounds (Laurence & Pickett, 1982, 1985).

3.2.1.2 Cues associated with larvae

The choice of oviposition site by gravid mosquitoes is based on the level of intraspecific (Allan & Kline, 1998; Boullis *et al*., 2021; Gonzalez *et al*., 2015; Khan *et al*., 2022, 2023; Mokany & Shine, 2003; Mwingira *et al*., 2020b; Soman & Reuben, 1970; Suh *et al*., 2016; Sumba *et al*., 2008; Wong *et al*., 2011; Xia, 2021a; Zahiri & Rau, 1998; Zahiri *et al*., 1997) and interspecific larval competition (Allan & Kline, 1998; Gonzalez *et al*., 2015; Khan *et al*., 2022; Rey & O'Connell, 2014; Shragai *et al*., 2019; Wachira *et al*., 2010; Zahiri *et al*., 1997) at the breeding site, a choice that has been demonstrated to be odour-mediated. Gravid mosquitoes regulate oviposition site choice depending on the presence of intra- and interspecific immatures at various developmental stages and densities in a species-dependent manner (Dhileepan, 1997; Gonzalez *et al*., 2015; Khan *et al*., 2023; Mwingira *et al*., 2020b; Shragai *et al*., 2019; Zahiri & Rau, 1998). In addition, female mosquitoes avoid egg laying in breeding sites where infected larvae are present (Zahiri & Rau, 1998; Zahiri *et al*., 1997), suggesting that larvae release VOCs that deter females from ovipositing.

Mosquitoes prefer to lay eggs in water where conspecific larvae are present (Allan & Kline, 1998; Boullis *et al*., 2021; Fonseca *et al*., 2015; Khan *et al*.,

2023; Mwingira *et al*., 2020b; Onyabe & Roitberg, 1997; Soman & Reuben, 1970; Shragai *et al*., 2019; Wachira *et al*., 2010; Xia, 2021a; Zahiri & Rau, 1998; Zahiri *et al*., 1997). Oviposition site choice of gravid mosquitoes in response to odours associated with intraspecific larvae is regulated in accordance with species- and taxon-specific strategies (Boullis *et al*., 2021; Faierstein *et al*., 2019; Gonzalez *et al*., 2014; Khan *et al*., 2023; Mwingira *et al*., 2021). For example, gravid *Ae. aegypti* and *Culex annulirostris* are attracted to water containing conspecific 4th instar larvae (Dhileepan, 1997; Khan *et al*., 2023), whereas *Anopheles* mosquitoes select oviposition sites containing conspecific 1st instar larvae (Mwingira *et al*., 2020b). The ecological rationale that may explain the culicine response to 4th instar larvae is that the late-stage larvae will soon transform to the non-feeding pupal stage and thus not compete with young larvae hatching from the laid eggs (Dhileepan, 1997; Khan *et al*., 2023). In addition, the 4th instars likely indicate that the breeding site contains sufficient food resources for the young larvae, as a result of the previous oviposition event modifying the microbial community (Bedhomme *et al*., 2005; Wong *et al*., 2011). *Anopheles* mosquitoes avoid breeding sites containing 4th instar larvae, which preys on the 1st instar larvae of the same species (Koenraadt & Takken, 2003; Shoukry, 1980). Thus, mosquito taxa use different mechanisms to evaluate potential oviposition sites, which are regulated by the volatile profile emitted from the breeding water, which changes over time due to larval development (Khan *et al*., 2023; Schoelitsz *et al*., 2020). Both culicine and anopheline mosquitoes often select oviposition sites that contain interspecific larvae (Shragai *et al*., 2019; Wachira *et al*., 2010; Zahiri *et al*., 1997).

The cues associated with heterospecific larvae regulate the oviposition site choice of gravid mosquitoes in a density- and species-specific mechanisms (Khan *et al*., 2022; Paper III; Shragai *et al*., 2019; Wachira *et al*., 2010; Zahiri *et al*., 1997). Restricted space and food promote interspecific competition and affect the feeding mode of the weaker species (Costanzo *et al*., 2005; Koenraadt *et al*., 2004; Santana-Martínez *et al*., 2017). As a result, this affects the development and survival of larvae, body size and emergence of adults (Ahmad *et al*., 2022; Araujo *et al*., 2012; Kweka *et al*., 2012; Schneider *et al*., 2004). Over time, the dominant species will replace the weaker one in a habitat, through a mechanism referred to as competitive

exclusion (Costanzo *et al*., 2005; Santana-Martínez *et al*., 2017). As a result, gravid mosquitoes rely on VOCs associated with the density of interspecific larvae to reduce competition among larvae (Paper III; Shragai *et al*., 2019; Wachira *et al*., 2010). For example, *An. gambiae* and *Ae. albopictus* select oviposition sites in a habitat where there are a low number of larvae of *Cx. quinquefasciatus* and *Ae. aegypti*, respectively (Shragai *et al*., 2019; Wachira *et al*., 2010).

In interspecific interactions, *Aedes* species *i.e.*, *Ae. aegypti* and *Ae. albopictus*, are considered dominant species that actively evaluate the level of competition and rapidly convert food resources into biomass, resulting in faster growth and development, which inadvertently depletes food resources in a short period of time, leading to competitive exclusion of other species, *e.g.*, members of the *Cx. pipiens* species complex, from oviposition sites (Allgood & Yee, 2014; Santana-Martínez *et al*., 2017). In contrast, gravid members of the *Cx. pipiens* species complex use a different strategy known as niche separation to avoid the egg-laying sites containing competitors, when possible (Burke *et al*., 2010; Costanzo *et al*., 2005; Leisnham *et al*., 2014; Marini, 2017). Moreover, females of the *Cx. pipiens* species complex may occupy breeding sites earlier in the season (Burke *et al*., 2010; Costanzo *et al*., 2005; Leisnham *et al*., 2014; Marini, 2017) or use other food resources that are available in the breeding site (Costanzo *et al*., 2005; Skiff & Yee, 2014).

Culicine mosquitoes respond to VOCs associated with conspecific and heterospecific larvae in a dose-dependent and taxon-specific manner (Afify & Galizia., 2015; Dormont *et al*., 2021; Khan *et al*., 2022, 2023; Mwingira *et al*., 2020a; Navarro-Silva *et al*., 2009). Among the culicines, *Ae. aegypti* respond to a wide range of different classes of compounds associated with the conspecific larvae, including long-chain fatty acids and fatty acid esters, straight-chain aldehydes, hydrocarbons and monoterpenes, and ketones as well as sulphides (Boullis *et al*., 2021; Hwang *et al*., 1982; Khan *et al*., 2022, 2023; Ikeshoji, 1968; Wang *et al*., 2019). Two classes of compounds have been identified from the larvae of *Ae. aegypti* and *Cx. quinquefasciatus* to which interspecific gravid females respond, including methyl and methylated hydrocarbons and ketones (Paper III). Gravid *Ae. aegypti* are attracted to the interspecific blend of hydrocarbons, while *Cx. quinquefasciatus* are deterred

from ovipositing in response to the ketones associated with *Ae. aegypti* larvae (Paper III). In contrast, the larval pheromone component of *Ae. aegypti* and *Ae. albopictus*, *n*-heneicosane, (Gonzalez *et al*., 2014; Mendki *et al*., 2000; Seenivasagan *et al*., 2009), elicits a similar behavioural response in both species (Gonzalez *et al*., 2014; Seenivasagan *et al*., 2009).

Similar to culicines, the anopheline mosquitoes, *An. coluzzii* and *An. gambiae*, also respond in a dose-dependent manner to VOCs associated with intra- and interspecific larvae when choosing oviposition sites (Schoelitsz *et al*., 2020; Suh *et al*., 2016). The compounds dimethyl disulphide and dimethyl trisulphide are identified in water conditioned with crowded 4th instar larvae of *An. coluzzii* and *An. gambiae*, to which both species, as well as *Cx. quinquefasciatus*, are deterred (Mwingira *et al.*, 2020b; Schoelitsz *et al*., 2020; Suh *et al*., 2016). Nonane, associated with 1st instar larvae of *An. gambiae*, elicits oviposition in conspecific females in the laboratory (Schoelitsz *et al*., 2020), and strongly attracts and stimulates oviposition in *An. gambiae* and *Cx. quinquefasciatus* under semi-field conditions (Mwingira *et al*., 2021; Schoelitsz *et al*., 2020). Thus, despite their divergent ancestry, *Cx. quinquefasciatus* have evolved to respond to interspecific VOCs (Mwingira *et al*., 2021; Schoelitsz *et al*., 2020). The ecological rationale for this requires further investigation.

3.2.1.3 Cues associated with pupae and pupae exuviae

Gravid culicine mosquitoes differentially respond to VOCs associated with pupae and pupal exuviae of conspecifics and heterospecifics in a density-dependent and species-specific manner, whereas in anophelines this behaviour has not yet been determined (Andreadis, 1977; Dhileepan, 1997; Faierstein *et al*., 2019; Khan *et al*., 2023; Marques & Miranda, 1992; Soman & Reuben, 1970). Khan *et al*., (2023) demonstrated that gravid *Ae. aegypti* are indifferent to pupae-conditioned water, suggesting that pupae do not emit attractive VOCs, likely due to low levels of associated microbiota (Moll *et al*., 2001). This is supported by Faierstein *et al*., (2019), who demonstrated that extracts of ground pupae of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*, which release the gut microbiome and associated VOCs, attract gravid females.

Aedes aegypti behaviourally respond to cues associated with pupal exuviae (Khan *et al*., 2023). The preference of gravid females to lay eggs in water associated with pupal exuviae may be partly related to the microbiota excreted in the meconium during adult emergence (Trimble & Wellington, 1980). During pupation, the peritrophic membrane, *i.e.*, the noncellular, chitoprotein-containing layer that forms around the ingested food in the midgut of most insects, to protect the epithelium of the midgut from food-borne pathogens, forms the meconium (Lehane, 1997). During adult emergence, the peritrophic meconium membrane of pupae is ruptured releasing gut microbiota and uric acid waste into the aquatic habitat (Gao *et al*., 2020; Moll *et al*., 2001), which may alter the microbiotic diversity of the breeding sites (Moll *et al*., 2001). When selecting a breeding site, gravid females likely use these microbial VOCs to detect and discriminate among the potential breeding sites. Recently, gravid *Ae. aegypti* showed a high preference for oviposition and responded in a dose-dependent manner to a VOC blend associated with pupal exuviae (Khan *et al*., 2023). Future studies are required to identify the source of the bioactive VOCs associated with the pupal exuviae.

3.2.2 Cues associated with non-mosquito competitors and predators

According to the natural selection theory, selection should maximise the life-long reproductive fitness of mosquito species (Kershenbaum *et al*., 2012; Resetarits *et al*., 1996). For this reason, female mosquitoes must choose an oviposition location that reduces the risk of non-mosquito competitors and predators (Blaustein *et al*., 2004; Chobu *et al*., 2015; Kershenbaum *et al*., 2012; Van Dam & Walton, 2008). Available studies have demonstrated that female mosquitoes are able to detect non-mosquito competitors and predators in the aquatic environment using olfaction (Blaustein *et al*., 2004; Duquesne *et al*., 2011; Pamplona *et al*., 2009).

Most gravid mosquitoes do not prefer to lay their eggs in breeding sites when interspecific competitors of other non-mosquito species are present due to competition for food resources and the potential for competitive exclusion (Duquesne *et al*., 2011; Knight *et al*., 2004). For example, the presence of *Daphnia magna* inhibits the oviposition of *Cx. pipiens*, as well as leading to a reduction in the size of their larvae (Duquesne *et al*., 2011). The presence of other competitors, such as tadpoles, also affect *Cx. quinquefasciatus* and

Aedes australis by reducing larval growth, development and survival (Mokany & Shine, 2002). Even when physically separated from the tadpoles, *Cx. quinquefasciatus* larvae changed their behaviour and migrate to the surface of the water to avoid competition for food, and potential predation (Mokany & Shine, 2002).

Gravid mosquitoes may avoid oviposition at a breeding site in a species-specific manner when the female perceives an odour associated with a predator (Blaustein *et al*., 2004; Chobu *et al*., 2015; Eitam *et al*., 2002; Munga *et al*., 2006; Pamplona *et al*., 2009; Silberbush *et al*., 2010; Stav *et al*., 2000; Walton *et al*., 2009). For example, *Culex* species avoid oviposition at breeding sites when detecting the VOCs released by the predators *Notonecta irrorate*, *Notonecta maculata* and *Gambusia affinis* (Blaustein *et al*., 1999, 2004, 2005; Eitam & Blaustein, 2004). The site selection of other mosquito species depends on the type of predator and the signals received by the female mosquito during oviposition site selection (Pamplona *et al*., 2009). For example, gravid *Ae. aegypti* do not avoid water conditioned with *G. affinis* and are strongly attracted to the odour of the predator *Mesocyclops longisetus* under field conditions (Torres-Estrada *et al*., 2001). The ecological reason for this could be that gravid mosquitoes avoid oviposition only when there is a high probability that all the young offspring of a single reproductive batch will be lost through predation (Chesson, 1984; Petranka & Fakhoury, 1991; Saha *et al*., 2007), and that the presence of the predator indicates a productive larval site in which the time to emergence is faster, shortening the developmental time. For this reason, mosquito species, such as *Culex* and *Culiseta*, which lay the entire clutch of eggs in one site, avoid oviposition sites associated with predators and predator-associated cues to protect their offspring from predators (Blaustein *et al*., 2004; Eitam *et al*., 2002; Stav *et al*., 2000). In contrast, *Ae. aegypti* follows a skip oviposition strategy, which allows a female to spread the risk across several different breeding sites to avoid the loss of all offspring to predation (Vonesh & Blaustein, 2010). However, both competitors and predators generally have a negative effect on the body size of mosquito larvae and adult (Duquesne *et al*., 2011; Roberts, 2018; Silberbush *et al*., 2019). In the presence of predators, mosquito larvae spend more time at the water surface and at the edge of the container, and avoid risky habitats, *e.g*., the bottom and the open water, to avoid being preyed upon, as predators usually stay below the water

surface. As a result, larvae change their feeding behaviour, gather food from the surface and eat less, resulting in a shorter development time, at the cost of other life history traits, such as adult body size (Dieng *et al*., 2003; Roberts, 2018; Roux *et al*., 2021). Smaller adults have shorter life expectancy and lower fecundity, which in turn affects the size and fitness of the next generation (Roberts, 2018; Silberbush *et al*., 2019).

A limited number of VOCs associated with predators have been identified and demonstrated to influence egg-laying site selection in mosquitoes. The compounds *n*-heneicosane and *n*-tricosane have been identified from *N. maculata* and are shown to repel gravid *Cx. longiareolota* under field conditions (Silberbush *et al*., 2010). Further studies are needed to identify additional VOCs associated with predators.

3.2.3 Cues associated with microorganisms

One of the most important biotic factors that gravid females must consider is the availability of food resources, *e.g.*, bacteria, algae and fungi (Diaz-Nieto *et al*., 2016; Gao *et al*., 2020; Merritt *et al*., 1992; Mosquera *et al*., 2021; Souza *et al*., 2019). The main food source for mosquito larvae is bacteria, which can be actively transferred to, and inoculated into, the breeding sites during oviposition (Akhouayri *et al*., 2013; Diaz-Nieto *et al*., 2016; Merritt *et al*., 1992; Souza *et al*., 2019). To increase their fitness, female mosquitoes thus rely on VOCs associated with microbes to find potential breeding sites (Mosquera *et al*., 2023a).

The diversity of microbiota at a breeding site varies considerably between sites, habitats and geographical locations (Bennett *et al*., 2019; Caragata *et al*., 2021; Mosquera *et al*., 2023b; Nilsson *et al*., 2018, 2019), with Proteobacteria and Firmicutes dominating the sites (Mosquera *et al*., 2023b; Nilsson *et al*., 2019; Ponnusamy *et al*., 2008a; Scolari *et al.,* 2021). Mosquito larvae feed on this microbiota, as demonstrated using 16S ribosomal RNA sequencing, identifying Proteobacteria and Firmicutes as the most abundant bacterial phyla, but also Bacteroidetes, and Actinobacteria (Boissière *et al*., 2012; Mancini *et al*., 2018; Terenius *et al*., 2012). The density of microbiota depends on various features of the breeding site, such as the presence and abundance of detritus, as well as biotic and abiotic factors (Herrera-Varela

et al., 2014; Hery *et al*., 2021; McCrae, 1984; Ponnusamy *et al*., 2015). Among the wide diversity of microbiota in the midgut of larvae and breeding sites, there is strong support that specific bacterial species, *e.g.*, *Klebsiella* and *Elizabethkingia* spp., are symbiotic and affect oviposition site selection (Chandel *et al*., 2013; Huang *et al*., 2006; Lindh *et al*., 2008a; Mosquera *et al*., 2021; Rocha *et al*., 2021; Yadav *et al*., 2015; Wang *et al*., 2011).

Select species of bacteria have been shown to be horizontally transferred by gravid mosquitoes, and as part of this commensal association, the bacteria are dispersed to different habitats during oviposition (Akhouayri *et al*., 2013; Kämpfer *et al*., 2011; Lindh *et al*., 2008a; Mosquera *et al*., 2021). For example, female *Ae. aegypti* may inoculate the breeding site with *Klebsiella* sp., prior and/or during oviposition (Mosquera *et al*., 2021). The growth of the *Klebsiella* sp., community increases over time by actively modifying the greater microbial composition at the breeding site (Coon *et al*., 2016; Mosquera *et al*., 2021; Scolari *et al*., 2021). To further strengthen this association *Klebsiella* sp., produce VOCs that are sensed by gravid *Ae. aegypti* and used to locate these breeding sites in the habitat (Mosquera *et al*., 2023a).

The density and diversity of microbiota at a breeding site affects mosquito egg laying, as a higher diversity of microbes increases the positive or negative effect on mosquito oviposition (Gerhardt, 1959; Hasselschwert & Rockett, 1988; Huang *et al*., 2006; Poonam *et al*., 2002; Ponnusamy *et al*., 2015; Rejmánková *et al*., 2005; Trexler *et al*., 2003), which likely is reflected in the VOCs emitted. Only a limited number of microbial VOCs, from different species of microbiota associated with culicine and anopheline mosquitoes, have been identified and demonstrated to influence oviposition site choice in gravid mosquitoes (Eneh *et al*., 2016; Ikeshoji *et al*., 1979; Lindh *et al*., 2008b, 2008b; Mosquera *et al*., 2023a; Melo *et al*., 2020; Ponnusamy *et al*., 2008b; Rejmankova *et al*., 2000). Shared across culicines and anophelines, the most common classes of microbial VOCs identified at breeding sites to which gravid mosquitoes respond are fatty acids, alcohols and sesquiterpenes (Khan *et al*., 2022). Of these chemical classes, two sesquiterpenes, emitted by either fungi or cyanobacteria, have been shown to strongly attract *An. gambiae* and *Ae. aegypti*, respectively, under laboratory and field conditions (Eneh *et al*., 2016; Lindh *et al.*, 2015; Melo *et al*., 2020).

Classes specific to each subfamily include fatty acid esters and phenolic compounds for the culicines, and pyrazine, ketones and sulphides for the anophelines (Khan *et al*., 2022, Mosquera *et al*., 2023a).

4. Oviposition odour detection

4.1 The olfactory system of mosquito

Olfaction is the most important sensory modality regulating the location and selection of potential breeding sites of female mosquitoes (Afify & Galizia, 2015; Day, 2016; Khan *et al*., 2022, 2023). Odours associated with oviposition are detected by chemosensory receptors expressed on the dendrites of olfactory sensory neurons (OSNs) (Mclver, 1982). The OSNs are housed in sensory hairs, sensilla, which are located on peripheral olfactory appendages, for which the antennae are the principal organs (Mclver, 1979, 1982). The VOCs pass through the wall pores or the slits of the olfactory sensilla (Cribb & Jones, 1995), after which the odorant molecules are selectively bound to water-soluble odorant binding proteins (*OBPs*) (Sánchez-Gracia *et al*., 2009). The *OBPs* act as chaperone molecules that deliver and release the compounds to the receptors (Wogulis *et al*., 2006; Yin *et al*., 2015) and may induce structural changes in the membrane of OSNs (Manoharan *et al*., 2013; Suh *et al*., 2014). The auxiliary cells at the base of sensilla synthesise and secrete *OBPs*, which may indicate a role in hygrosensation (Suh *et al*., 2014). The odorant molecules bind selectively to the olfactory receptors (Sánchez-Gracia *et al*., 2009), which transduce the chemical signals into electrical signals (Guidobaldi *et al*., 2014). The passive electrical signals are transmitted to the axon hillock, where action potentials are generated and then conveyed to the primary olfactory centre, the antennal lobe, of the brain via the OSN axon (Hansson & Christensen 1999; Singh *et al*., 2023). In the antennal lobe, as well as in higher olfactory centres, the information conveyed are then processed, measured, transformed and finally

expressed in the form of a specific behaviour (Singh *et al*., 2023), *i.e.,* oviposition site selection.

4.2 The major olfactory organ – the antenna of mosquito

The antenna of mosquito is attached to the head by a cup-shaped structure, the scape, by which the antenna is able to move (McIver & Siemicki, 1975). The second segment, called the Johnston's organ, is used for the mechanical sensation of vibrations (Mclver, 1982; McIver & Siemicki, 1975). The main part of the antenna, the flagellomeres, consists of 13 segments that in female mosquitoes are covered by various morphological types of sensilla (McIver & Siemicki, 1975). The following section provides a general overview of the antennal sensilla, focusing on their function in relation to oviposition site selection.

4.3 Antennal sensilla and their function

The antennae of mosquitoes bear five types of sensilla: 1) trichodea, 2) grooved pegs, 3) coeloconica, 4) ampullacea and 5) chaetica (Mclver, 1982). Of these, sensilla trichodea, grooved begs and coeloconic sensilla have been shown to have an olfactory function (Davis, 1977; Ghaninia *et al*., 2008; Hill *et al*., 2009; Mclver, 1982; Siju *et al*., 2010). Mosquitoes display a distinct sexual dimorphism in their olfactory sensilla repertoires, mainly due to the fact that males only bear sensilla on the distal three segments (McIver, 1982). There are two classes of olfactory sensilla based on their morphology, single-walled (sensilla trichodea) and double-walled (grooved peg and sensilla coeloconica), which either have pores or slits, respectively, that allow odorants to pass into the sensillum (Cribb & Jones, 1995; Mclver, 1974, 1982). Sensilla trichodea is the most abundant type of sensilla on the female antennae of disease vector species, increasing distally along the flagellomeres, and grooved pegs are the second most abundant sensilla observed distally on flagellomeres with nearly half of the pegs found on the last flagellomeres of antennae (McIver, 1982; Pitts & Zwiebel, 2006). These two sensilla types, trichodea and grooved peg account for approximately 90% of the total antennal sensilla population (McIver, 1978, 1982). Of the mosquito species analysed, *Cx. quinquefasciatus* has a higher mean number of trichoid sensilla (ca. 1124), compared to 642 sensilla in *Ae. aegypti* and

564 in *Anopheles* mosquitoes (McIver, 1978, 1982). The mean number of grooved pegs sensilla of *Cx. quinquefasciatus*, *Ae. aegypti* and *Anopheles* are 234, 105, 114, respectively (McIver, 1978, 1982). Based on their external morphology and function, these sensilla can be further subdivided.

In culicine mosquitoes, the morphology of trichoid sensilla appears to be conserved, with four subtypes, short sharp-tipped (sst), short blunt-tipped I (sbtI), short blunt-tipped II (sbtII) and long sharp-tipped (lst), described for both *Ae. aegypti* and *Cx. quinquefasciatus* (Ghaninia *et al*., 2007; Hill *et al*., 2009). Based on their physiological response to behaviourally active odorants, 11 and 17 functional subtypes of trichoid sensilla have been described in *Ae. aegypti* and *Cx. quinquefasciatus,* respectively (Ghaninia *et al*., 2007; Hill *et al*., 2009). Sensilla trichodea are innervated by two OSNs (Ghaninia *et al*., 2007; McIver, 1982), which selectively respond to VOCs emitted from oviposition sites, such as 4-methylcyclohexanol and 2-butoxyethanol, in *Ae. aegypti*, *Cx. quinquefasciatus* and *Anopheles* species (Davis & Bowen, 1994; Ghaninia *et al*., 2007; Hill *et al*., 2009; Siju *et al*., 2010). Moreover, other classes of compounds, including ketones, alcohols, terpenes, aldehydes, as well as phenolic and indolic compounds, have been shown to elicit responses in OSNs innervating the trichoid sensilla (Chen *et al*., 2018; Ghaninia *et al*., 2007; Ghaninia *et al*., 2008; Hill *et al*., 2009; Siju *et al*., 2010; Syed & Leal, 2008), some of which have been identified from oviposition sites. Of these, the response to phenolic and indolic compounds appears to be highly conserved across culicine and anopheline mosquitoes (Bentley *et al*, 1982; Blackwell & Johnson, 2000; Blackwell *et al*, 1993; Collins & Blackwell, 1998; Hill *et al*., 2009; Qiu *et al*., 2006; Siju *et al*., 2010), which emphasises a strong selection pressure on the olfactory system of mosquitoes to respond to these VOCs (Bentley *et al*, 1982; Blackwell & Johnson, 2000; Blackwell *et al*, 1993; Bohbot *et al*., 2011; Collins & Blackwell, 1998; Hill *et al*., 2009; Qiu *et al*., 2006; Siju *et al*., 2010). This is further supported by the fact that the sensitivity of the OSNs to these compounds increases post-blood meal (Qiu *et al*., 2006; Siju *et al*., 2010). Further studies are required to identify the cognate OSNs for additional VOCs associated with oviposition site selection.

Grooved peg sensilla are innervated by two OSNs (Mclver, 1982), which respond to other chemical classes than trichoid sensilla, mainly carboxylic

acids and amines (Davis, 1977; Davis & Sokolove 1976; Mclver, 1982; Pitts *et al*., 2022). Moreover, mosquitoes are able to detect the humidity level by receptors expressed in these sensilla, through which females are able to locate oviposition sites (Laursen *et al*., 2023; Matthews *et al*., 2019). While a previous study indicated that the sensitivity of OSNs, housed in grooved pegs, to an oviposition site VOC may increase in gravid mosquitoes (Davis, 1984), additional research is required to elucidate their role in regulation in oviposition site seeking and selection.

4.4 Olfactory receptors tuned to VOCs associated with oviposition

A number of studies have investigated the function of olfactory receptors in mosquitoes in heterologous expressions systems, using either "off-the-shelf" VOCs or behaviourally relevant VOCs (Carey *et al*., 2010; Omondi *et al*., 2019; Wang *et al*., 2010). To date, only a few studies have investigated the function of olfactory receptors in relation to oviposition site selection in mosquitoes (Bohbot & Dickens, 2009; Bohbot *et al*., 2011; Carey *et al*., 2010; Laursen *et al*., 2023; Liu *et al*., 2018; Wang *et al*., 2010).

Indole is an aromatic heterocyclic VOC associated with oviposition in *Ae. aegypti*, *Cx. quinquefasciatus* and *An. gambiae*, which is detected by a homologous receptor, OR2, expressed in all these species (Bohbot *et al*., 2011; Carey *et al*., 2010; Dekel *et al*., 2019; Pelletier *et al*., 2010; Scialo *et al*., 2012). Similarly, 3-methylindole, another VOC described to regulate oviposition across mosquitoes, is detected by the conserved OR10 (Bohbot *et al*., 2011; Carey *et al*., 2010; Liu *et al*., 2018). As opposed to most other *ORs, OR2* and *OR10* display 70-79% amino acid identity across these species (Bohbot *et al*., 2007). As opposed to indolic compounds, phenolic compounds appear to be detected by non-homologous *ORs* across these species and in *Drosophila melanogaster* (Bohbot *et al*., 2011; Dekel *et al*., 2019; Liu *et al*., 2018; Pelletier *et al*., 2010; Ruel *et al*., 2019, 2021). For example, 4-ethylphenol elicits a response in OR1 in *An. gambiae* (Carey *et al*., 2010; Khan *et al*., 2022; Wang *et al*., 2010) and OR37 in *Cx. quinquefasciatus* (Zhu *et al*., 2013).

Besides *ORs*, ionotropic receptors (*IRs*) have been demonstrated to bind VOCs emanating from potential breeding sites. For example, IR41a is sensitive to polyamines, which are emitted by bacteria, and regulate oviposition in *Ae. aegypti* (Hussain *et al*., 2016; Pitts *et al*., 2017). Transcriptome analyses have demonstrated that the expression level of both *OR*s and *IR*s is regulated in response to a change in the physiological state of female mosquitoes (Hill *et al*., 2021; Rinker *et al*., 2013; Santos *et al*., 2022), which is correlated with a change in both behavioural and physiological sensitivity to ecologically-relevant VOCs (Davis, 1984; Omondi *et al*., 2019; Qiu *et al*., 2006; Rinker *et al*., 2013; Siju *et al*., 2010). Further research is needed to decipher the function of additional *ORs* and *IRs*, using relevant oviposition-associated VOCs, to increase our understanding of the molecular mechanisms regulating odour-driven oviposition in mosquitoes.

5. Application

5.1 Integrated vector management

Gravid mosquitoes represent one of the important stages in the mosquito life cycle but have received little attention in the development of control strategies (Takken & Knols, 1999; WHO, 2022). While MOP is commercially available, and is a potential lure for *Culex* mosquitoes this pheromone component has not gained wide use, likely due to the cost for biosynthesis (Leal *et al*., 2008). Recent development in the field, however, may change this. Available trapping systems, especially for culicine mosquitoes, are on the other hand, making use of the semiochemicals associated with vegetation fermentation infusions, as well as mosquito aquatic stages, to lure gravid mosquitoes (Burkett-Cadena & Mullen, 2007; Schorkopf *et al*., 2016). Gravid-traps of various designs are often used in combination with fermentation infusions to lure and monitor gravid *Culex* (Takken & Knols, 1999). Examples of trapping systems relying on the initial passive attraction of mosquitoes and the subsequent release of semiochemicals from aquatic stages are the In2Care mosquito station (Autry, 2021; Buckner *et al*., 2017, 2021) and the BioGents BG-GAT (Cilek *et al*., 2017; 2023; Eiras *et al*., 2021), which are currently being used successfully to monitor and control *Ae. aegypti* and *Ae. albopictus* (Buckner *et al.,* 2021; Eiras *et al*., 2021). These trapping systems may also be combined with a contamination agent, such as pyriproxyfen and *Beauveria bassiana*, which will be carried to other breeding sites by egg-laying mosquitoes (Buckner *et al*., 2017, 2021). Policy makers, particularly the WHO, are keen to exploit additional odour-mediated technology for the surveillance and control of

mosquito populations, particularly by targeting gravid mosquitoes (WHO, 2022).

6. Aims and objectives

The overall aim of this thesis was to identify the odour-mediated mechanisms regulating oviposition preference of *Ae. aegypti* and *Cx. quinquefasciatus* in response to intra- and interspecific aquatic stages, as well as to the commensal bacteria, *Klebsiella* sp..

Specific objectives

The first objective was to assess the density-dependent behavioural response of gravid *Ae. aegypti* to water conditioned with intraspecific aquatic stages, and to identify the associated VOCs that regulate oviposition site choice and egg laying (**Paper II**).

The second objective was to identify the oviposition preference of gravid mosquitoes of two sympatric species, *Ae. aegypti* and *Cx. quinquefasciatus*, to water conditioned with heterospecific 4th instar larvae, and to identify the associated VOCs regulating the observed differential behavioural responses (**Paper III**).

The third objective was to investigate the dose-dependent preference of gravid *Ae. aegypti* to the commensal bacteria *Klebsiella* sp., and to identify the olfactory pathway detecting the associated VOCs regulating this choice (**Paper IV**).

7. Methodology

In this chapter, I briefly present background information on the two sympatric mosquito species used in the various projects of this thesis. The behaviour and physiological responses of the two species of mosquitoes were evaluated under laboratory conditions, and the VOCs collected using different methods described below. A detailed description of the methodology can be found in the corresponding papers.

7.1 *Aedes aegypti* and *Culex quinquefasciatus*

Both *Ae. aegypti* and *Cx. quinquefasciatus* are vectors of many important diseases, such as dengue, Zika fever, yellow fever, West Nile fever, human filariasis and Japanese encephalitis, and are a growing concern for public health (WHO, 2022). Like all mosquitoes, both species undergo complete metamorphosis. Mosquitoes pass through four larval stages and a pupal stage in an aquatic environment, and then emerge as a terrestrial imago (Christophers, 1960). Both *Ae. aegypti* and *Cx. quinquefasciatus* are opportunistic feeders as larvae, but utilise different feeding strategies (Carrieri *et al*., 2003; Costanzo *et al*., 2014; Skiff & Yee, 2014; Yee *et al*., 2015). *Aedes aegypti* larvae feed by shredding the food into small pieces, while *Cx quinquefasciatus* feed by collecting and filtering the food from the water column (Merritt *et al*., 1992). The larvae of both species can be found coexisting in the same breeding sites, often in drains, ditches or septic tanks (Barrera *et al*., 2008; Burke *et al*., 2010; Santana-Martinez *et al*., 2017). The preferred breeding sites of both species, however, are man-made containers (Powell & Tabachnick 2013), such as flower vases, which are often found in urban and suburban habitats. *Aedes aegypti* often lay eggs on substrates that

are close to the edge of water, while *Cx. quinquefasciatus* deposit eggs on the water surface (Vezzani, 2007).

The two sympatric species use different strategies for oviposition. *Aedes aegypti* uses skip oviposition, in which individual eggs are spread over several sites to increase the chances of survival of the offspring (Reinbold-Wasson & Reiskind, 2021). In contrast, *Cx. quinquefasciatus* lay an entire clutch of eggs in one site (Day, 2016). The eggs of *Ae. aegypti* are able to tolerate desiccation for up to many months (Kramer *et al*., 2020), whereas *Cx. quinquefasciatus* must be reared continuously to maintain egg production.

The mosquito strains used in the papers included in this thesis were the Rockefeller (**Paper II** / Khan *et al*., 2023) and Orlando (**Paper IV** / Mosquera *et al*., 2023a) of *Ae. aegypti*, in addition to the Johannesburg strain of *Cx. quinquefasciatus* (**Paper III**). Moreover, $Orco^{5/16}$ mutants (DeGennaro *et al*., 2013) were used to assess their behavioural response (**Paper IV** / Mosquera *et al*., 2023a). The behavioural experiments were planned to match the time of peak oviposition activity (Farnesi *et al*., 2018), with mosquitoes being released 2 h prior to the onset of the scotophase for 19 ± 1 h and 45 ± 1 h, respectively. The physiological response of gravid mosquitoes was tested 2 h prior to the onset of the scotophase.

7.1.1 Analysing oviposition site selection

A multi-choice oviposition assay was used to examine the effect of VOCs emanating from water conditioned with conspecific and heterospecific aquatic stages on the oviposition site selection and egg laying of *Ae. aegypti* and *Cx. quinquefasciatus* (**Papers II, III**). One artificial oviposition site was placed in each of the four corners of a Bugdorm-1 cage (L: 30 cm × W: 30 cm × H: 30 cm; MegaView Science, Taichung, Taiwan), ca. 8-10 cm away from the cage wall, and one was placed in the centre of the cage (Fig. 1a). Each artificial oviposition site was composed of three cups assembled one inside the other. The con-/heterospecific conditioned or control water (40 ml) was added into a blue plastic cup (250 ml; Duni, Malmö, Sweden). The blue colour was chosen because it was previously shown to positively affect egg laying in *Cx. quinquefasciatus* (Moazzeni *et al*., 2023). Besides being dark in colour, blue is the second-most preferred colour demonstrated to

positively affect oviposition in mosquitoes (Dhileepan, 1997; Moazzeni *et al*., 2023). A second transparent cup (120 ml; ÖoB, Lund, Sweden), with six 2.5 mm diameter perforations in the bottom, was placed within the blue plastic cup to greatly reduce inputs from sensory modalities other than olfaction (Fig. 1b). A third cup (30 ml), holding 20 ml distilled water, was placed carefully inside the second cup, making sure that the perforations were not covered (Fig. 1b). For oviposition assays with *Ae. aegypti*, a filter paper (90 mm diameter; Ahlstrom, Munksjö, Finland) was placed in the top cup, and served as an egg-laying substrate, while no filter paper was provided in the uppermost container for *Cx. quinquefasciatus*. Each artificial oviposition site, which held water conditioned with a distinct density of specific con-/heterospecific aquatic stages, or their associated control, were assigned randomly in the Bugdorm-1 cage. Each treatment was rotated within the Bugdorm-1 cage, in between trials, to reduce any position bias.

Five-to-seven days post-blood feeding, a single gravid *Ae. aegypti* or *Cx. quinquefasciatus* was released 2 h prior to scotophase in a Bugdorm-1 cage. *Aedes aegypti* and *Cx. quinquefasciatus* were permitted to select among the five artificial oviposition sites for 19 ± 1 h and 45 ± 1 h, respectively. *Aedes aegypti* were provided access to 10% sucrose solution *ad libitum* during the period of the oviposition assay. In contrast, *Cx. quinquefasciatus* were deprived access to sugar, 24 h before and throughout the duration of the egg-laying bioassay, since gravid females deferred egg laying when given access to sucrose solution. The behavioural assay was maintained under 27 ± 1 °C, 65 ± 5% relative humidity, and at a 12 h: 12 h light-dark cycle. The number of eggs (*Ae. aegypti*) or rafts (*Cx. quinquefasciatus*) laid per female in each artificial oviposition site was noted at the end of the bioassay. The assay was repeated three-to-five times (N=3-5) for *Ae. aegypti*, with at least 30 replicates (n=90-150), while, for *Cx. quinquefasciatus*, the assay was repeated five times (N=5) with 25 replicates (n=125). The behavioural response of *Ae. aegypti* to different densities of conspecific aquatic stages, and to the most preferred density of each aquatic stage, was analysed by comparing the mean total number of eggs laid per female using an ANOVA followed by a Tukey *post-hoc* test. Similarly, the oviposition preference of *Ae. aegypti* towards water conditioned with different densities of 4th instar *Cx. quinquefasciatus* was analysed using an ANOVA followed by a Tukey *post-hoc* test. The oviposition response of *Cx. quinquefasciatus* to water

conditioned with different densities of 4th instar *Ae. aegypti* larvae was determined by a multinominal logistic regression, using maximum likelihood analysis. Multinomial logistic regression was used to describe the relationship between nominal dependent and independent variables. The dependent variable is binary, *i.e.*, presence or absence of a raft, which means that there are only two outcomes, since *Cx. quinquefasciatus* only lays a single egg raft at one oviposition site and not at another.

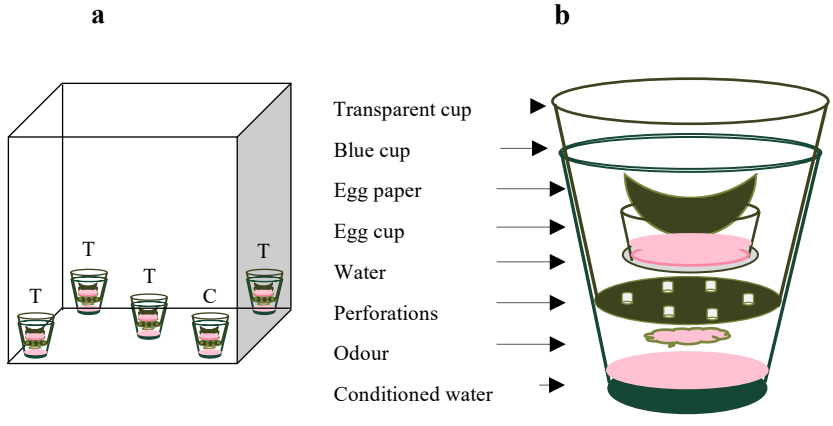

Figure 1. Multi-choice oviposition assays were used to evaluate the oviposition preference of *Aedes aegypti* and *Culex quinquefasciatus* to water conditioned with conspecific and heterospecific aquatic stages (a). The position of the artificial oviposition site (triple cups) within the Bugdorm-1 cage is indicated (b). The composition of the triple cups, allowing olfactory cues, but not other sensory cues, to stimulate the gravid mosquitoes is depicted.

7.1.2 *Klebsiella* species cultures

The bacteria *Klebsiella* sp. was cultured and plated on tryptic soy agar (VWR, Stockholm, Sweden) using the streak plate technique. Plates were incubated overnight. Single colony was transferred from tryptic soy agar culture plates to tubes containing 6 ml of 0.01%, 0.1%, 1% or 10% tryptic soy broth (TSB) for 24 h at 200 rpm on a shaker at 25 °C. After centrifugation, at 4 °C for 15 min at 3000 rpm, the bacterial pellet was collected. The supernatant was removed and the pellet resuspended in 0.085% NaCl. This step was repeated two times. The bacterial cells were

resuspended in different TSB dilutions (0.01%, 0.1%, 1%, or 10%) in order to obtain a stock suspension. In 30 ml of TSB, 15 µl stock solution was transferred and incubated for 24 h at 25 °C on a shaker at 200 rpm. Once the bacterial culture reached the stationary phase, an aliquot was serially diluted and plated to count the colony forming units (CFUs). The different bacterial loads were investigated for the behavioural response of *Ae. aegypti* to the odour released, as well as to the control TSB alone.

7.1.3 Dual choice oviposition assay with *Klebsiella* cultures

The oviposition site selection of *Ae. aegypti* was assessed in response to VOCs emitted from the bacteria in a dual choice assay. For this purpose, the Bugdorm cages were modified (for detail see section 7.1.1 1st paragraph and section 7.4). The two oviposition cups were placed in opposite corners of the cage (Fig. 3). The TSB (30 ml) with and without *Klebsiella* sp. (control) were transferred into individual 100 ml glass bottles (Simax, VWR) fitted with lids containing two ports to permit air to flow in and out of the bottle. A filter (0.22 µm; polyethersulfone, ThermoFisher, Stockholm, Sweden) was employed at the outflow port to isolate the bacteria from the assay. Charcoal filtered air was passed through either of the bottles (0.1 l min^{-1}) to two 12-channel flowmeters (Kytola Instruments, Muurame, Finland), which were connected via Teflon™ tubing to the bottom cup in the artificial oviposition site in the12 experimental cages. A single gravid female was released 2 h prior to scotophase for 22 ± 1 h, under the same climatic conditions as described above. The number of eggs laid on the oviposition paper in the test and control was then counted (N>30 females per bacterial load). Four different bacterial loads were tested ($10^{6.3}$ CFU ml^{-1}, $10^{7.3}$ CFU ml^{-1}, $10^{8.3}$ CFU ml^{-1}, and $10^{9.2}$ CFU ml^{-1}) against their controls (0.01%, 0.1%, 1%, and 10% TSB, respectively). The position of the cups was not changed between the experiment due to the risk of contamination. However, before the start of the bioassays, two choice assays with controls were run in order to rule out any positional bias in the experimental setups.

7.2 Odour extract collections

Two different methods were used for VOC collections. The static odour headspace was collected by solid-phase microextraction (SPME) from water conditioned with different aquatic stages of *Ae. aegypti*, and from *Klebsiella*

sp. cultures (**Papers II, IV**). The solid phase extraction (SPE) method was used to collect extracts from water conditioned with 4th instar larvae of either *Ae. aegypti* or *Cx. quinquefasciatus* (**Paper III**).

7.2.1 Solid-phase microextraction

The SPME fibres (divinylbenzene/carboxen/polydimethylsiloxane Supelco StableFlex^{TM}, 50/30 µm, 24 ga, 2 cm, Sigma Aldrich, Stockholm, Sweden) were conditioned for 30 min at 225 °C using a gas chromatograph (GC; Agilent Technologies 6890, Santa Clara, USA) prior to headspace collections (**Papers II, IV)**. The glassware used for SPME headspace collections were cleaned and heated at 200 °C for 8 h before use. Before headspace collection, the water was conditioned with each of the densities of *Ae. aegypti* aquatic stages that induced the highest egg-laying response in the multi-choice assay, along with their respective controls. In addition, *Klebsiella* sp. was cultured and diluted in TSB (1%), as described above, prior to headspace collections, with TSB serving as a control. The headspace of the bacterial culture, 2 ml in a 10 ml sterile vial fitted with a PTF-E lined septum (Supelco, VWR), was collected for 4 h. The treated (400 ml) or control water (400 ml) was dispensed into 1 l glass bottles (VWR). Sodium chloride (≥ 99%), was then dissolved into each bottle at a concentration experimentally determined to the enhance release of volatiles (255 mg ml^{-1}) (Lindh *et al.*, 2015; Mozūraitis *et al.,* 2010). After 5 min of incubation, the conditioned SPME fibre was inserted into a small drilled hole (1.4 mm diameter) in the polypropylene lid of the glass bottle (Fig. 2). The headspace was collected for 17 h. Thereafter, the SPME was injected directly for combined GC and electroantennographic detection (EAD) analysis, or for the identification of bioactive compounds onto a combined GC-mass spectrometer (MS).

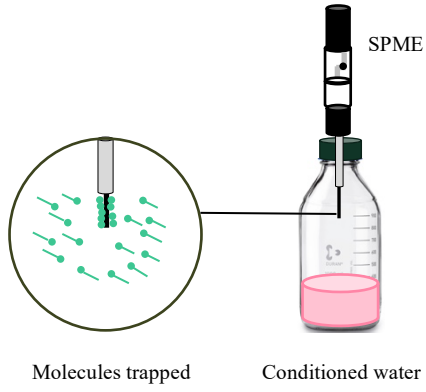

Figure 2. Solid phase microextraction (SPME) was used to collect odour from water conditioned with various *Aedes aegypti* aquatic stages and *Klebsiella* sp., as well as their respective controls.

7.2.2 Solid-phase extraction

The SPE method was used to collect the VOCs from water conditioned with *Ae. aegypti* and *Cx. quinquefasciatus* 4th instar larvae, as well as control water (treated with the same food regime provided to larvae) (**Paper III**). Larval conditioned or control water was first filtered through a single layer of "folded" filter paper (18.5 cm; Whatman Int Ltd, Maidstone, England) and then the filtered water (150 ml) was passed once over a Chromafix C18 cartridge (VWR). The C18 column was dried by passing nitrogen for 2 min. Afterward, the collected VOCs were eluted with 500 µl dichloromethane (99.9%, Merck) and the extract concentrated to 40 µl and stored at -80 °C for future analysis. The SPE extract (2 µl) was injected onto the GC-EAD, and for the identification of compounds onto a GC-MS.

7.3 Electrophysiological analysis

The combined GC-EAD technique is used to detect bioactive VOCs in complex odour extracts, by registering the antennal response of insects to VOCs eluting individually from the GC. This technique is limited and does not offer evidence on how the VOCs influence the behavioural response, including whether the VOCs are attractive, repellent or behaviourally indifferent, to the insects. This method was used to screen for bioactive

compounds present in the headspace collection and pooled SPE extracts odour blends in this thesis (**Papers II, III, IV**).

7.3.1 Screening for bioactive volatile organic compounds in odour extracts

For the GC-EAD analysis, the GC (Agilent technologies 6890) was equipped with a fused silica capillary HP-5 column (30 m length × 0.25 mm i.d. × 0.25 µm film thickness), and hydrogen was used as a carrier gas at a linear flow rate of 45 cm s^{-1}. For the SPME headspace injections, the GC oven temperature was programmed from 50 °C (hold for 1 min), and then increased at an incremental unit of 8 °C min^{-1} to 275 °C (10 min hold). For the SPE extract injections (2 µl), the GC oven temperature was set from 40 °C (hold for 1 min), to 275 °C (10 min hold), at an incremental rate of 8 °C min^{-1}. At the GC effluent splitter, nitrogen was added and split in a 1:1 volume four-way-cross splitter (Gerstel, Mülheim, Germany), so that half of the injected sample was delivered to the flame ionization detector (FID) and half to the EAD. The GC effluent delivered to the EAD passed through a Gerstel ODP-2 transfer line, which tracked the GC oven temperature, into a glass tube (10 cm × 8 mm), where it was mixed with charcoal-filtered humidified air (1.5 l min^{-1}). The antennal preparation was positioned ca. 0.5 cm at a distance from the opening of the glass tube. The corresponding signals of the GC and the EAD were matched during the analysis in order to identify the VOCs eliciting an electrical signal in the antenna.

For the EAD screening, 5 days post-blood meal *Ae. aegypti* (**Papers II, IV**) and 7 d post-blood meal *Cx. quinquefasciatus* (7 d post-blood meal) (**Paper III**) were used. A female mosquito was cold anesthetized (2-3 minutes on ice), and the head excised under a stereomicroscope. Afterward, the distal end (1-2 flagellomeres) of both antennae was removed. Two glass electrodes, made from microcapillaries (borosilicate, o.d 1.5 mm × i.d 0.86; Harvard Apparatus, Stockholm, Sweden), were filled with Beadle-Ephrussi ringer solution (Ephrussi & Beadle, 1936). One electrode served as the reference, and was inserted into the foramen of the head capsule, whereas the second electrode was inserted over the tip of one of the antennae and served as the recording electrode. Both glass electrodes contained a chlorinated silver wire to establish the electrical contact. The recording electrode was connected to a pre-amplifier (10×; Syntech, Buchenbach, Germany), connected to a digital

analogue signal converter interface box (IDAC-2, Syntech), which was connected to a computer for signal recording, visualization and storage of the data. Three-to-five recordings were performed for each odour extract. All the data were analysed using GC-EAD software 2011 (v.1.2.3, Syntech).

7.3.2 Identification of bioactive volatile organic compounds

Combined GC-MS is an analytical technique, which can be used to separate, identify and quantify different chemical compounds within a complex odour blend. The MS works by measuring the mass-to-charge ratio of a molecule, broken down into its ionized fragments (Bouchonnet, 2013). In this thesis, a GC (6890, Agilent Technologies) coupled with an MS (5975, Agilent Technologies), was used for identifying the bioactive VOCs present in the odour extracts from aquatic stages of *Ae. aegypti* and *Cx. quinquefasciatus*, as well as *Klebsiella* sp.. The GC-MS was operated in the electron ionization mode at 70 eV (**Papers II, III, IV**). The GC-MS used the same type of capillary HP-5 column (60 m length × 0.25 mm i.d. × 0.25 µm film thickness) and oven temperature gradient program, as described above. Helium was used as the mobile phase, at a constant flow rate of 34 cm s^{-1}. The identification of VOCs was determined according to the retention indices using retention times (Kovats' indices) and mass spectra, by matching with the National Institute of Standards and Technology-17 (NIST-17 library). The area of each total ion chromatogram was compared to determine the relative abundance of bioactive VOCs in each headspace or extract. The synthetic blends were developed based on the detected ratio of the physiologically active VOCs. The synthetic blend of the identified compounds was validated by a co-injection onto the GC-MS and GC-EAD. The purity of each chemical compounds was verified by injection of 10^{-2} dilution into the GC-MS.

7.4 Behavioural response to synthetic odour blends

A dual-choice assay was used to evaluate the oviposition preference of gravid mosquitoes to the synthetic blends generated from the bioactive VOCs identified from 1) water conditioned with different aquatic stages of *Ae. aegypti,* at densities eliciting the highest behavioural response in conspecific gravid females (**Paper II**), 2) water conditioned with 4^{th} instar larvae of either *Ae. aegypti* or *Cx. quinquefasciatus*, at densities eliciting the

highest behavioural response in heterospecific gravid females, or the lowest density, respectively (**Papers III**), and 3) mosquito-associated bacteria of *Ae. aegypti* (**Papers IV**) (Fig. 3). Two artificial oviposition sites, described above, were placed in opposite corners of a Bugdorm-1 cage, ca. 8-10 cm from the cage wall. The synthetic odour blends tested, along with a solvent (Sigma-Aldrich) control, was introduced into either of these sites, in a total of 12 Bugdorm cages, via Teflon^{TM} tubing connected to either of two 250 ml glass wash bottles (VWR) and two 12-channel flow meters (Kytola Instruments), using charcoal filtered air (0.1 l min^{-1}), as described above. The synthetic odour blends, delivered at different dilutions (6 ml) and the control (6 ml hexane) were released via wick dispensers (Fig. 3), which allowed for a controlled release and ratio maintenance of the blends (Karlsson et al., 2017). The wick dispenser was constructed from a 12 ml glass vial (Genetec, Stockholm, Sweden), with a 2 mm diameter perforation drilled in the lid, and a wick made of Teflon tubing (75 mm length × 1.68 mm i.d. × 0.30 mm wall thickness) with a piece of unbleached cotton string threaded through the tube (Karlsson *et al*., 2017). The wick dispensers were positioned standing upright into one of the two glass bottles, and then the lid was tightly closed.

Gravid *Ae. aegypti* and *Cx. quinquefasciatus* were prepared, and the assays run, as described above. After completion of the experiment, the number of eggs (*Ae. aegypti*), or egg rafts (*Cx. quinquefasciatus*), was recorded. The multi-choice oviposition assay datasets for each of the different densities for each aquatic stage, and across the most preferred densities of a conspecific aquatic stage, were compared using the average total number of eggs laid per female by analysis of variance (ANOVA) followed by a Tukey post-hoc test. For the dual choice assays, the oviposition choice indices for the behavioural data in response to bacteria and synthetic blend were calculated: (C / C + T) and (T / T + C), in which C is the number of eggs or rafts laid in the control and T is the number of eggs or rafts laid in the test cup. The behavioural data of the dual-choice oviposition assays, comparing the behavioural response to the synthetic odour blend was analysed using binary logistic regression followed by an odds ratio comparison (JMP Pro v. 16; **Papers II, III**), whereas the behavioural response to the larval conditioned water and odour extracts against the solvent control (hexane; **Paper III**) was analysed using binomial logistic regression analysis. The oviposition choice indices of the

experiment with *Klebsiella* sp. were calculated using the Wilcoxon-matched pair test (JMP Pro v. 16; **Paper IV**).

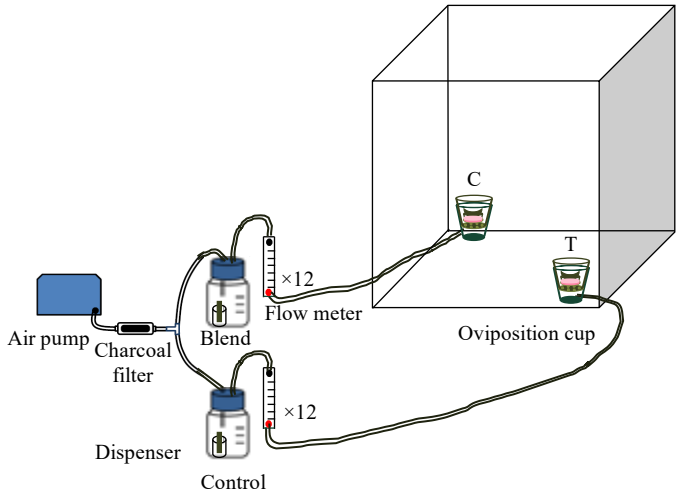

Figure 3. Dual-choice oviposition assays were used to assess the oviposition site preference and egg-laying of *Aedes aegypti* and *Culex quinquefasciatus* to synthetic blends.

8. Results and Discussion

8.1 Aquatic stage-dependent oviposition site selection of *Aedes aegypti* (**Paper II**)

Mosquitoes are differentially attracted to oviposition sites, and respond to odours associated with water conditioned with intraspecific aquatic stages (Boullis *et al*., 2021; Ganesan *et al*., 2006; Gonzalez *et al*., 2015; Khan *et al*., 2022, 2023; Laurence & Pickett, 1982; Mwingira *et al*., 2020b, 2021; Soman & Reuben, 1970; Suh *et al*., 2016; Wong *et al*., 2011; Zahiri *et al*., 1998). Moreover, gravid mosquitoes appear to use stage-specific volatiles, in a species- and taxon-specific manner, to discriminate among potential breeding sites (Allan & Kline, 1998; Boullis *et al*., 2021; Gonzalez *et al*., 2014, 2015; Khan *et al*., 2022, 2023; Mwingira *et al*., 2020b, 2021; Schoelitsz *et al*., 2020). This project aimed at identifying the mechanism regulating the oviposition preference of *Ae. aegypti* in response to VOCs released from water conditioned with different aquatic stages of conspecifics (**Paper II**).

Gravid *Ae. aegypti* demonstrated a density-dependent response to water conditioned with eggs, 2nd instar larvae, 4th instar larvae and pupae exuviae, but not pupae, in a multi-choice assay. In the same assay, and when given a choice between water conditioned with the most preferred density of each aquatic stage and a control, gravid females preferentially laid more eggs in water conditioned with 4th instar larvae, which has also been demonstrated in the field (Wong *et al*., 2011). The results of these experiments emphasise a change in odour profile during the development of the aquatic stages from egg to adult emergence (pupal exuviae) (**Paper II**) (Fig. 4). The preference of gravid females for late-stage larvae has also been reported in other mosquito species, *e.g., Cx. annulirostris* and *Cx. molestus* (Dhileepan, 1997).

Such preference has until now not been described for anopheline mosquitoes, as available data from *An. coluzzii* demonstrate that these attracted to breeding sites containing 1st stage larvae (Mwingira *et al*., 2020b). Whether this change is due to VOCs produced directly by the aquatic stages or is a result of changes in the microbial diversity (McCrae, 1984; Nilsson *et al*., 2018, 2019) remains to be investigated.

What is the ecological rationale for gravid *Ae. aegypti* to preferentially lay eggs where late-instar larvae are present? Available studies on culicine mosquitoes emphasised that gravid mosquitoes are guided to these sites by a reliable signal, which should provide information concerning the suitability of the site for the growth and development of their offspring (Kershenbaum *et al*., 2012; Silberbush & Blaustein, 2011). The most important factors here are likely the level of competition and food availability (Boullis *et al*., 2021; Do Nascimento *et al*., 2022; Gonzalez *et al*., 2014; Mwingira *et al*., 2021; Wong *et al*., 2011; Xia *et al*., 2021b; Yoshioka *et al*., 2012). In that regard, the observed preference of gravid *Ae. aegypti* to water conditioned with 4th instar larvae (**Paper II**) may initially appear counter-intuitive, as late-stage larvae would be more fierce competitors, not least for food resources (Bedhomme *et al*., 2005; Wong *et al*., 2011). However, mature larvae will soon convert into non-feeding pupae, which will not compete with the newly hatched larvae (Dhileepan, 1997; Khan *et al*., 2023; Wong *et al*., 2011). Moreover, by the time that mosquitoes reach the 4th instar, the larvae are mainly feeding on communities of well-developed microbiota (Diaz-Nieto *et al*., 2016; Souza *et al*., 2019). This microbiota is, to a large extent, likely to have been inoculated by gravid females, which have been demonstrated to actively transfer specific bacterial species, *e.g.*, *Klebsiella spp.,* and *Elizabethkingia spp.*, to the breeding site during egg laying (Kämpfer *et al*., 2011; Mosquera *et al*., 2021). This not only enhance available food resources over time for larval growth and development, but also reduce intraspecific competition. That said, in the field gravid *Ae. aegypti* often lay eggs synchronously so that all aquatic larval stages develop at the same time, which significantly reduces the risk of intraspecific competition (**Paper II**; Wong *et al*., 2011).

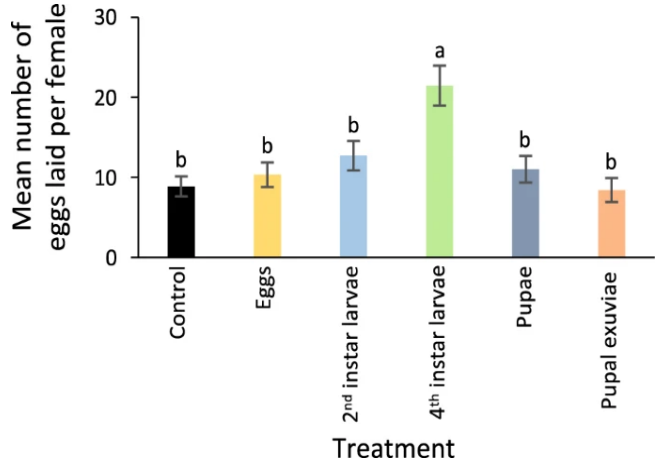

Figure 4. *Aedes aegypti* prefer to oviposit in response to volatiles associated with late-stage larvae in comparison to other aquatic stages. The different lowercase letters denote significant differences ($P < 0.05$), as determined by ANOVA followed by a Tukey *post-hoc* test. Error bars represent standard error of the mean (**Paper II**).

8.2 Density-dependent oviposition site selection of *Aedes aegypti* and *Culex quinquefasciatus* (**Papers II, III**)

Odours associated with intra- and interspecific aquatic stages of mosquitoes regulate oviposition site selection in mosquitoes in a density- and species-specific manner (**Papers II, III**; Shragai *et al*., 2019; Wachira *et al*., 2010; Zahiri & Rau, 1997). Gravid *Ae. aegypti* appear to use similar strategies when choosing egg-laying sites, preferring low or intermediate densities, and avoiding sites associated with high densities, of both intra- and interspecific aquatic stages (**Papers II, III**). The behavioural response of *Ae. aegypti* to heterospecific 4th instar larvae (**Paper III**), however, suggests that gravid mosquitoes use different mechanisms when selecting breeding sites containing con- or heterospecific aquatic stages (Fonseca *et al*., 2015; Gonzalez *et al*., 2015; Lyimo *et al*., 1992; Mwingira *et al*., 2020b; **Papers II, III**; Shragai *et al*., 2019; Suh *et al*., 2016a; Sumba *et al*., 2008; Wachira *et al*., 2010; Yadav *et al*., 2017; Zahiri & Rau, 1998). In contrast to *Ae.*

aegypti, gravid *Cx. quinquefasciatus* laid the majority of their egg rafts in control water rather than in water conditioned with 4th instar *Ae. aegypti* larvae (**Paper III**). The ecological rationale for the observed species-dependent preferences, is likely due to the fact that mosquito species are differentially able to compete for resources (niches), such as food and habitat (Costanzo *et al*., 2005; Koenraadt *et al*., 2004; Santana-Martinez *et al*., 2017). This competition may lead to competitive exclusion of the weaker species (Santana-Martinez *et al*., 2017). Weaker species may, however, be able to share breeding sites with more dominant species using different mechanisms, such as differential use of nutrient resources or by being spatially or temporally segregated within the habitat (Burke *et al*., 2010; Carrieri *et al*., 2003; Leisnham *et al*., 2014; Skiff & Yee, 2014). The findings from my studies (**Paper III**) demonstrate that VOCs associated with heterospecific aquatic stages are able to regulate niche segregation and competitive exclusion in egg-laying sites shared by sympatric species.

Gravid *Ae. aegypti* must evaluate and select the level of intraspecific competition in potential oviposition sites, as this decision directly regulates the development and survival of the offspring, and thus the fitness of the female (Kesavaraju *et al*., 2014; Novak *et al*., 1993; Yoshioka *et al*., 2012). *Aedes aegypti* deposit more eggs in water conditioned with intermediate densities of intraspecific aquatic stages in order to increase the larval hatching (Chadee *et al*., 1990), as well as the growth, development and survival of the offspring, by minimising intraspecific competition (Gonzalez *et al*., 2015; Lyimo *et al*., 1992; Suh *et al*., 2016; Wada, 1965; Yadav *et al*, 2017; Zahiri & Rau, 1998). In contrast, high densities of larvae lead to an increased consumption of the surface bacteria of eggs by the larvae and a decrease in the concentration of dissolved oxygen, resulting in reduced egg hatching and increased dormancy for surviving eggs (Edgerly *et al*., 1998; Fischer *et al*., 2011; Livdahl, 1982; Livdahl *et al*., 1984). The effect of overcrowding at breeding sites is not limited to *Ae. aegypti,* as demonstrated for both *Cx. quinquefasciatus* and *An. coluzzii*, in which high densities and restricted space promote competition, leading to larval starvation and a release of specific VOCs that deter oviposition of gravid mosquitoes (Ikeshoji & Mulla, 1974; Suh *et al*., 2016). The identification of VOCs from high densities of aquatic stages of *Ae. aegypti* needs to be explored in a future study.

In contrast to the response to presence of intraspecific larvae, gravid *Ae. aegypti* selected, and deposited more eggs, in sites associated with the odour of water conditioned with the lowest relative density of 4th instar *Cx. quinquefasciatus* larvae (**Paper III)**. Similar preferences have been described for culicine and anopheline mosquitoes, *e.g*., *An. gambiae vs. Cx. quinquefasciatus* and *Ae. albopictus vs. Ae. aegypti* (Gonzalez *et al*., 2015; Wachira *et al*., 2010). Confounding factors contributing to the choice of *Ae. aegypti* and other mosquito species could be that the breeding sites contain well-established communities of microbiota (Diaz-Nieto *et al*., 2016; Scolari *et al*., 2019; Souza *et al*., 2019).

To investigate the mechanism underlying the trade-off between interspecific competition and densities, the effect of *Ae. aegypti* 4th instar conditioned water on the oviposition site choice of gravid *Cx. quinquefasciatus* was studied. Gravid *Cx. quinquefasciatus* preferred to lay egg rafts in the control water, when presented together with water conditioned with different densities of *Ae. aegypti* larvae (**Paper III**). Moreover, gravid *Cx. quinquefasciatus* were prone to retain eggs when exposed to a high dose of a synthetic odour blend based on the bioactive VOCs identified in the headspace of *Ae. aegypti* larval conditioned water (**Paper III**, for details see sections 7.4.2 and 8.4). These findings suggest that the olfactory system of *Cx. quinquefasciatus* is under strong selection to avoid interspecific competition at breeding sites (Carrieri *et al.,* 2003; Santana-Martínez *et al*., 2017) and thus competitive exclusion (Santana-Martínez *et al*., 2017). The overall result emphasises that *Cx. quinquefasciatus* use VOCs to avoid interspecific competition.

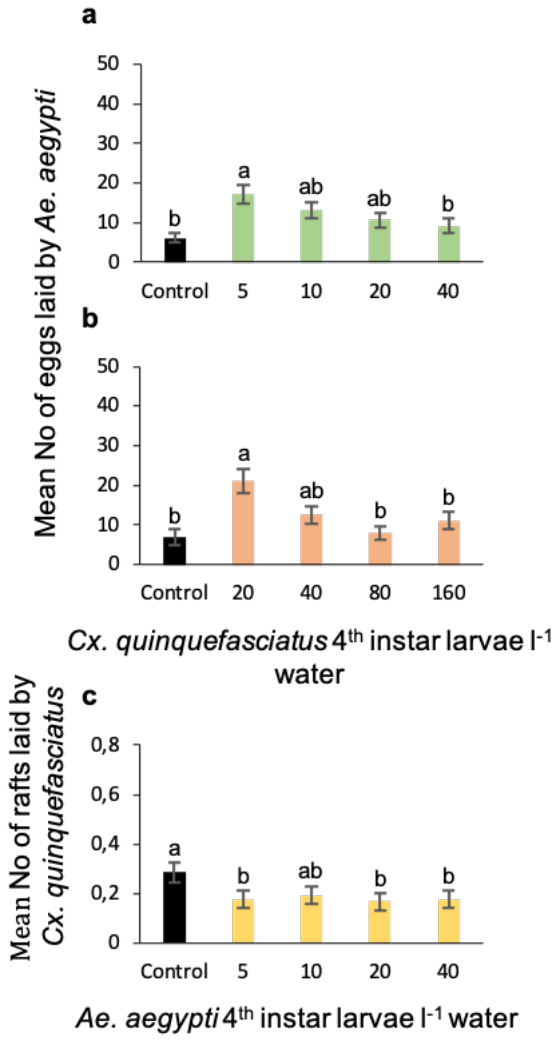

Figure 5. Gravid *Aedes aegypti* and *Culex quinquefasciatus* respond differentially to volatiles associated with water conditioned with heterospecific 4th instar larvae. The number of eggs laid by gravid *Ae. aegypti* (a-b) and egg rafts laid by *Cx. quinquefasciatus* (c) in response to water conditioned with various densities of heterospecific 4th instar larvae in multi-choice assays are presented. The error bars depict the standard error of the mean. Differences in lowercase letters indicate significant differences ($P < 0.05$) (**Paper III**).

8.3 Bacterial density-dependent oviposition site selection (**Paper IV**)

The presence and density of microbiota at the oviposition site affect oviposition site selection of gravid mosquitoes (Caragata *et al*., 2021; Eneh *et al*., 2016; Melo *et al*., 2020; Nilsson *et al*., 2018, 2019). In this study, gravid *Ae. aegypti* were attracted and stimulated to oviposit in a dose-response manner to odorants released from different doses of a bacterium, *Klebsiella* sp., which is inoculated into breeding sites during oviposition, providing a food resource to larvae (Akhouayri *et al*., 2013; Kämpfer *et al*., 2011; Lindh *et al*., 2008a-b; Mosquera *et al*., 2021). Emerging adults pick up the bacteria from the breeding sites, the bacteria then develop in the mosquito gut and subsequently transfer these to the next breeding site (Lindh *et al*., 2008a, Mosquera *et al*., 2021; Rocha *et al*., 2021).

The close association between *Ae. aegypti* and *Klebsiella* sp. suggests a symbiotic association (Girard *et al*., 2021; Mosquera *et al*., 2021). Support for this hypothesis comes from previous studies demonstrating that *Klebsiella* sp. benefit by being transferred between sites by *Ae. aegypti* (Coon *et al*., 2016; Mosquera *et al*., 2021; Scolari *et al*., 2021). The purpose of this study was to investigate the role of *Klebsiella* sp. in providing a reliable signal of a potential oviposition site for *Ae. aegypti*. I demonstrate that gravid *Ae. aegypti* make use of VOCs produced by *Klebsiella* sp. to locate potential breeding sites, and that this response is conserved across at least two wildtype strains (Figs 6-7). These VOCs are detected by the OR pathway, as demonstrated by the lack of oviposition preference and physiological response in *Orco* mutants.

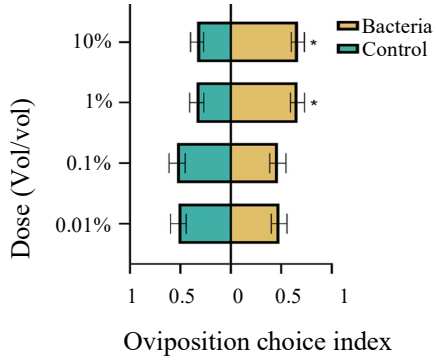

Figure 6. Oviposition response of gravid *Aedes aegypti* to volatile organic compounds emitted by *Klebsiella* sp., culture in a dual choice assay. The *Klebsiella* sp. volatiles emanated from bacteria cultured in 1% TSB and 10% TSB were tested against the respective TSB controls (*, $P < 0.05$). Error bars indicate the standard error of the mean (**Paper IV**).

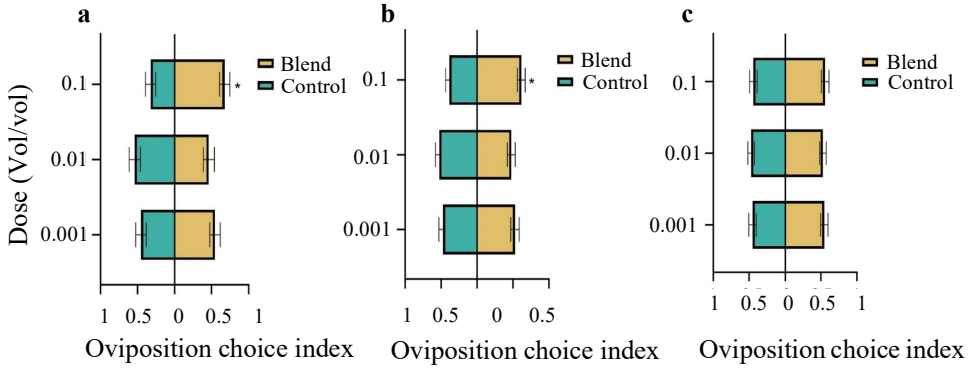

Figure 7. Behavioural response of gravid *Aedes aegypti* to a synthetic binary odour blend associated with *Klebsiella* sp.. At the highest dose tested, the binary blend significantly increased the number of eggs laid by two strains of *Ae. aegypti* (a) Rockefeller and (b) Orlando compared to the solvent (pentane) control in a two-choice assay (*, $P < 0.05$). (c) No preference was observed in *orco* mutant mosquitoes. Error bars denote the standard error of the mean (**Paper IV**).

8.4 Volatile profile dependent on the aquatic stages and density (**Papers II, III, IV**)

Volatile organic compounds associated with intraspecific (Boullis *et al*., 2021; Ganesan *et al*., 2006; Gonzalez *et al*., 2015; Laurence & Pickett, 1982; Mendki *et al*., 2000; Mwingira *et al*., 2020b; **Paper II**; Soman & Reuben, 1970; Suh *et al*., 2016; Sumba *et al*., 2008) and interspecific (Gonzalez *et al*., 2014, Gonzalez *et al*., 2015; Khan *et al*., 2022; Mwingira *et al*., 2021; **Paper III**; Shragai *et al*., 2019; Sumba *et al*., 2008; Zahiri & Rau, 1998) aquatic stages, as well as bacteria (Melo *et al*., 2020; Mosquera *et al*., 2023a, **Paper IV**; Ponnusamy *et al*., 2008a) differentially influence oviposition site choice and egg-laying behaviour of mosquitoes. This thesis presents the systematic workflow to identify the bioactive VOCs associated with intra- and interspecific aquatic stages of *Ae. aegypti* and *Cx. quinquefasciatus*, as well as the commensal bacteria, *Klebsiella* sp., and demonstrates how synthetic blends of these VOCs regulate oviposition site selection and egg laying (**Papers II, III, IV**).

Gravid *Ae. aegypti* responded predominantly to two different classes of compounds, including straight-chain aldehydes and monoterpenes, associated with intraspecific aquatic stages (**Paper II**) (Table 1). Of these, nonanal and decanal have previously been identified from 4th instar *Ae. aegypti* larvae in the field (Xia *et al*., 2021b). These aldehydes, as well as the monoterpene camphor, have also been identified in emanates of grass infusions, rice plants and grass pollen, which are closely associated with the breeding sites of *Cx. quinquefasciatus* and *An. arabiensis*, respectively (Du & Millar, 1999; Millar *et al*., 1992; Wondwosen *et al*., 2017, 2018). While previous studies have identified straight-chain fatty acids and esters to be associated with intraspecific eggs and larvae of *Ae. aegypti* (Ganesan *et al*., 2006; Ong & Jaal, 2015; Wang *et al*., 2019), these were not found to elicit an electrophysiological response in this study. One reason for this could be differences in methodology, as earlier studies used the eggs and larvae directly for the identification of VOCs (Ganesan *et al.*, 2006; Ong & Jaal, 2015; Wang *et al*., 2019), whereas in this study water conditioned with the aquatic stages was used (**Paper II**).

The VOCs associated with the water conditioned with 4th instar larvae affect the oviposition site choice of interspecific gravid *Ae. aegypti* and *Cx.*

quinquefasciatus in a species-specific manner (**Paper III**) (Table 2-3). The abundance of VOCs emitted by 4th instar *Cx. quinquefasciatus* larvae increased in a density-dependent manner, resulting in differential detection of these VOCs by the antenna of *Ae. aegypti*. Out of the ten bioactive VOCs identified to be associated with *Ae. aegypti* larvae, only five (C_{7-11}) were found in extracts collected from larvae at low density. The remaining VOCs were C_{7-14} straight-chain and methyl-branched alkanes. *Culex quinquefasciatus* responded to ketones present in the extracts collected from 4th instar larvae of *Ae. aegypti* (**Paper III**). Of the C_{7-14} VOCs identified in this study, the straight-chain and methyl-branched alkanes (4-methyldecane, undecane, 2,6-dimethylundecane, methyl dodecane and tetradecane) have previously been identified from breeding water containing conspecific larvae of *Ae. aegypti* in the field (Xia *et al*, 2021b), and from their microbial communities (Heenan-Daly *et al*., 2021). Of note, the position of the methyl group in these compounds has not been confirmed, which requires further investigation.

The VOCs produced by the commensal *Klebsiella* sp. likely provide an honest signal of food availability for the offspring of gravid *Ae. aegypti*. Gravid *Ae. aegypti* transfer *Klebsiella* sp., both inactively and actively to a breeding site prior to or during oviposition (Mosquera *et al*., 2021). A binary odour blend of the two compounds, 2-ethyl hexanol and 2,4-di-tert-butylphenol, identified to be detected by the female antenna was sufficient to elicit oviposition in gravid *Ae. aegypti* of two wild-type strains, Rockefeller and Orlando (**Paper IV**). Knockout of the odorant receptor co-receptor, *orco*, revealed that these VOCs are detected by the OR pathway similar to what has been shown by Melo *et al*., (2020).

Of the rich repertoire of ORs in *Ae. aegypti*, only two, OR49 and OR117, are known to bind to one of the VOCs identified in this thesis, camphor (Nalikkaramal *et al*, *in prep*; Vainer *et al*., 2023). There is therefore a need to deorphanise additional olfactory receptors, starting off with those that are highly expressed following a blood meal (Hill *et al*., 2021), to elucidate the molecular mechanisms regulating oviposition site selection and choice.

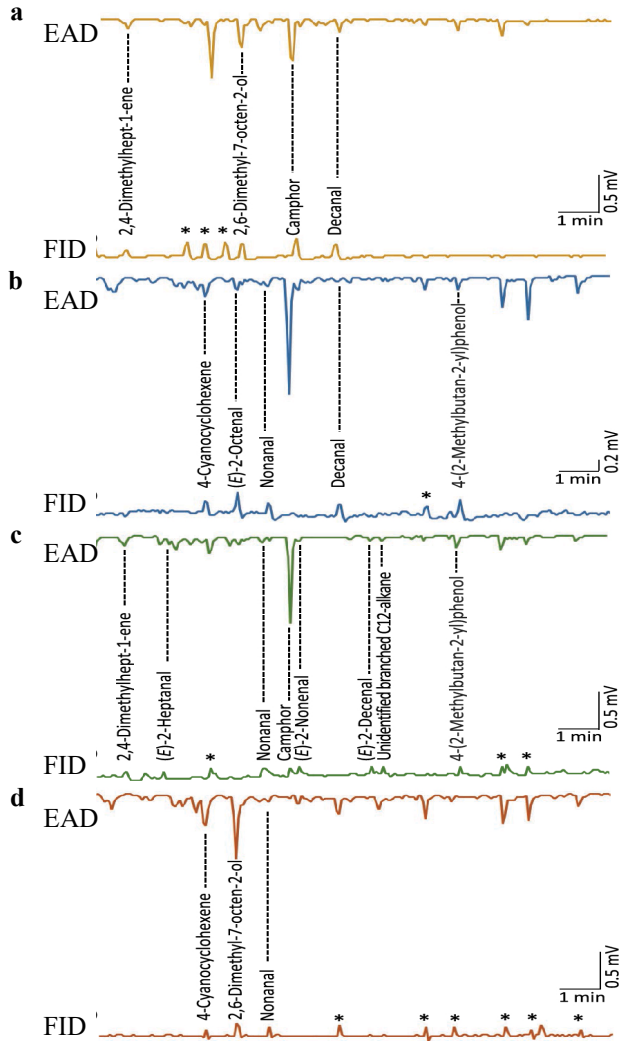

Figure 8. Physiological responses of gravid *Aedes aegypti* to bioactive volatile organic compounds associated with water conditioned with conspecific aquatic stages. Gas chromatography, using flame ionization detection (FID) and electroantennographic detection (EAD) analyses of eggs (a), 2nd instar larvae (b), 4th instar larvae (c) and pupal exuviae (d), demonstrate antennal responses of *Ae. aegypti* (mV) to bioactive volatile organic compounds in the headspace of conspecific immature-conditioned water eluting over time (min) from the gas chromatograph. Asterisks in the EAD traces represent compounds that were also present in the control SPME headspace.

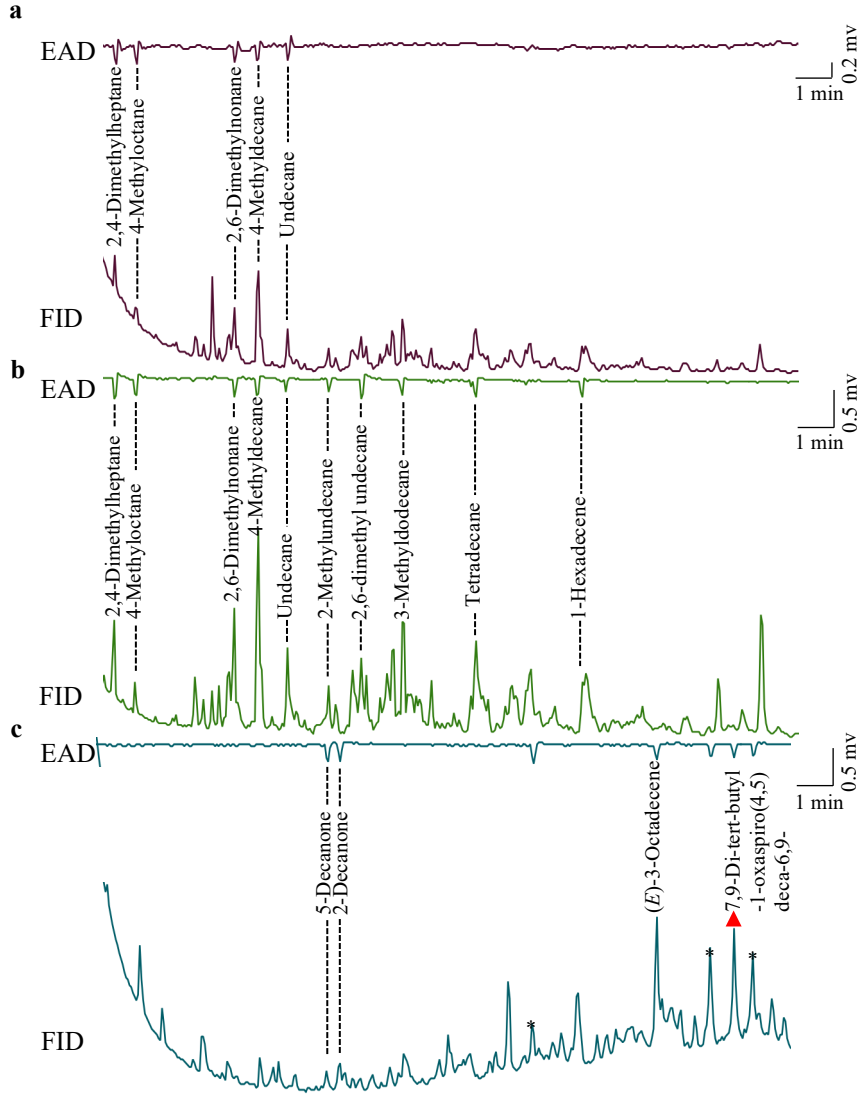

Figure 9. Physiological responses of *Aedes aegypti* and *Culex quinquefasciatus* to volatile organic compounds associated with heterospecific 4th-instar larvae. Gas chromatography, using flame ionization detection (FID) and electroantennographic detection (EAD) analyses demonstrate antennal responses of *Ae. aegypti* (a-b) and *Cx. quinquefasciatus* (c) (mV) in response to the bioactive compounds from solid phase extraction of water conditioned with heterospecific 4th instar larvae, eluting over time (min). Asterisks in the GC-EAD traces represent compounds that were also present in the control extracts.

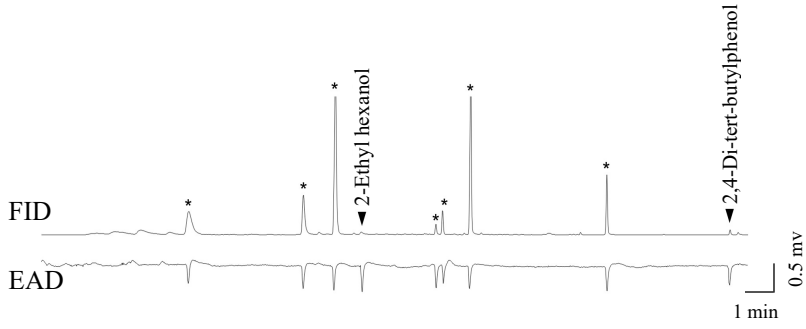

Figure 10. Physiological responses of gravid *Aedes aegypti* to volatile organic compounds emitted from *Klebsiella* sp.. Gas chromatography, using flame ionization detection (FID) and electroantennographic detection (EAD) analyses demonstrate antennal responses of *Ae. aegypti* (mV) in response to the bioactive compounds in the headspace of *Klebsiella* sp., eluting over time (min) from the gas chromatograph. Asterisks represent the VOCs that were also present in the control SPME headspace.

9. Concluding remarks and future perspectives

In this thesis project, I have shown that *Ae. aegypti* and *Cx. quinquefasciatus* regulate oviposition site choice and egg laying in a density-, stage- and species-specific manner in response to qualitative and quantitative differences in the VOCs emanating from water conditioned with intra- and interspecific aquatic stages, as well as to VOCs released by commensal *Klebsiella* sp., (**Papers II-IV**). There are still many open questions that need to be investigated to understand the behavioural, chemical and molecular basis of mosquito oviposition. Of key interest for me would be to elucidate the physiological mechanism regulating the retainment of eggs in *Cx. quinquefasciatus* when exposed to the VOCs of *Ae. aegypti* aquatic stages. From a chemical analytical perspective, additional analysis is required to identify the regioisometry of the branched hydrocarbons, to gain an insight into the function of these novel VOCs in regulating mosquito behaviour. Moreover, further analysis of the VOCs emanating from aquatic stages, particularly at high densities, may allow for an additional understanding of how oviposition site selection is regulated. Based on the findings from the studies presented in this study, along with these focal experiments, we may be able to develop efficient lures that can be used in combination with other tools in future integrated vector control programmes.

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Popular science summary

Mosquitoes are considered the deadliest animals in the world, and may transmit serious diseases, such as malaria, dengue fever, chikungunya, Zika and West Nile fever, when they bite humans and other vertebrates to obtain blood meal needed for egg development. The yellow fever mosquito, *Aedes aegypti* alone, puts ca. 3.9 billion people at risk of serious illness or death from mosquito-borne viral diseases, of which there are approximately 100 million new cases reported each year. Finding new ways to reduce the risks associated with these diseases include targeting mosquitoes ready to lay their eggs near human habitation, and requires a better understanding of the biology and chemical ecology of these, and other, mosquito species, in order to interrupt the mosquito life cycle.

Egg-laying mosquitoes use physical and chemical signals to detect and discriminate among potential breeding sites. The chemical substances to which egg-laying are attracted and stimulated to lay eggs, called attractants and stimulants, induce gravid females to move towards the egg-laying substrate, located above or adjacent to the water sources and then triggers egg laying. In contrast, other signals may deter or repel mosquitoes away from potential breeding sites. These signals could potentially be used for developing novel mosquito control tools.

The oviposition preference of mosquitoes varies and depends on the presence and abundance of certain biotic factors including intra- and interspecific aquatic stages as well as bacteria in the breeding site. In this thesis, I demonstrate that *Ae. aegypti* preferentially lay eggs in sites with low densities of intraspecific aquatic stages and are differentially stimulated to lay eggs in response to specific odours associated with the various aquatic

stages. Similarly, gravid *Ae. aegypti* preferred to lay eggs in sites releasing odours associated with low densities of late-stage larvae of the Southern house mosquito, *Culex quinquefasciatus.* In contrast, *Cx. quinquefasciatus* were deterred to lay eggs, and even retained from egg laying, in the presence of odours release from late-stage *Ae. aegypti* larvae. These studies emphasise that egg-laying mosquitoes respond in a species-specific manner to intra- and interspecific odours that directly signal the level of competition, and regulate niche separation and competitive exclusion.

Bacteria constitute the major food resource for mosquitoes, and may be actively or passively transferred to a breeding site prior to or during egg laying. Egg laying *Ae. aegypti* have co-evolved with bacteria of the genus *Klebsiella*. In this study, I demonstrate that one species of *Klebsiella* emits odours that attract and stimulate females searching for a site to lay eggs. These odours strengthen the commensal relationship between mosquito and bacteria. Of the three possible olfactory pathways detecting these odorants, the odorant receptor pathway was determined to regulate this behaviour.

Overall, this PhD thesis sheds light on several biotic aspects of odour-mediated oviposition site selection in mosquitoes. These findings provide novel information on the role of the volatile organic odour compounds regulating this behaviour. Some of these compounds may be used to improve future vector control programmes by targeting egg-laying mosquitoes, and thereby limit vector populations and reduce the burden of mosquito-borne diseases.

Populärvetenskaplig sammanfattning

Myggor anses vara de dödligaste djuren i världen och kan överföra allvarliga sjukdomar, såsom malaria, denguefeber, chikungunya, Zika och West Nile-feber, när de biter människor och andra ryggradsdjur för att få blod som mat, som behövs för äggutveckling. Gula febermyggan, *Aedes aegypti* ensam, riskerar ca. 3,9 miljarder människor att drabbas av allvarlig sjukdom eller att dö av myggburna virussjukdomar, varav cirka 100 miljoner nya fall rapporteras varje år. Att hitta nya sätt att minska riskerna förknippade med dessa sjukdomar inkluderar inriktning på myggor som är redo att lägga sina ägg nära människors bostad, och kräver en bättre förståelse av biologin och den kemiska ekologin hos dessa och andra myggarter för att kunna avbryta mygglivet cykel.

Äggläggande myggor använder fysiska och kemiska signaler för att upptäcka och särskilja potentiella häckningsplatser. De kemiska ämnen som äggläggning lockas till och stimuleras att lägga ägg, som kallas lockmedel och stimulanser, får gravida honor att röra sig mot äggläggningssubstratet, som ligger ovanför eller intill vattenkällorna och utlöser sedan äggläggning. Däremot kan andra signaler avskräcka eller stöta bort myggor från potentiella häckningsplatser. Dessa signaler kan potentiellt användas för att utveckla nya myggkontrollverktyg.

Äggläggningspreferensen för myggor varierar och beror på förekomsten och förekomsten av vissa biotiska faktorer inklusive intra- och interspecifika akvatiska stadier samt bakterier i häckningsplatsen. I denna avhandling visar jag att *Ae. aegypti* lägger företrädesvis ägg på platser med låg täthet av intraspecifika akvatiska stadier och stimuleras differentiellt att lägga ägg som svar på specifika lukter associerade med de olika akvatiska stadierna. Likaså

gravid *Ae. aegypti* föredrog att lägga ägg på platser som släppte ut lukter associerade med låg täthet av larver i sent skede av den södra husmyggan, *Culex quinquefasciatus*. Däremot har *Cx. quinquefasciatus* avskräcktes från att lägga ägg, och till och med behölls från äggläggning, i närvaro av lukt som frigjordes från sent stadium av *Ae. aegypti* larver. Dessa studier betonar att äggläggande myggor svarar på ett artspecifikt sätt på intra- och interspecifika lukter som direkt signalerar konkurrensnivån och reglerar nischseparation och konkurrensutslagning.

Bakterier utgör den största födotillgången för myggor och kan aktivt eller passivt överföras till en häckningsplats före eller under äggläggning. Äggläggning *Ae. aegypti* har utvecklats tillsammans med bakterier av släktet *Klebsiella*. I den här studien visar jag att en art av *Klebsiella* avger lukter som lockar och stimulerar honor som söker efter en plats för att lägga ägg. Dessa lukter stärker det kommensala förhållandet mellan mygga och bakterier. Av de tre möjliga luktvägarna som upptäcker dessa luktämnen, bestämdes luktämnesreceptorvägen för att reglera detta beteende.

Sammantaget belyser denna doktorsavhandling flera biotiska aspekter av luktmedierat val av äggläggningsplatser hos myggor. Dessa fynd ger ny information om rollen av de flyktiga organiska luktföreningarna som reglerar detta beteende. Vissa av dessa föreningar kan användas för att förbättra framtida vektorkontrollprogram genom att rikta in sig på äggläggande myggor och därigenom begränsa vektorpopulationer och minska bördan av myggburna sjukdomar

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Appendix

Table 1. Physiologically bioactive volatile organic compounds identified from conspecific stage-conditioned water through GC-EAD and GC-MS analyses, and used for bioassays.

Retention time	Retention indices	Compound	Purity (%)
6.56	839	2,4-Dimethylhept-1-ene	99.4
8.76	957	(*E*)-2-Heptenal	95.3
10.04	1023	4-Cyanocyclohexene	97.9
10.74	1059	(*E*)-2-Octenal	95
10.89	1072	2,6-Dimethyl-7-octen-2-ol	100
11.64	1105	Nonanal	99.4
12.50	1156	Camphor	99
12.70	1162	(*E*)-2-Nonenal	97.6
13.54	1207	Decanal	99.5
14.57	1264	(*E*)-2-Decenal	96.4
14.88	1281	Unknown	
16.93	1402	4-(2-Methylbutan-2-yl)phenol	99.8

Table 2. Bioactive VOCs in *Culex quinquefasciatus* 4th instar conditioned water detected by *Aedes aegypti* using GC-EAD and GC-MS.

Retention time	Retention index	Compound	Purity (%)
6.98	818	2,4-Dimethylheptane	98
7.77	861	4-Methyloctane	99.7
10.95	1024	2,6-Dimethylnonane	–
11.71	1063	4-Methyldecane	99.5
12.48	1101	Undecane	99.9
13.69	1166	2-Methylundecane	99.5
14.63	1217	2,6-Dimethylundecane	97.3
15.79	1282	3-Methyldodecane	99.3
17.83	1402	Tetradecane	99.9
20.86	1594	1-Hexadecene	99.8

Table 3. Bioactive VOCs in *Aedes aegypti* 4th instar conditioned water detected by *Culex quinquefasciatus* using GC-EAD and GC-MS.

Retention time	Retention index	Compounds	Purity (%)
13.92	1174	Decan-5-one	99
14.28	1192	Decan-2-one	98.2
23.55	1786	(*E*)-3-Octadecene	–
25.57	1943	7,9-Ditert-butyl-1-oxaspiro [4,5] deca-6,9-diene-2,8-dione*	93

*Likely a contaminant

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Odour-mediated oviposition-site selection by mosquitoes

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Running header: Odour-mediated oviposition-site selection by mosquitoes

Abstract
The exploration of the chemical ecology of oviposition behaviour in mosquitoes provides the means to describe the role of volatile organic compounds in securing intrinsic fitness by gravid mosquitoes. This is done by using odour-mediated selection for sites, which minimises larval mortality and maximises growth rate through decreased competition and predation, as well as increased access to food resources. Oviposition sites and their surrounding habitats are rich sources for odours. Identifying which of these odorants gravid mosquitoes are using as oviposition site cues, and in which combinations, has been under investigation for almost a century. With the advent of techniques, including combined chemical and electrophysiological detection, functional genomics and reverse chemical ecology, the screening of these vast natural resources has accelerated and provided the approaches necessary to unravel the mechanisms that regulate mosquito oviposition-site selection. The use of attractive and stimulating odorant blends as lures to surveille and control gravid and ovipositing mosquitoes has long been advocated, and with the recent advancements in chemical ecology, may soon become a reality.

Keywords: Culicidae, olfaction, semiochemicals, behaviour, vector control

Introduction

Oviposition-site selection is an essential stage in the life history of mosquitoes, and a critical factor for the development and survival of offspring, and for the maintenance of mosquito populations (Bentley and Day, 1989; Day, 2016). Selection and assessment of suitable oviposition sites are dependent on maternal choice of breeding sites, which decide the fate of the next-generation, as immature stages of mosquitoes are limited in their ability to migrate between sites. Life history theory suggests that female fitness is maximised by female mosquitoes selecting sites that minimise larval mortality and maximise growth rate (Silberbush *et al.,* 2019; Vonesh and Blaustein, 2010). To do so, gravid females locate and discriminate among potential oviposition sites by refining their search over multiple spatial scales. The ultimate selection of an oviposition site is dependent on the availability of larval nutrients, as well as the level of competition and predators. In this chapter, we present the current knowledge on the odour-mediated oviposition behaviour in mosquitoes, and how this information can be used to develop novel vector control tools.

Notes on terminology and assays

To gain an accurate understanding of the state of field of odour-driven oviposition in mosquito research, a description of the predominant methods and terminology used, as well as the limitations of these, requires explication. The most common workflow, which was established by Crumb in 1924, and subsequently refined, begins with the collection of volatile extracts in or surrounding a natural source, *e.g*., eggs, breeding water and breeding site, and confirming the oviposition response of a single mosquito species. Subsequently, chemical analysis identifies the individual volatile organic compounds (VOCs) present in the extract. These VOCs, with priority often given to those in high abundance, are then individually tested behaviourally in laboratory or field oviposition assays, on several mosquito species, often by different researchers. The measure of oviposition response is most often the presence of deposited eggs in a no-choice or a two-choice assay. Long-range and short-rang attraction are usually described as the difference in the arrival of mosquitoes to the oviposition site in the field or in a cage in the laboratory, respectively, in response to the tested VOC. Stimulation differs from attraction as the contact chemosensory event that leads to egg laying. It is important to note that discrimination between attraction and stimulation is not often clarified in the literature. The repellence of a compound is deemed as the avoidance of odours from an oviposition site in the assays described above, while deterrence is the avoidance of contact stimuli. Since these are the general terms used in this research field, we will continue to use them in this chapter. For a more in-depth description of the terminology surrounding attraction, stimulation, repellence and deterrence, please see Hinze *et al*. (2022) and Carrasco *et al*. (2022). The benefits and drawbacks of this workflow are discussed below.

Cues regulating oviposition-site selection

Gravid mosquitoes select an oviposition site in the landscape depending on innate preferences, regulated by internal and external chemical signals (*e.g*., Afify *et al*., 2015; Day, 2016; Xia *et al.,* 2021). Different genera of mosquitoes occupy different niches, encompassing a wide range of breeding sites, *e.g.*, tree-holes, artificial water containers, discarded tires, stagnant pools, irrigation ditches and marshes (*e.g.,* Gimnig *et al.,* 2001; Lounibos, 1981; Xia *et al.,* 2021). Such sites emit both habitat- and fitness-related volatile signals for the gravid mosquito. Food resources within these sites affect larval development and survival, as well as adult behaviour and associated modulation of vectorial capacity (Araújo *et al.,* 2012; Kebede

et al., 2005; Merritt *et al.,* 1992; Moller-Jacobs *et al.,* 2014; Ye-Ebiyo *et al.,* 2000, Ye-Ebiyo *et al.,* 2003). Furthermore, natural and artificial breeding sites are often found in proximity with vegetation, as well as containing conspecific aquatic stages, competitors and predators, which may modulate the decision of a gravid mosquito (*e.g*., Mwingira *et al.,* 2020a). Here, we present current information about the source, role and nature of the VOCs that have been identified for the major disease vector mosquitoes.

Cues associated with conspecific and heterospecific aquatic stages

Gravid female mosquitoes use chemical cues emitted by immature conspecific and heterospecific stages to locate and discriminate among breeding sites (Allan and Kline, 1998; Faierstein *et al.,* 2019; Gonzalez *et al.,* 2015; Mwingira *et al.,* 2020b; Shragai *et al.,* 2019; Soman and Reuben, 1970; Wong *et al.,* 2011; Zahiri and Rau, 1998). While the act of assessing the breeding site for suitability, over-crowding and competition from aquatic stages is conserved in mosquitoes, which stages and species contribute to the salient signals vary across taxa (McCrae, 1984; Williams *et al.,* 2008; Yadav *et al.,* 2017; Zahiri and Rau, 1998). Here, the life history traits of the mosquito appear to play a significant role in which aquatic stages provide salient information about the suitability of the breeding site (Ellis, 2008; Wong *et al.,* 2011).

Egg-associated cues appear to be used differentially by mosquito taxa (Ganesan *et al.,* 2006; Laurence and Pickett, 1982; Laurence and Pickett, 1985; Ong and Jaal, 2015; Roberts, 2021; Wang *et al.,* 2019). While gravid culicine mosquitoes discriminate and select oviposition sites based on the presence and density of conspecific and heterospecific mosquito eggs (Table 1) (Allan and Kline, 1998; Dhileepan, 1997; Nakamura, 1978; Onyabe and Roitberg, 1997; Roberts, 2021; Wachira *et al.,* 2010; Williams *et al.,* 2008), anopheline mosquitoes appear to disregard the presence of conspecific, but not all heterospecific, eggs during oviposition site selection (Sumba *et al.,* 2008; Wachira *et al.,* 2010).

Table 1: Effects of oviposition cues from the aquatic stages on the response of gravid females

Source of cue Stage	Species	OAI	Compounds	Cas number	Extract	References
	Cx. quinquefasciatus	+	(-)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide	85551-39-9	Synth	Laurence and Pickett, 1982[a]; Laurence and Pickett, 1985[a]; Laurance *et al*., 1985[a], Machiya *et al*., 1985[a], Dawson *et al*., 1990[a]
	Cx. tarsalis	+	(-)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide	85551-39-9	Synth	Hwang *et al*., 1987[a]
Eggs	*Ae. aegypti*	+	(Z)-9-hexadecenoic acid	373-49-9	MeOH	Ganesan *et al*., 2006[a]
		-	(Z)-9-octadecenoic acid	112-80-1	MeOH	Ganesan *et al*., 2006[a]
		+	6-hexanolactone	502-44-3	MeOH	Ganesan *et al*., 2006[a]
		+	Dodecanoic acid	143-07-7	MeOH	Ganesan *et al*., 2006[a]
		+	Hexadecanoic acid	1957-10-03	MeOH	Ganesan *et al*., 2006[a]
		-	Methyl (Z)-9-hexadecenoate	1120-25-8	MeOH	Ganesan *et al*., 2006[a]
		-	Methyl dodecanoate	111-82-0	MeOH	Ganesan *et al*., 2006[a]
		-	Methyl hexadecanoate	112-39-0	MeOH	Ganesan *et al*., 2006[a]
		-	Methyl octadecanoate	112-61-8	MeOH	Ganesan *et al*., 2006[a]
		-	Methyl tetradecanoate	124-10-7	MeOH	Ganesan *et al*., 2006[a]
		-	Methyl-(Z)-9-octadecenoate	112-62-9	MeOH	Ganesan *et al*., 2006[a]
		+	Octadecanoic acid	1957-11-04	MeOH	Ganesan *et al*., 2006[a]
		+	Tetradecanoic acid	544-63-8	MeOH	Ganesan *et al*., 2006[a]
		-	Hexadecanoic acid	1957-10-03	AOAC	Ong and Jaal, 2015[a]
		-	(Z)-9-hexadecenoic acid	373-49-9	AOAC	Ong and Jaal, 2015[a]
		+	Hexanoic acid	42-62-1	AOAC	Ong and Jaal, 2015[a]
		ns	Tridecanoic acid	638-53-9	AOAC	Ong and Jaal, 2015[b]; Hwang *et al*., 1982[c]
		ns	Decanoic acid	334-48-5	AOAC	Ong and Jaal, 2015[b]; Hwang *et al*., 1982[c]

			-	Butanoic acid	107-92-6	AOAC	Ong and Jaal, 2015[b]; Kramer *et al*., 1980[c]; Hwang *et al*., 1980[c]
			-	9-tetradecanoic acid	544-64-9	AOAC	Ong and Jaal, 2015[b]; Wang *et al*., 2019[b]; Boullis *et al*., 2021[c]
Larvae	L1-4	*Ae. aegypti*	-	3-methylbutanoic acid	503-74-2	Hexane	Wang *et al*., 2019[b]; Boullis *et al*., 2021[c]
	L1-4		+	Tetradecanoic acid	544-63-8	Hexane	Wang *et al*., 2019[b]; Boullis *et al*., 2021[c]
	L1-4		-	9-tetradecanoic acid	544-64-9	Hexane	Wang *et al*., 2019[b]; Boullis *et al*., 2021[c]
	L1-4		+	pentadecanoic acid	1002-84-2	Hexane	Wang *et al*., 2019[b]; Boullis *et al*., 2021[c]
	L1-4		+	dodecanoic acid	143-07-7	Hexane	Wang *et al*., 2019[b]; Ganesan *et al*., 2006[c]
	L1-4		ns	tridecanoic acid	638-53-9	Hexane	Wang *et al*., 2019[b]; Hwang *et al*., 1982[c]
Larval-conditioned water	L1-L4 (20 d)	*Ae. aegypti*	+	Heneicosane	629-94-7	Hexane	Mendki *et al*., 2000[a]; Seenivasigan *et al*., 2009[a]; Gonzalez *et al*., 2014[a]
	forest, periurban		+	Heneicosane	629-94-7	HS	Xia *et al*., 2021[b]; Mendki *et al*., 2000[c]; Seenivasigan *et al*., 2009[c]
	urban		+	Nonanal	124-19-6	HS	Xia *et al*., 2021[b]; Eiras *et al*., 2010[c]
	L3, L4	*Ae. albopictus*	+	Heneicosane	629-94-7	Hexane	Gonzalez *et al*., 2014[a]
	L3, L4	*An. coluzzii*	-	Dimethyldisulfide	624-92-0	HS	Suh *et al*., 2016[a]
	L3, L4		-	Dimethyltrisulfide	3658-80-8	HS	Suh *et al*., 2016[a]
	L3, L4		-	Sulcatone	110-93-0	HS	Suh *et al*., 2016[a]
	L1	*An. gambiae*	+	2,4-pentanedione	123-54-6	HS	Schoelitsz *et al*., 2020[a]
	L4		-	Dimethyldisulfide	624-92-0	HS	Schoelitsz *et al*., 2020[a]
	L4		-	Dimethyltrisulfide	3658-80-8	HS	Schoelitsz *et al*., 2020[a]
	L1		+	Nonane	111-84-2	HS	Schoelitsz *et al*., 2020[a]
	field	*Cx. p. fatigans* /* *Cx. p. pallens*	+	2-3-methyl butoxy ethanol	7521-79-1	Distillation	Ikeshoji, 1968[a]
	field		+	2-hexyloxy ethanol	112-25-4	Distillation	Ikeshoji, 1968[a]
	field		+	2-octyloxyethanol	27252-75-1	Distillation	Ikeshoji, 1968[a]
	field		+	1,2-diethoxyethane	629-14-1	Distillation	Ikeshoji, 1968[a]

L larval stage, d days, *species as reported, + attraction/stimulation, - aversion, ± attraction/stimulation with aversion at high concerntration, ns no significant response, C compound, B blend, E extract, HS head space, MeOH methanol extraction, AOAC fatty acid extraction (AOAC 1995), [a]includes identification and behaviour, [b]only identification, [c]only behaviour

The behavioural response of gravid culicines to eggs is plastic, *i.e.*, has the capacity for modulation, and is affected by site availability and pre-existing egg density (Allan and Kline, 1998; Apostol *et al.,* 1994; Chadee *et al.,* 1990; Nakamura, 1978; Williams *et al.,* 2008; Wachira *et al.,* 2010; Wasserberg *et al.,* 2014), which is, in turn, regulated by VOCs (Boullis *et al.,* 2021; Ganesan *et al.,* 2006; Hwang *et al.,* 1982; Hwang *et al.,* 1987; Laurence and Pickett, 1985; Ong and Jaal, 2015; Wang *et al.,* 2019) (Table 1).

Perhaps overly-ambitiously named, the 'mosquito oviposition pheromone' (MOP), (-)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide, is a major component of the *Cx. quinquefasciatus* oviposition-aggregation pheromone (Bruno and Laurence, 1979; Laurence and Pickett, 1982; Laurence and Pickett, 1985; Pickett and Woodcock, 1996). MOP was the first egg-associated VOC affecting oviposition site preference to be identified from the maternally derived apical droplet (Laurence and Pickett, 1985). MOP attracts and stimulates egg-laying in *Cx. quinquefasciatus* and other *Culex* species, albeit at higher thresholds of activity, suggesting that minor pheromone components may play a role in species specificity (Bruno and Laurence, 1979; Hwang *et al.,* 1987). While putative secondary components, including methyl esters, fatty acids and fatty acid esters, have been identified in active fractions collected from the apical droplet of *Culex* mosquitoes, these have yet to be assessed behaviourally (Starratt and Osgood, 1972). This potential missing piece to *Culex* oviposition pheromones may be due to the intractability of working with these secondary compounds (*e.g*., technical issues, including availability, synthesis and cost) or it may be that these components have been of less interest to researchers whose focus has been on identifying VOCs for inexpensive and broadly-tuned gravid mosquito lures.

There is no maternally derived apical droplet evident on the eggs from the genus *Aedes*, however, long-chain aliphatic acids and fatty acid esters associated with eggs laid by *Ae. aegypti* have been shown to regulate the attraction and stimulation of oviposition in conspecifics (Boullis *et al.,* 2021; Ganesan *et al.,* 2006; Hwang *et al.,* 1982; Ong and Jaal, 2015). Previously, Allan and Kline (1998) determined that gravid *Ae. albopictus* did not modulate their oviposition-site selection in the presence of *Ae. aegypti* eggs. Taken together these results suggest that egg-associated VOCs in *Ae. aegypti* may act as egg pheromones. However, these results should be considered with a critical eye when compared with the biological activity of MOP, which attracts gravid *Cx. quinquefasciatus* and stimulates oviposition at a threshold 10 000 times lower than that tested for the *Ae. aegypti* egg-associated VOCs, indicating that lower doses remain to be investigated. The demonstrated Allee effect, the loss of attraction and recruitment of deterrence with the increasing dose, of several of these compounds, correlates with the reduction in oviposition preference in response to sites overcrowded with eggs (Williams *et al.,* 2008).

Larvae-associated cues from conspecific larvae and larval-conditioned water affect the oviposition behaviour of gravid culicines (Allan and Kline, 1998; Boullis *et al.,* 2021; Fonseca *et al.,* 2015; Mokany and Shine, 2003; Onyabe and Roitberg, 1997; Soman and Reuben, 1970; Xia, 2021; Zahiri *et al.,* 1997; Zahiri and Rau, 1998) and anophelines (Mwingira *et al.,* 2020b; Schoelitsz *et al.,* 2020; Suh *et al.,* 2016; Sumba *et al.,* 2008) during oviposition-site selection (Table 1). Mosquitoes of both taxa display density- and stage-dependent responses to conspecific larvae, preferring to oviposit on water that contains/contained early instar conspecific larvae in conditions that are not overcrowded (Gonzalez *et al.,* 2015; Mwingira *et al.,* 2020; Rey and O'Connell, 2014; Shragai *et al.,* 2019; Zahiri and Rau, 1998). A similar effect has been observed in the response to heterospecific larvae and larval water (Gonzalez *et al.,* 2015; Shragai *et al.,* 2019; Wachira *et al.,* 2010; Zahiri *et al.,* 1997; Zahiri and Rau 1998). The behavioural response of gravid mosquitoes to conspecific larvae may, however, be affected by the presence of pathogens and parasites (Mitchell-Foster *et al.,* 2012; Schwab *et al.,* 2003; Zahiri *et al.,* 1997; Zahiri and Rau 1998). Both culicines and anophelines select potential breeding sites where immature larvae are, or have previously been, present, which suggests that larval-associated VOCs affect oviposition-site choice by gravid females. Whether the immediate presence of the larvae is required for some responses, compared with the larval-conditioned water, has not yet been rigorously investigated. Manipulation of potential breeding sites, however, suggests that the preference for oviposition with respect to conspecific and heterospecific larvae by different species of gravid mosquitoes is likely to be regulated through different channels in the olfactory system (Mokany and Shine, 2003).

Volatile organic compounds and volatile extracts associated with conspecific and heterospecific larvae (Table 1) regulate the oviposition behaviour of gravid culicine and anopheline females dose-dependently at ecologically relevant concentrations and in a taxon-specific manner, reflecting the natural behaviour of gravid mosquitoes to larvae and larval-conditioned water (Boullis *et al.,* 2021; Faierstein *et al.,* 2019; Gonzalez *et al.,* 2014; Mwingira *et al.,* 2021). Similar to that found for eggs, long chain fatty acids and fatty acid esters have been identified from larvae and larval-conditioned water (Boullis *et al.,* 2021; Hwang *et al.,* 1982; Ikeshoji, 1968; Wang *et al.,* 2019), in addition to long chain alkanes and alkane esters, as well as several ketones and sulphides (Ikeshoji, 1968; Suh *et al.,* 2016).

Gravid *Ae. aegypti* and *Ae. albopictus* respond dose-dependently to the larval pheromone component n-heneicosane, identified from the larval cuticle of both species (Gonzalez *et al.,* 2014; Mendki *et al.,* 2000; Seenivasagan *et al.,* 2009). While in these increasingly sympatric species, n-heneicosane is produced by late stage larvae of both species in similar amounts (Gonzalez *et al.,* 2014) and elicits similar dose-response profiles in gravid females (Gonzalez *et al.,* 2014; Seenivasagan *et al.,* 2009), the oviposition response of gravid females to conspecific and heterospecific larvae differ (Allan and Kline, 1998; Gonzalez *et al.,* 2015; Shragai *et al.,* 2019; Zahiri *et al.,* 1997). This indicates that additional larval-associated VOCs are used for species recognition in *Ae. aegypti* and *Ae. albopictus*. Several fatty acids isolated from the larvae of *Ae. aegypti* (Wang *et al.,* 2019) affect oviposition-site selection by gravid conspecifics (Boullis *et al.,* 2021; Hwang *et al.,* 1982), and may, therefore, be candidates for minor larval pheromone components conferring species-specificity for *Ae. aegypti* (Table 1). This is a common strategy employed to achieve species-specificity of a pheromone among closely-related sympatric species (Boullis *et al.,* 2021; Hwang *et al.,* 1982; Mwingira *et al.,* 2021; Schoelitsz *et al.,* 2020).

In distantly-related sympatric species, gravid females can assess the level of heterospecific competition at breeding sites by eavesdropping on the pheromone components released by heterospecific immature stages (Mwingira *et al.,* 2021; Schoelitsz *et al.,* 2020). Nonane, an early larval stage pheromone component of *An. gambiae* (Schoelitsz *et al.,* 2020), attracts and stimulates oviposition in *Cx. quinquefasciatus*, as well as conspecific gravid females, dose-dependently (Mwingira *et al.,* 2021; Schoelitsz *et al.,* 2020). Late larval stage associated components of *An. gambiae*, dimethyldisulphide and dimethyltrisulphide (Schoelitsz *et al.,* 2020), deter oviposition by both *An. gambiae* and *Cx. quinquefasciatus* (Lindh *et al.,* 2008a; Schoelitsz *et al.,* 2020), in a manner consistent with the observed larval densisty-dependent oviposition-site selection demonstrated by both these species (Wachira *et al.,* 2010). While nonane has not been identified as a VOC associated with the larvae of *Cx. quinquefasciatus*, several other VOCs identified from larval-conditioned water, along with several structural analogues, modulate the oviposition-site selection of gravid females (Table 1) (Ikeshoji, 1968; Ikeshoji and Mulla 1974a). Of these, gravid *An. gambiae* oviposits in response to 2-nonanone (Lindh *et al.,* 2008a). Both *An. gambiae* and *Cx. quinquefasciatus* can be found in the same natural breeding sites, which has led to the speculation that there may be competition among the larvae, and that the gravid females may assess the risk of inter-specific competition during oviposition-site selection. Kweka *et al*. (2012) directly assessed the effect of co-habitation in breeding sites by *An. gambiae* and *Cx. quinquefasciatus* in semi-field conditions, and determined that while there was no obvious deleterious effects on either species, *An. gambiae* adult body sizes were smaller in sites with co-habitation compared to those without. Together, the response profiles of these gravid mosquitoes, and the capacity to share breeding sites, indicates that while *An. gambiae* and *Cx. quinquefasciatus* are unlikely to share ancestrally derived larval components, these distantly related species detect heterospecific VOCs and modulate oviposition-site choices with respect to the density and larval stage of both conspecific and heterospecific larvae.

Pupae and pupal exuviae-associated cues elicit culicine, but not anopheline, mosquitoes to preferentially oviposit density-dependently in water (Andreadis, 1977; Consoli and Teixeira, 1988; Faierstein *et al.,* 2019; Soman and Reuben, 1970). In general, gravid females from the three model culicine mosquito species, *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*,

are attracted to water conditioned with extracts from low density of conspecific and heterospecific pupae, however a lack of response by *Ae. aegypti* to the pupal extract of *Ae. albopictus* and the differential responses of gravid females to the variously fractionated extracts, suggests a species-specific difference in the associated volatile profile of *Ae. albopictus* (Faierstein *et al.,* 2019; Marques and Miranda, 1992). In addition, the attraction and stimulation of oviposition in response to conspecific pupae and pupal casings may be, at least in part, a result of the VOCs released from bacteria associated with this non-feeding aquatic stage (Trimble and Wellington, 1980). In keeping with gravid female responses to the other aquatic states, current and/or recent overcrowding at breeding sites, as indicated by the presence of high densities of pupae or pupal exuviae, however, negatively affects culicine oviposition (Glasser, 2011). While these intra- and interspecific behavioural responses are likely regulated by the presence or absence of attractants and deterrents (Andreadis, 1977; Faierstein *et al.,* 2019; Marques and Miranda, 1992), the behaviourally active compounds are currently unidentified.

Competitors and predator associated cues

Ecological theory suggests that gravid mosquitoes select oviposition sites based on minimising larval mortality and maximising growth rate, which translates into choosing sites with high resources, low conspecific and competitor densities, and low predator abundance (Kershenbaum *et al.,* 2012). While this theory is supported by a substantial literature denoting that mosquitoes can detect the presence of larval competitors and predators in breeding sites (Blaustein *et al.,* 2004; Chobu *et al.,* 2015; Stav *et al.,* 2000; Van Dam and Walton, 2008; Why *et al.,* 2016; Why *et al.,* 2021), the interactions among all of these ecological factors is complex and interdependent, and capable of creating a paradox in which gravid mosquitoes are attracted to less optimal oviposition sites (Albeny-Simoes *et al.,* 2014).

Gravid mosquitoes from many species avoid ovipositing eggs in aquatic sites where interspecific competitors are present (Blaustein and Kotler, 1993; Mokany and Shine, 2003; Petranka and Fakhoury, 1991), however, not all species rely on this strategy (Mokany and Shine, 2003). In instances in which gravid females do not regulate oviposition-site selection based on competitor presence, competitor-free breeding sites may be rare to non-existent, *e.g*., *Cx. quinquefasciatus* and tadpoles, rendering competitor detection a potentially unproductive use of energy (Mokany and Shine, 2002). However, the presence of competitors in a breeding site, which also contains shared predators, has the potential to confer a modicum of protection to both competitor species through the increased availability of prey, suggesting that the detection of the cues from a combination of competitors and predators may be of benefit to ovipositing mosquitoes (Bentley and Day, 1989).

Chemosensory cues directly associated with predators deter oviposition by gravid mosquitoes in a species-specific manner (Blaustein *et al.,* 2004; Chobu *et al.,* 2015; Eitam *et al.,* 2002; Munga *et al.,* 2006; Pamplona *et al.,* 2009; Silberbush *et al.,* 2010; Stav *et al.,* 2000; Walton *et al.,* 2009) (Table 2). Predators, however, indirectly modify breeding site environments by reducing competitors and increasing carcasses, thereby increasing the abundance and changing the diversity of microbes via trophic cascade (Carpenter *et al.,* 1985; Stav *et al.,* 2000). In turn, this can signal a site with abundant resources to a gravid mosquito (Hasselschwert and Rockett, 1988; Huang *et al.,* 2006a; Ponnusamy *et al.,* 2015), and induce a paradoxical effect of predator presence on oviposition-site section, particularly in species

which use small and/or ephemeral breeding sites (Albeny-Simoes *et al.,* 2014)**.** Under these conditions, gravid mosquitoes prefer breeding sites with predators, *e.g.*, *Ae. aegypti* and the predatory mosquito larvae *Toxorhynchites theobaldi*, over other sites with lower natural microbial abundance or antibiotic-treated sites containing predators (Albeny-Simoes *et al.,* 2014). As such, larval detection of the chemical signals associated with competitors and predators may induce changes in behaviour and physiology, *e.g.,* increased time near water surface and shorter times to adult emergence, respectively (Silberbush *et al.,* 2019), but a discussion on such adaptations is beyond the scope of this chapter. Increased understanding of the odour-mediated ecological interactions among conspecifics, competitors and predators with the other biotic and abiotic factors contributing to the oviposition-site selection of a gravid mosquito will foster the development of improved vector control tools and enhance our control strategies.

Table 2: Effects of oviposition cues from predators on the response of gravid females

Source of oviposition cue		Species	Bioactivity	Tested	Compounds	Cas number	Extraction	References (a,b,c)
Source	Predator							
Backswimmer conditioned water	*Notonecta maculata*	*Culiseta longiareolata*	-	C	n-heneicosane	629-94-7	HS-SPME	Silberbush *et al.,* 2010[a]
			-	C	n-tricosane	638-67-5	HS-SPME	Silberbush *et al.,* 2010[a]
		Anopheles gambiae G3	ns	C	n-heneicosane	629-94-7	HS-SPME	Silberbush *et al.,* 2010[b], Warburg *et al*., 2011[c]
			ns	C	n-tricosane	638-67-5	HS-SPME	Silberbush *et al.,* 2010[b], Warburg *et al*., 2011[c]
Copepod conditioned water	*Mesocyclopes longisetus*	*Aedes aegypti*	-	P	α-terpinene	99-86-5	Hexane	Torres-Estrada *et al.,* 2001[a]
			-	P	α-copaene	3856-25-5	Hexane	Torres-Estrada *et al.,* 2001[a]
			-	P	α-longipinene	5989-08-02	Hexane	Torres-Estrada *et al.,* 2001[a]
			-	P	α-cedrene	11028-42-5	Hexane	Torres-Estrada *et al.,* 2001[a]
			-	P	δ-cadinene	16729-01-4	Hexane	Torres-Estrada *et al.,* 2001[a]
			-	P	3-carene	13466-78-9	Hexane	Torres-Estrada *et al.,* 2001[a]

- aversion,ns no significant response, C individual compound, P predator, HS head space, SPME solid phase microextraction, [a]includes identification and behaviour, [b]only identification, [c]only behaviour

Habitat and food cues from vegetation

For most mosquito species, the presence of vegetation, in proximity with water, signals a potentially suitable habitat for their offspring (Bentley and Day, 1989; Day, 2016). Vegetation associated with larval habitats can provide the larvae with shelter, nutrition, shade, mitigation against drought and protection from predators (Asmare *et al.,* 2017a; Asmare *et al.,* 2017b; Bentley and Day, 1989). For some mosquito species, vegetation also provides an oviposition substrate (Huang *et al.,* 2006b; Orr and Resh, 1992). Since larvae are unable to migrate across habitats, the selection by a gravid female of an oviposition site is an essential aspect of mosquito fitness. To this end, odours emitted from floral and vegetative plant tissues provide reliable signals for gravid mosquitoes across taxa in locating oviposition sites within a habitat over a range of spatial scales (Asmare *et al.,* 2017a; Torres-Estrada *et al.,* 2005; Torres-Estrada *et al.,* 2007; Wondwosen *et al.,* 2016; Wondwosen *et al.,* 2017; Wondwosen *et al.,* 2018).

Different classes of oviposition VOCs have been identified from vegetation, including aldehydes, esters and terpenoids (Bokore *et al.,* 2021; Wondwosen *et al.,* 2016; Wondwosen *et al.,* 2017; Wondwosen *et al.,* 2018) (Table 3). Gravid mosquitoes can use volatile cues from vegetation in the landscape to orient and move into areas that are likely to include larval habitats, *e.g*., irrigated crops, wetlands and marshes, microdams, phytotelmata and urban

slum zones (Asmare *et al.,* 2017a; Ayllón *et al.,* 2018; Dejenie *et al.,* 2011). Moreover, the association of diverse mosquito disease vector species with humans has been attributed to changes in vegetation and water distribution in the landscape (Rose *et al.,* 2020; White *et al.,* 2011; Ye-Ebiyo *et al.,* 2000). Many landscape modifications meant to improve human quality of life, *e.g*., agriculture, water-sensitive urban design, water treatment and water flow regulation, create and enhance existing vegetation-associated aquatic habitats that are highly suitable as mosquito breeding sites. Anopheline larvae are often found in habitats dominated by vegetation, particularly wild and domesticated grasses belonging to the Poaceae family (Asmare *et al.,* 2017a; Bokore *et al.,* 2021; Hernandez *et al.,* 1997; Rodriguez *et al.,* 1993; Wondwosen *et al.,* 2016; Wondwosen *et al.,* 2017; Wondwosen *et al.,* 2018; Ye-Ebiyo *et al.,* 2003). The VOCs associated with the grasses from these habitats have been shown to act over a range of scales, attracting gravid anophelines and stimulating oviposition in the laboratory (Asmare *et al.,* 2017a; Torres-Estrada *et al.,* 2005; Wondwosen *et al.,* 2016; Wondwosen *et al.,* 2017; Wondwosen *et al.,* 2018), and lure both *Anopheles* and *Culex spp.* in the field (Wondwosen *et al.,* 2021) (Table 3). Macrophytes also regulate oviposition-site selection and discrimination by culicines and anophelines (Eid *et al.,* 1992; El Maghrbi and Hosni, 2014; Torres-Estrada *et al.,* 2007; Turnipseed *et al.,* 2018; Webb *et al.,* 2013), with macrophytic VOCs modulating gravid mosquito attraction and oviposition stimulation in a compound-, concentration-, and species-dependent manner (Torres-Estrada *et al.,* 2007; Turnipseed *et al.,* 2018).

Vegetation can provide a source of nutrition for mosquito larvae, either directly, in the form of tissues, *e.g*., pollen, that can be consumed by the larvae, or indirectly, by providing a substrate for the microorganisms on which the larvae feed (Ye-Ebiyo *et al.,* 2000; Asmare *et al.,* 2017b). Pollen grains of a suitable size for mosquito larvae to imbibe (≥50 µm; Merritt *et al.,* 1992), *e.g*., the size of most grass pollen (Jones and Newell, 1948), can provide a rich nutrient source for mosquito larvae that enhance fitness (Asmare *et al.,* 2017; Bentoy *et al.,* 2015; Ye-Ebiyo *et al.,* 2000; Ye-Ebiyo *et al.,* 2003). Similar to grass foliage, VOCs from grass pollen attract and stimulate oviposition in gravid *An. arabiensis*, indicating that odour-driven oviposition-site selection plays a role in assuring that the resulting larvae have access to nutrient-rich habitats (Asmare *et al.,* 2017b; Wondwosen *et al.,* 2016; Wondwosen *et al.,* 2017; Wondwosen *et al.,* 2018). In general, while anophelines make use of olfactory cues from fresh sources of vegetation to indicate food-rich larval habitats, culicines are more apt to use cues from decaying and fermenting vegetation (*e.g*., Asmare, *et al.,* 2017; Du and Millar, 1999; Wondwosen *et al.,* 2017).

Table 3: Effects of oviposition cues from vegetation on the response of gravid females

Habitat and food cues from detritus

Detritus in aqueous environments is composed of organic debris, including the remains and waste products from plants and animals, which is suspended in, and accumulating at the bottom of, the water column. Anopheline and culicine larvae are filter feeders, imbibing both living and non-living organic micro-particulates from within, and/or from the bottom, of the water column (Merritt *et al.,* 1992). The quantity and quality of organic matter preferred in the water column of the breeding sites differs among species, with anophelines generally preferring clear water with loam bottoms and surface feeding, while culicines are found in more turbid water and bottom feed (Merritt *et al.,* 1992). Detritus composes a significant

portion of the nutrients in the diet of mosquito larvae, and, in general, VOCs from this organic matter signal the potential quality of the oviposition site to gravid females (*e.g*., Bentely and Day, 1989; Wondwosen *et al.,* 2016; Wondwosen *et al.,* 2017; Wondwosen *et al.,* 2018).

Gravid anopheline and culicine mosquitoes distinguish among the potential breeding habitats in a landscape, based at least in part, on olfactory cues from detritus (Bentely and Day, 1989; Wondwosen *et al.,* 2016; Wondwosen *et al.,* 2017; Wondwosen *et al.,* 2018). While anopheline larvae typically have ca. 75% detritus content in their guts (Piyaratne *et al.,* 2005; Walker *et al.,* 1988,) gravid females avoid oviposition sites with high detritus content in the water column and prefer sites with surface detritus (*e.g.,* pollen) and soil containing high amounts of organic matter (Asmare *et al.,* 2017; Herrera-Varela *et al.,* 2014). In contrast, culicines frequently lay eggs in substrates that contain high levels of suspended organic matter with associated active microbial communities, mainly derived from natural and agricultural activities (Day, 2016). The VOCs associated with natural and artificial infusions of organic matter, made from a large number of sources, attract and stimulate gravid culicine oviposition in a species-dependent manner (*e.g*., Du and Millar, 1999; Lewis *et al.,* 1974; McPhatter and Debboun, 2009; O'Gower, 1963; Reisen and Meyer, 1990; Reiter and Colon, 1991) (Table 3). The attraction and oviposition behaviours of gravid culicines to the VOCs emanating from such infusions are likely to be in response to a blend of odorants emitted from both the detritus and the associated microbiota (Du and Millar, 1999; Millar *et al.,* 1992). The species-specific behaviours of gravid anophelines and culicines in response to detritus-based infusions are reflected in the oviposition-site seeking and egg-laying responses of such females to the phenolic and indolic compounds commonly associated with these infusions, when presented as individual compounds or binary blends (Bentley *et al.,* 1979; Du and Millar, 1999; Eneh *et al.,* 2016a) (Table 3). While the anophelines avoid oviposition sites with common infusion VOCs, *e.g*., indole, 3-methylindole and 4-methylphenol, the response of the culicines appears to be species- and dose-dependent (Allan and Kline, 1995; Beehler *et al.,* 1994; Bentley *et al.,* 1979; Bentley *et al.,* 1981; Du and Millar, 1999; Eneh *et al.,* 2016a; Millar *et al.,* 1992; Trexler *et al.,* 2003a).

Food cues from microbiota

The presence of microorganisms in potential breeding habitats differentially affects and mediates oviposition-site selection by gravid culicine and anopheline mosquitoes (Eneh *et al.,* 2019; Hasselschwert and Rockett, 1988; Huang *et al.,* 2006a; Ponnusamy *et al.,* 2015). As mosquito larvae rely on microbial resources as a main food source for growth and development (Merritt *et al.,* 1992; Rejmánková *et al.,* 1996; Souza *et al.,* 2019), microbial kairomones provide potentially reliable information for the gravid mosquito to identify and discriminate among suitable breeding habitats for oviposition (Hazard *et al.,* 1967; Ponnusamy *et al.,* 2015; Melo *et al.,* 2020).

The microbial profiles in larval breeding sites and oviposition sites preferred by gravid mosquitoes are highly variable, and differ among mosquito species, site history, breeding habitat and geographic distribution (Caragata *et al.,* 2021; Nilsson *et al.,* 2018; Nilsson *et al.,* 2019). Despite the variability in microbial communities at larval breeding sites, some bacterial species have been repetitively identified from breeding sites and the guts of immature stage mosquitoes, and shown to attract and stimulate oviposition in gravid females (Table 4) (Chandel *et al.,* 2013; Huang *et al.,* 2006a; Lindh *et al.,* 2008b; Mosquera *et al.,* 2021;

Ponnusamy *et al.,* 2015; Rocha *et al.,* 2021; Trexler *et al.,* 2003b; Wang *et al.,* 2011). The density of the microbiota affects the species diversity, as well as the composition and intensity of microbial volatile emissions from a breeding site, in turn affecting the mosquito species selecting these sites for oviposition (Gerhardt, 1959; Hasselschwert and Rockett, 1988; Huang *et al.,* 2006a; Poonam *et al.,* 2002; Ponnusamy *et al.,* 2015; Rejmánková *et al.,* 2005). The microorganism diversity at the breeding sites is, itself, dependent on the biotic and abiotic conditions of the sites, including clarity of the water column, as well as the quality and quantity of the detritus and other trophic sources (Day, 2016; McCrae, 1984). As such, the habitats, in which the microbial species identified as attracting and eliciting oviposition in gravid mosquitoes are found, reflect the oviposition-site selection of gravid females, which may be modulated by other trophic and abiotic factors (Day, 2016; Ponnusamy *et al.,* 2008b). For example, gravid *An. gambiae* prefer oviposition sites emitting the volatiles of soil fungi, *Fusarium spp.*, which thrive in association with the rhizomes of grasses in the loamy soil of shallow clear pools (Eneh *et al.,* 2016b). Such trophic interactions increase the complexity of oviposition-site selection in mosquitoes.

Table 4: Effects of oviposition cues from microbes on the response of gravid females

Further increasing the complexity of microbial effects on mosquito oviposition, various mosquito species may be commensal with the consistently persistent larval habitat-associated microbes, *e.g*., *Klebsiella spp.* and *Elizabethkingia spp.*. Adult females carry these microbes in their guts, and gravid individuals can modify the microbial communities in a breeding site to benefit their larvae, by inoculating the site with microbes during oviposition (Akhouayri *et al.,* 2013; Kämpfer *et al.,* 2011; Lindh *et al.,* 2008b). The modification of the microbial communities in the larval breeding sites, aside from providing a guaranteed food source for their larvae, has the potential to change other biotic and abiotic factors in these sites to reduce larval competition and predation (Xia, 2021). In particular, as many mosquito species are early colonisers of ephemeral breeding sites (Duchet *et al.,* 2020), the modified odour signature in these sites results in oviposition-site avoidance by competitors, and a dose-dependent attraction and oviposition stimulation in gravid conspecifics and closely-related sympatric species, to lessen the risk of predation for each individual larva (Eneh *et al.,* 2019).

Microbial VOCs involved in the signalling of the quality of an oviposition site for various species of mosquitoes have been identified from microbiota, *e.g*., fungi and bacteria (Table 4). Two of the most potent microbial VOCs in attracting gravid anophelines and culicines identified so far are sesquiterpene derivatives (Eneh *et al.,* 2016b; Melo *et al.,* 2020). Both cedrol, a sesquiterpene alcohol identified from the endophytic fungi, *Fusarium fujikuroi* complex and *F. falciforme*, associated with sedge rhizomes in the soil of *Anopheles* larval breeding sites, and geosmin, an irregular bicyclic sesquiterpene identified from cyanobacteria in *Aedes* guts and breeding sites, are individually sufficient to attract gravid *An. gambiae* and *Ae. aegypti* dose-dependently to water, respectively (Eneh *et al.,* 2016b; Lindh *et al.,* 2015; Melo *et al.,* 2020). In laboratory and semi-field experiments, these compounds elicit attraction individually, but under field conditions, more complex odour blends have been found to be more successful at attracting gravid mosquitoes.

Rational screens of synthetic compounds

Rational screens of off-the-shelf VOCs have been used for almost a century to identify attractants and repellent compounds that could potentially be used for mosquito control (Crumb, 1924) (Table 5). The approach to determine which compounds to assay varies and has historically begun with subset of compounds previously identified from the larval habitats of both conspecific and heterospecific mosquitoes (Bentley *et al.,* 1981; Bentley *et al.,* 1982; Gjullin, 1961; Gjullin and Johnsen, 1965; Ikeshoji, 1968). These compounds are then assayed individually for attracting and stimulating oviposition in one or more, often sympatric, mosquito species (Bentley *et al.,* 1981; Bentley *et al.,* 1982; Gjullin, 1961; Gjullin and Johnsen, 1965; Ikeshoji, 1968). In the event that a compound is deemed to induce sufficient behavioural activity, more potent agonists are sought, through screening analogues by adjusting *e.g*., carbon chain lengths, functional groups and the number and location of double bonds (Bandyopadhyay *et al.,* 2011; Bentley *et al.,* 1981; Guha *et al.,* 2012; Ikeshoji and Mulla, 1974ab; Perry *et al.,* 1967; Seenivasagan *et al.,* 2010; Seenivasagan *et al.,* 2012; Sharma *et al.,* 2008; Sharma *et al.,* 2009).

Table 5: Effects of synthetic compounds on the response of gravid females

Large scale rational screens often identify only a handful of potentially bioactive compounds, *e.g*., screening almost 450 compounds in laboratory oviposition bioassays against *Cx. quinquefasciatus* and *Cx. tarsalis* yielded only seven compounds with bioactivity, and those only against *Cx. quinquefasciatus* (Gjullin, 1961; Gjullin *et al.,* 1965). Moreover, while such screens can identify agonists, the rate of identification is low and the potency of the agonists does not substantially outperform that of the initial compound (Guha *et al.,* 2012; Ikeshoji and Mulla, 1974a; Perry *et al.,* 1967; Sharma *et al.,* 2008; Sharma *et al.,* 2009). As a result, these screens are time-consuming endeavours that most often ignore the increased potency and robustness of blends (Baak-Baak *et al.,* 2013). Moreover, the identification of oviposition cues can be accelerated and refined by identifying physiologically-active compounds from natural sources relevant to the species under investigation using combined chemical and electrophysiological detection screens, *e.g*., combined gas chromatography and electroantennographic detection (GC-EAD), followed by behavioural bioassays with the blends (Du and Millar, 1999; Wondwosen *et al.,* 2016; Wondwosen *et al.,* 2017; Wondwosen *et al.,* 2018). While rational screens identify compounds of interest, few, if any, of the identified compounds have been adopted for use in surveillance and control programmes, which continue to rely on natural odour sources*, e.g*., infusions (Du and Millar, 1999; Millar *et al.,* 1992). The obstacles to overcome between the identification and development of a potential synthetic lure and the implementation of the lure in monitoring and control programmes by the stakeholders are largely a matter of regulation and policy, and economics. A lure must be approved and then recommended by policy-setting bodies (*e.g*., the World Health Organization; WHO), often before business enterprises are willing to fund commercial development to fit the new lure into tools in which it can be affordable, stable and safe. These are challenges that need to be addressed in a wider discussion.

Sensory and molecular correlates for oviposition-site selection

The mechanism by which olfactory cues, such as those associated with oviposition sites, are detected by gravid female mosquitoes is via olfactory sensory neurons (OSNs) housed in sensory hairs called sensilla found primarily on the antennae (McIver, 1980) (for more information see Pitts *et al.* (2022). The VOCs enter the sensillum lymph through sensillum wall

pores where the odorants encounter variably selective binding proteins, which transport salient compounds to the surface of the OSNs to bind with increasingly selective olfactory receptors to transduce the chemical into an electrical signal (Hallem *et al.,* 2006) (for more information see Ruel and Bohbot, 2022). The coincident activation of various classes of selective OSNs by the odorants in the VOC blend emanating from a potential oviposition site conveys the suitability of the site to the higher brain centres, ultimately culminating in oviposition-site selection behaviours (Ignell *et al.,* 2010). The modulation of the neural and molecular components within this signalling pathway by internal factors, *e.g.*, reproductive state, further regulates oviposition-site selection.

Physiological response to oviposition cues

While subsets of mosquito OSNs are tuned to oviposition site cues (Davis, 1976; 1977; Qiu *et al.,* 2006; Siju *et al.,* 2010), most of the VOCs eliciting neuronal responses have been identified during screens of panels of odorants whose bioactivity was inferred from their presence at breeding habitats, often of heterospecific larvae (*e.g.,* Blackwell and Johnson, 2000; Hill *et al.,* 2009). As mentioned above, some classes of compounds elicit behavioural responses in gravid mosquitoes across species, *e.g*., phenolic and indolic compounds, and as such, these compounds are often identified as bioactive in the antennal screens of various species (Bentley *et al.,* 1982; Blackwell *et al.,* 1993; Blackwell and Johnson, 2000; Collins and Blackwell, 1998; Hill *et al.,* 2009; Qiu *et al.,* 2006; Siju *et al.,* 2010). Direct interrogation of the antennae of gravid mosquitoes with the volatiles collected from oviposition sites and their associated resources using GC-EAD, has led to the identification of bioactive odorants (Du and Millar, 1999; Wondwosen *et al.,* 2016; Wondwosen *et al.,* 2017; Wondwosen *et al.,* 2018), and the subsequent capability to connect these VOCs with their specific OSNs, either from previous panel-based studies (Ghaninia *et al.,* 2007; Ghaninia *et al.,* 2008; Hill *et al.,* 2009; Qiu *et al.,* 2006; Siju *et al.,* 2010), or in future rational screens using single sensillum recording techniques.

Molecular determinants of oviposition-site volatile detection of gravid mosquitoes

Few large-scale screens of mosquito olfactory receptors have been made, and these have used either panels of odorants with presumed bioactivity, similar to those used in physiological screens, or volatile headspace extracts from hosts (Carey *et al.,* 2010; Omondi *et al.,* 2019; Wang *et al.,* 2010). In addition, more receptors have been functionally characterised, either using heterologous expressions systems or gene-editing techniques (Bohbot *et al.,* 2011; Hughes *et al.,* 2010; Pelletier *et al.,* 2010; Zhu *et al.,* 2013). While the focus of most of these studies has not been on identifying receptors involved in oviposition-site selection, there are several odorant receptors (ORs) and ionotropic receptors (IRs) identified, which interact with oviposition-relevant volatile ligands (Table 6). The oviposition response to indolic compounds across mosquito species is underpinned by the conservation of OR gene lineages, indole-sensitive *OR2* and 3-methylindole-sensitive *OR10* (Bohbot *et al.,* 2011; Dekel *et al.,* 2019; Liu *et al.,* 2018; Pelletier *et al.,* 2010; Ruel *et al.,* 2019; Ruel *et al.,* 2021), and the response to 1-octen-3-ol is regulated by the conserved *OR8* lineage (Dekel *et al.,* 2016).

Table 6: Mosquito odorant receptors functionally characterised with ligands from oviposition-related resources.

Lineage	Species	Name	Permanent identifier[1]	Ligands	References
Indole-sensitive					
OR2	*Anopheles gambiae*	OR2	AGAP009519	indole	Carey *et al*., 2010
	Aedes aegypti	OR2	AAEL005999	indole	Bohbot *et al*., 2011
	Aedes albopictus	OR2	AALF011758	indole	Scialo *et al*., 2012
	Culex quinquefasciatus	OR121	CPIJ014392	indole	Pelletier *et al*., 2010
	Toxorhynchites amboinensis	OR2	m.10291[2]	indole	Dekel *et al*., 2019
	Drosophila melanogaster	OR30a	FBgn0032096	indole	Ruel *et al* 2021
OR10	*Anopheles gambiae*	OR10	AGAP009520	3-methylindole	Carey *et al*., 2010
	Anopheles sinensis	OR10	ASIC007209	3-methylindole	Liu *et al*., 2018
	Aedes aegypti	OR10	AAEL006003	3-methylindole	Bohbot *et al*., 2011; Ruel *et al*., 2019
	Culex quinquefasciatus	OR21	CPIJ002479	3-methylindole	Hughes *et al*., 2010
	Toxorhynchites amboinensis	OR10	m.26775*	3-methylindole	Dekel *et al*., 2019
	Drosophila melanogaster	OR43a	FBgn0026389	3-methylindole	Ruel *et al* 2021
1-octen-3-ol-sensitive					
OR8	*Anopheles gambiae*	OR8	AGAP001912	(R)-1-octen-3ol	Lu *et al*., 2007
	Aedes aegypti	OR8	AAEL012254	(R)-1-octen-3ol	Bohbot and Dickens 2009
	Culex quinquefasciatus	OR118	CPIJ013954	(R)-1-octen-3ol	Hill *et al*., 2015; Xu *et al*., 2015
		OR114	CPIJ013945	(R/S)-1-octen-3ol	Xu *et al*., 2015
		OR113	CPIJ013944	nr (R/S)-1-octen-3ol	Hill *et al*., 2015
	Toxorhynchites amboinensis	OR8	m.134699[2]	(R)-1-octen-3ol	Dekel *et al*., 2016
	Drosophila melanogaster	OR85c	FBgn0037591	(R/S)-1-octen-3ol	Galizia *et al*., 2010
Geosomin-sensitive					
OR56a	*Drosophila melanogaster*	OR56a	FBgn0034473	geosomin	Stensmyr *et al*., 2012
	Aedes aegypti	OR11	AAEL011583	nd	Melo *et al*., 2019
Natural resource ligand-sensitive					
	Anopheles gambiae	OR1	AGAP009640	phenol	Carey *et al*., 2010
			AGAP009640	4-ethylphenol	Carey *et al*., 2010
			AGAP009640	4-methylphenol	Carey *et al*., 2010
		OR9	AGAP008333	4-methylphenol	Carey *et al*., 2010
		OR30	AGAP009391	6-methyl-5-hepten-2-one	Carey *et al*., 2010
		OR48	AGAP006666	2-ethyl-1-hexanol	Carey *et al*., 2010
			AGAP006666	nonanal	Carey *et al*., 2010
		OR57	AGAP004357	3-methyl-1-butanol	Carey *et al*., 2010
	Aedes aegypti	OR4	AAEL015147	6-methyl-5-hepten-2-one	McBride *et al*., 2014
	Culex quinquefasciatus	OR1	CPIJ000541	1-octen-3-ol	Xu *et al*., 2013
		OR37	CPIJ004163	4-ethylphenol	Zhu *et al*., 2013
			CPIJ004163	4-methylphenol	Zhu *et al*., 2013
		OR99	CPIJ011787	4-ethylphenol	Zhu *et al*., 2013
			CPIJ011787	4-methylphenol	Zhu *et al*., 2013

nd not yet demonstrated, [1]from VectorBase (vectorbase.org), with the exception of [2], which are from Dekel *et al*., 2019; 2021

Unlike the vast majority of mosquito OR lineages, these lineages of indole- and octenol-sensitive receptors are conserved within the dipterans, indicating a pervasive requirement for indole and octenol detection within this highly ecologically diverse class of insects (Dekel *et al.,* 2016; Ruel *et al.,* 2021). Moreover, the detection of select polyamines by gravid females through the other olfactory receptor family, IR41a, also appears to be a conserved trait in dipterans (Hussain *et al.,* 2016; Pitts *et al.,* 2017). Whether the mechanism underlying the detection of phenolics is conserved across species appears less clear. While there are receptor lineages sensitive to phenolic compounds in mosquitoes, these appear, for the most part, to be multigenic, with independent radiations within each species, resulting in divergent sensitivities to the various phenolic compounds and different key ligands (Carey *et al.,* 2010;

Wang *et al.,* 2010; Xu *et al.,* 2013). More olfactory receptors in mosquitoes will need to be functionally characterised with known oviposition site cues before the implications of the putative conserved receptor-ligand interacting lineages can be profitably discussed.

Plasticity

Mosquitoes detect a variety of chemical cues in the environment, which affect, and are affected by, both the overall physiological state of the female, and thus, its modulation of the sensitivity and specificity of the peripheral olfactory system (Davis, 1984; Omondi *et al.,* 2019; Qiu *et al.,* 2006; Siju *et al.,* 2010). The response of select OSNs are regulated after a blood meal, during the period in which gravid females seek an oviposition site (Qiu *et al.,* 2006; Siju *et al.,* 2010). The sensitivity of OSNs to select odorants within emanations associated with oviposition sites, *e.g*., indolic and phenolic compounds, increases in gravid *Ae. aegypti* compared to nulliparous host-seeking females (Qiu *et al.,* 2006; Siju *et al.,* 2010), while the sensitivity to select host odours, *e.g*., lactic acid, decreases (Davis, 1984; Qiu *et al.,* 2006). Modulation of neural sensitivity is classically considered to be a result of neuromodulators and hormones acting on the target neurons. Several neuropeptides and biogenic amines, together with their receptors and synthetic enzymes, have been shown to change gene and/or protein expression in the antennae of gravid mosquitoes (Hill *et al.,* 2021; Matthews *et al.,* 2016). In addition, the expression of the female-specific splice variant of the transcription factor with cis-regulatory elements upstream of ORs in *Ae. aegypti*, fruitless, is sufficient to induce host seeking in males, indicating its role in regulating host seeking in mosquitoes (Basrur *et al.,* 2020). Moreover, *fruitless* transcript abundance, significantly reduced post-blood meal, is upregulated during, and for 24 h after, oviposition (Hill *et al.,* 2021), further indicating a role in regulating the potentially OR-dependent re-establishment of host seeking post-oviposition.

Modulation of select members of the chemosensory gene families is demonstrated by their differential regulation in the antenna and maxillary palps of mosquitoes during pre-oviposition and oviposition (Hill *et al.,* 2019; Hill *et al.,* 2021; Matthews *et al.,* 2016; Rinker *et al.,* 2013). Paradoxically, the transcription of the genes encoding the receptors sensitive to the indolics and 1-octen-3-ol, *OR2, OR10* and *OR8* orthologues, are not regulated post-blood meal, while the receptor sensitive to the polyamines, *IR41a*, appears to increase in *Ae. aegypti*, but not *An. gambiae* (Hill *et al.,* 2021; Matthews *et al.,* 2016; Rinker *et al.,* 2013). This likely indicates that the switch of pre-oviposition and oviposition behaviours in mosquitoes is regulated by other chemosensory genes, either by upregulating transcription of other select genes sensitive to oviposition cues, and/or the downregulation of genes involved in host seeking. The post-blood meal refractoriness of female mosquitoes to host odours (Chadee, 2012; Klowden and Lea, 1984), is reflected in the lower relative transcript abundance in select chemosensory genes sensitive to host odour cues, *e.g.,* CO_2-sensitive Grs (*Ae. aegypti*: Hill *et al.,* 2019; *Culex quinquefasciatus*: unpublished data) and ORs sensitive to human odorants (Omondi *et al.,* 2019; Rinker *et al.,* 2013). It is clear, however, that further functional characterisation and expression analyses of the mosquito chemosensory genes is required for a basic understanding of the regulation of the mosquito gonotrophic cycle.

Vector control perspectives

Integrated vector management programmes rely on our ability to monitor and intervene at multiple different stages in the mosquito life cycle (Mwingira *et al.,* 2020a; WHO *et al.,* 2017; Wooding *et al.,* 2020). Intense selection pressure applied by control programmes on one stage, and through a single channel, leads to populations that can escape the control measure, as evident in the development of physiological and behavioural resistance to our pesticide-based indoor control measures, revealing, and in part contributing to, residual, uncontrolled, pathogen transmission (Mwingira *et al.,* 2020a; WHO *et al.,* 2017). Control strategies that target the mosquitoes, which could be infected, *i.e*., those with a previous blood meal, are likely to reduce disease transmission with a lower input of resources (WHO *et al.,* 2017). While several active control strategies targeting larvae and host-seeking females are widely used, gravid vectors have been an under-targeted stage in the mosquito life cycle, with some successful tools developed and implemented for select culicines, and few tools and lures available for anophelines (Dormont *et al*., 2021; Dugassa *et al.,* 2016; Snetselaar *et al.,* 2014; Xie *et al.,* 2019).

Gravid- and ovi-traps of various types, baited with various lures, have been used successfully for more than 30 years in mosquito population monitoring programmes, particularly with regards to culicines (*e.g*., Mboera *et al.,* 2000; McHugh and Hanny, 1990; Mwingira *et al.,* 2020a). The efficiency of the different trap types is, in general, subfamily-specific, with side-draft traps, *e.g.,* OviArt (Dugassa *et al.,* 2016), being much more effective for anophelines than updraft traps, *e.g*., CDC gravid trap, which are particularly adept at collecting culicines (Li *et al.,* 2016; Reiter and Colon, 1991). Previous 3D-tracking studies describing the movement of mosquitoes around host-odour baited traps that enabled species-oriented design improvements, will likely lead to further ovi-trap designs in the near future. Moreover, odour-based lures increase the efficacy of traps, which is also divided along subfamily lines, with, *e.g*., fermenting vegetation infusions attracting many species of gravid culicines, while repelling anophelines, which are in turn attracted to *e.g*., lake water (Dugassa *et al.,* 2016; Freier and Francy, 1991; Mboera *et al.,* 2000). As can be inferred from the diversity and varying species-specificity of the oviposition attractants described for mosquitoes above (*e.g.,* Mwingira *et al.,* 2020a), there is substantial room available for developing and fine-tuning lures, both natural and synthetic, for the different trap types, in order to improve surveillance for species that are currently under-represented, and increase their potential for use in control programmes.

Integrated vector management provides communities with a mosquito control toolbox with which to tailor the strategy to local needs and conditions at a variety of levels. Increasing the flexibility of ovi-traps to target and collect more species, and the further incorporation of these traps into surveillance and control programmes, requires an improved understanding of the ecology of these vectors. There is also the potential of amplifying the effects resulting from the above innovations by combining gravid and ovi-traps with other control tools. The effective deployment of tools to collect blood fed and gravid mosquitoes is heavily dependent on location (Debebe *et al.,* 2020). While the behaviour of vectors around their hosts has received intense attention (Wooding *et al*., 2020), behaviours further afield in the landscape are less well known. Indeed, factors affecting trap placement are numerous and species-dependent, including distance from habitation, amount of shading, trap height and location of potential oviposition sites (Dugassa *et al.,* 2013; Debebe *et al.,* 2020), and likely also

dependent on the deployment of other control tools. Strategies relying on two or more control tools interacting, *e.g*., push-pull, are starting to be investigated for mosquito control, using spatial repellents around human-habitation (push) and odour-based ovi-traps (pull) (Herrera-Varela *et al.,* 2014; Schoelitsz *et al.,* 2020). Push-pull systems, and the like, are likely to receive much more attention in the future. Ongoing research to increase and refine the individual tools, the efficacy of various tool combinations and the effective deployment of the tools within the landscape, will ensure that integrated vector management continues to be the most effective approach to vector control.

Conclusion

The increased demand for outdoor control intervention of disease vectors to integrate into existing strategies (Barreaux *et al.,* 2017; WHO, 2017) has spurred the research community into exploring novel ways of targeting gravid mosquitoes. By targeting blood-fed females using odour-based tools, the likelihood of removing infected individuals from the population increases, reducing disease transmission. Research on the odour-mediated mechanisms regulating mosquito oviposition began in the 1920s and persisted into the current century, when either the natural resource itself, or individual VOCs identified as present within these sites, were assayed for behavioural effect (Crumb, 1924). Recent improvements in the workflow, including refinement of the chemical analyses, in combination with electrophysiological recordings to identify bioactive VOCs from natural sources prior to behavioural assays, have reduced, if not eliminated, the need for large rational screens of individual off-the-shelf synthetic compounds. Furthermore, this development has led to the identification of odour blends, based on the bioactive compounds from within a natural resource, that can compete with the cues present in the natural environment (*e.g*., Wondwosen *et al.,* 2021), and are less likely than individual compounds to elicit behavioural resistance in mosquitoes (Mwingira *et al.,* 2020a; Dormont *et al.,* 2021). The mechanisms regulating pre-oviposition and oviposition are currently under investigation using functional genomics, and in combination with reverse chemical ecology, additional bioactive compounds will be identified, which will contribute to the future development of tools for vector surveillance and control.

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Table 3: Effects of oviposition cues from Poacaea vegetation on the response of gravid female *Anopheles gambiae*

Source of oviposition cue		Bioactivity	Tested	Compounds	Cas number	Extraction	References (a,b,c)
Source	Strain/biotype/ stage						
Cynodon dactylon	Plant	+	P	2-ethyl-1-hexanol	104-76-7	Hexane	Bokore *et al.,*2021[a]
		+	P	6-methyl-5-hepten-2-one	110-93-0	Hexane	Bokore *et al.,*2021[a]
		+	P	4-hexen-1-ol acetate	928-91-6	Hexane	Bokore *et al.,*2021[a]
		+	P	Cyclohexanone, 2,2,6-trimethyl	2408-37	Hexane	Bokore *et al.,*2021[a]
		+	P	Isophorone	78-59-1	Hexane	Bokore *et al.,*2021[a]
		+	P	α-pinene	80-56-8	Hexane	Bokore *et al.,*2021[a]
		+	P	β-pinene	127-91-3	Hexane	Bokore *et al.,*2021[a]
		+	P	Limonene	138-86-3	Hexane	Bokore *et al.,*2021[a]
		+	P	4-thujanol	546-79-2	Hexane	Bokore *et al.,*2021[a]
		+	P	1,1-dimethyl-3-methylene-2-vinyl cyclohexane	95452-08-7	Hexane	Bokore *et al.,*2021[a]
		+	P	Camphor	76-22-2	Hexane	Bokore *et al.,*2021[a]
		+	P	β-cyclocitral	432-25-7	Hexane	Bokore *et al.,*2021[a]
		+	P	Cyclosativene	22469-52-9	Hexane	Bokore *et al.,*2021[a]
		+	P	Copaene	3856-25-5 (α) 317819-78-6 (β)	Hexane	Bokore *et al.,*2021[a]
		+	P	β-elemene	515-13-9	Hexane	Bokore *et al.,*2021[a]
		+	P	α-gurjunene	489-40-7	Hexane	Bokore *et al.,*2021[a]
		+	P	β-caryophyllene	87-44-5	Hexane	Bokore *et al.,*2021[a]
		+	P	α-bergamotene	17699-05-7	Hexane	Bokore *et al.,*2021[a]
		+	P	β-Ionone	79-77-6	Hexane	Bokore *et al.,*2021[a]
		+	P	δ-guaiene	3691-11-0	Hexane	Bokore *et al.,*2021[a]
		+	P	Germacrene D	37839-63-7	Hexane	Bokore *et al.,*2021[a]
		+	P	α-guaiene	3691-12-01	Hexane	Bokore *et al.,*2021[a]
		+	P	α-muurolene	10208-80-7	Hexane	Bokore *et al.,*2021[a]
		+	P	Hexahydrofarnesyl acetone	502-69-2	Hexane	Bokore *et al.,*2021[a]
	infusion	-	C	3-methyl-1-butanol	123-51-3	HS / themal desorption	Eneh *et al.*, 2016a[a]
		ns	C	4-hepten-1-ol	20851-55-2	HS / themal desorption	Eneh *et al.*, 2016a[a]
		-	C	Phenol	108-95-2	HS / themal desorption	Eneh *et al.*, 2016a[a]
		ns	C	Phenylmethanol	93821-04-6	HS / themal desorption	Eneh *et al.*, 2016a[a]
		-	C	4-methylphenol	106-44-5	HS / themal desorption	Eneh *et al.*, 2016a[a]
		ns	C	2-phenylethanol	1960-12-08	HS / themal desorption	Eneh *et al.*, 2016a[a]
		-	C	Nonanal	124-19-6	HS / themal desorption	Eneh *et al.*, 2016a[a]
		ns	C	4-ethylphenol	123-07-9	HS / themal desorption	Eneh *et al.*, 2016a[a]
		-	C	Indole	120-72-9	HS / themal desorption	Eneh *et al.*, 2016a[a]
		-	C	3-methylindole	83-34-1	HS / themal desorption	Eneh *et al.*, 2016a[a]
Panicum repens	Plant	+	P	Limonene	138-86-3	Hexane	Bokore *et al.*, 2021[a]
		+	P	Eucalyptol	470-82-6	Hexane	Bokore *et al.*, 2021[a]
		+	P	β-cyclocitral	432-25-7	Hexane	Bokore *et al.*, 2021[a]
		+	P	Ylangene	14912-44-8	Hexane	Bokore *et al.*, 2021[a]
		+	P	β-elemene	33880-83-0	Hexane	Bokore *et al.*, 2021[a]
		+	P	α-guaiene	3691-12-01	Hexane	Bokore *et al.*, 2021[a]
Cenchrus setaceus	Plant	+	P	1,4-diethylbenzene	105-05-5	Hexane	Bokore *et al.*, 2021[a]
		+	P	Cymene	99-87-6	Hexane	Bokore *et al.*, 2021[a]
		+	P	2,4-dimethyl-acetophenone	89-74-7	Hexane	Bokore *et al.*, 2021[a]
		+	P	β-hydroxyethyl phenyl ether	9004-78-8	Hexane	Bokore *et al.*, 2021[a]
		+	P	1H-indene, 1-ethylidene	2471-83-2	Hexane	Bokore *et al.*, 2021[a]
		+	P	4,6-decadiyne	16387-71-6	Hexane	Bokore *et al.*, 2021[a]
		+	P	4-isopropylbenzyl alcohol	536-60-7	Hexane	Bokore *et al.*, 2021[a]
		+	P	1,1-dimethyl-3-methylene-2-vinyl cyclohexane	95452-08-7	Hexane	Bokore *et al.*, 2021[a]

+ attraction/stimulation, - aversion, ± attraction/stimulation with aversion at high concerntration, ns no significant response, C compound, B blend, E extract, HS head space, SPME solid phase microextraction, [a]includes identification and behaviour, [b]only identification, [c]only behaviour

Table 4: Effects of oviposition cues from Poacaea vegetation on the response of gravid female *Anopheles arabiensis*

Source of oviposition cue		Bioactivity	Tested	Compounds	Cas number	Extraction	References (a,b,c)
Source	**Strain/biotype/ stage**						
Oryza sativa	Tillering, booting & flowering	+	B	ß-caryophyllene	87-44-5	HS / hexane	Wondwosen *et al.*, 2016[a]
		+	B	Nonanal	124-19-6	HS / hexane	Wondwosen *et al.*, 2016[a]
		+	B	Decanal	112-31-2	HS / hexane	Wondwosen *et al.*, 2016[a]
		+	B	6-methyl-5-hepten-2-one	110-93-0	HS / hexane	Wondwosen *et al.*, 2016[a]
		+	B	Limonene	5989-27-5	HS / hexane	Wondwosen *et al.*, 2016[a]
		+	B	ß-pinene	18172-67-3	HS / hexane	Wondwosen *et al.*, 2016[a]
		+	B	α-pinene	7785-70-8	HS / hexane	Wondwosen *et al.*, 2016[a]
		+	B	3-carene	13466-78-9	HS / hexane	Wondwosen *et al.*, 2016[a]
Zea mays	Male flower	+	B	α-pinene	7785-70-8	HS / hexane	Wondwosen *et al.*, 2017[a]
		+	B	Nonanal	124-19-6	HS / hexane	Wondwosen *et al.*, 2017[a]
		+	B	Benzaldehyde	100-52-7	HS / hexane	Wondwosen *et al.*, 2017[a]
		+	B	p-cymene	99-87-6	HS / hexane	Wondwosen *et al.*, 2017[a]
		+	B	Limonene	5989-27-5	HS / hexane	Wondwosen *et al.*, 2017[a]
Saccharum officinarum	Male flower	+	B	O-xylene	95-47-6	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	Styrene	100-42-5	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	1,8-cineole	470-82-6	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	Undecane	1120-21-4	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	N-ethylbenzenamine	103-69-5	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	Dibutyl phthalate	84-74-2	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	Eicosane	112-95-8	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	α-pinene	7785-70-8	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	Nonanal	124-19-6	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	p-cymene	99-87-6	HS / DCM	Wondwosen *et al.*, 2018[a]
		+	B	N-ethyl aniline	103-69-5	HS / DCM	Wondwosen *et al.*, 2018[a]

+ attraction/stimulation, - aversion, ± attraction/stimulation with aversion at high concerntration, ns no significant response, C compound, B blend, E extract, HS head space, DCM dichloromethane, [a]includes identification and behaviour, [b]only identification, [c]only behaviour

Table 5: Effects of oviposition cues from Poacaea vegetation on the response of gravid female *Anopheles albimanus*

Source of oviposition cue		Bioactivity	Tested	Compounds	Cas number	Extraction	References (a,b,c)
Source	Tissue						
Cynodon dactylon	Plant	±	E	2,3-butandiol	513-85-9	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Butyl acetate	123-86-4	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	2,6-dimethyl-5-heptenal	106-72-9	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Tricyclene	508-32-7	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Camphene	79-92-5	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Phenol	108-95-2	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	ß-pinene	18172-67-3	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	3-carene	13466-78-9	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Benzyl alcohol	100-51-6	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Phenol, 2-methoxy-	1990-05-01	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	α-campholenal	91819-58-8	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Phenol,-4-ethyl	123-07-9	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Longifolene	475-20-7	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
Fimbristylis spadicea	Plant	±	E	Butyl acetate	123-86-4	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	2,6-dimethyl-5-heptenal	106-72-9	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	4-hydroxybenzenesulfonic acid	98-98-67-9	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Benzyl alcohol	100-51-6	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Phenol, 2-methoxy-	1990-05-01	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Phenylethyl alcohol	60-12-8	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	o-tolualdehyde	529-20-4	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
Ceratophyllum demersum	Plant	±	E	Butyl acetate	123-86-4	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	4-methylphenol	106-44-5	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Phenol, 2-ethyl	90-00-6	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Phenol, 4-ethyl-2-methoxy	2785-89-9	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	1,3-benzenediol,4-ethyl	2896-60-8	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
Brachiaria mutica	Plant	±	E	Guaiacol	1990-05-01	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Phenylethyl alcohol	1960-12-08	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Cinnamaldehyde	14371-10-9	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)- (isosylvestrene)	1461-27-4	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Isoeugenol	5932-68-3	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Methyl p-anisate	121-98-2	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Longifolene	475-20-7	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Caryophyllene	87-44-5	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
Jouvea straminea	Plant	±	E	Ethyl acetate	141-78-6	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	2,6-dimethyl-5-heptenal	106-72-9	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Benzyl alcohol	100-51-6	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Acetic acid, 2-methylphenyl ester	533-18-6	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	2-t-butyl-4-(dimethylbenzyl)phenol	1706-65-6	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Tricosane	638-67-5	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Tetracosane	646-31-1	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Pentacosane	629-99-2	Ethyl ether	Torres-Estrada *et al.*, 2005[b]
		±	E	Hexacosane	630-01-3	Ethyl ether	Torres-Estrada *et al.*, 2005[b]

+ attraction/stimulation, - aversion, ± attraction/stimulation with aversion at high concerntration, ns no significant response, C compound, B blend, E extract, HS head space, SPME solid phase microextraction, [a]includes identification and behaviour, [b]only identification, [c]only behaviour

Table 6: Effects of oviposition cues from Poacaea infusion on the response of gravid female *Culex spp.*

Source of oviposition cue	Species	Bioactivity	Tested	Compounds	Cas number	Extraction	References (a,b,c)
Cynodon dactylon	*Cx. tarsalis*	+	C	3-methylindole	83-34-1	HS / Ether, commercial	Du and Millar, 1999b[a]; Beehler *et al.*, 1994[c]
		+	C	4-methylphenol	108-39-4	HS / Ether	Du and Millar, 1999b[a]
		+	C	Nonanal	124-19-6	HS / Ether	Du and Millar, 1999b[a]
		ns	C	Dimethyltrisulfide	3658-80-8	HS / Ether	Du and Millar, 1999b[a]
		ns	C	Phenol	108-95-2	HS / Ether	Du and Millar, 1999b[a]
		ns	C	4-ethylphenol	123-07-9	HS / Ether	Du and Millar, 1999b[a]
		+	C	Indole	120-72-9	HS / Ether	Du and Millar, 1999b[a]
		ns	C	2-undecanone	112-12-9	HS / Ether	Du and Millar, 1999b[a]
		ns	C	2-tridecanone	593-08-8	HS / Ether	Du and Millar, 1999b[a]
		+	C	Naphthalene	91-20-3	HS / Ether	Du and Millar, 1999b[a]
	Cx. quinquefasciatus	±	C	3-methylindole	83-34-1	HS / Ether, commercial	Du and Millar, 1999[a]; Millar *et al.*, 1992[a]; Beehler *et al.*, 1994[c]
		+	C	4-methylphenol	108-39-4	HS / Ether, commercial	Du and Millar, 1999[a]; Millar *et al.*, 1992[a]; Zhu *et al.*, 2013[c]
		+	C	Nonanal	124-19-6	HS / Ether	Du and Millar, 1999[a]
		-	C	Dimethyltrisulfide	3658-80-8	HS / Ether	Du and Millar, 1999[a]
		ns	C	Phenol	108-95-2	HS / Ether	Du and Millar, 1999[a]; Millar *et al.*, 1992[a]
		ns	C	4-ethylphenol	123-07-9	HS / Ether, commercial	Du and Millar, 1999[a]; Millar *et al.*, 1992[a]; Zhu *et al.*, 2013[c]
		-	C	Indole	120-72-9	HS / Ether	Du and Millar, 1999[a]; Millar *et al.*, 1992[a]
		ns	C	2-undecanone	112-12-9	HS / Ether	Du and Millar, 1999[a]
		+	C	2-tridecanone	593-08-8	HS / Ether	Du and Millar, 1999[a]
		ns	C	Naphthalene	91-20-3	HS / Ether	Du and Millar, 1999[a]
		+	B	Phenol	108-95-2	Ether	Beehler et al., 1994[c]
		+	B	4-methylphenol	106-44-5	Ether	Beehler et al., 1994[c]
		+	B	4-ethylphenol	123-07-9	Ether	Beehler et al., 1994[c]
		+	B	Indole	120-72-9	Ether	Beehler et al., 1994[c]
		+	B	3-methylindole	83-34-1	Ether	Beehler et al., 1994[c]
	Cx. stigomatosma	+	C	3-methylindole	83-34-1	commercial	Du and Millar, 1999[a]; Millar *et al.*, 1992[a]

+ attraction/stimulation, - aversion, ± attraction/stimulation with aversion at high concerntration, ns no significant response, C compound, B blend, E extract, HS head space, SPME solid phase microextraction, [a]includes identification and behaviour, [b]only identification, [c]only behaviour

Table 7: Effects of oviposition cues from Cyperaceae vegetation on the response of gravid female *Anopheles gambiae*

Source of oviposition cue							
Source	**Strain/biotype/ stage**	**Bioactivity**	**Tested**	**Compounds**	**Cas number**	**Extraction**	**References (a,b,c)**
Cyperus rotundus	Fungal rhizome, soil infusion	+		Cedrol	77-53-2	HS / themal desorption	Lindh *et al.*, 2015[a]; Eneh *et al.*, 2016b[a]
	Plant	+	P	2,4-dimethyl-acetophenone	89-74-7	Hexane	Bokore *et al.*, 2021[a]
		+	P	4-isopropylbenzyl alcohol	536-60-7	Hexane	Bokore *et al.*, 2021[a]
		+	P	β-pinene	127-91-3	Hexane	Bokore *et al.*, 2021[a]
		+	P	Myrcene	123-35-3	Hexane	Bokore *et al.*, 2021[a]
		+	P	Limonene	138-86-3	Hexane	Bokore *et al.*, 2021[a]
		+	P	1,1-dimethyl-3-methylene-2-vinyl cyclohexane	95452-08-7	Hexane	Bokore *et al.*, 2021[a]
		+	P	Copaene	3856-25-5 (α) 317819-78-6 (β)	Hexane	Bokore *et al.*, 2021[a]
		+	P	γ-elemene	30824-67-0	Hexane	Bokore *et al.*, 2021[a]
		+	P	β-elemene	515-13-9	Hexane	Bokore *et al.*, 2021[a]
		+	P	Cyperene	2387-78-2	Hexane	Bokore *et al.*, 2021[a]
		+	P	β-caryophyllene	87-44-5	Hexane	Bokore *et al.*, 2021[a]
		+	P	Humulene	6753-98-6	Hexane	Bokore *et al.*, 2021[a]
		+	P	α-guaiene	3691-12-01	Hexane	Bokore *et al.*, 2021[a]
		+	P	Caryophyllene oxide	1139-30-6	Hexane	Bokore *et al.*, 2021[a]
		+	P	Humulene epoxide II	19888-34-7	Hexane	Bokore *et al.*, 2021[a]
Cyperus exaltus	Plant	+	P	1-isobutyl-1-cyclohexene	3983-03-07	Hexane	Bokore *et al.*, 2021[a]
		+	P	β-pinene	127-91-3	Hexane	Bokore *et al.*, 2021[a]
		+	P	Limonene	138-86-3	Hexane	Bokore *et al.*, 2021[a]
		+	P	1,1-dimethyl-3-methylene-2-vinyl cyclohexane	95452-08-7	Hexane	Bokore *et al.*, 2021[a]
		+	P	β-elemene	515-13-9	Hexane	Bokore *et al.*, 2021[a]
		+	P	Cyperene	2387-78-2	Hexane	Bokore *et al.*, 2021[a]
		+	P	Cedrene	469-61-4	Hexane	Bokore *et al.*, 2021[a]
		+	P	β-caryophyllene	87-44-5	Hexane	Bokore *et al.*, 2021[a]
		+	P	Humulene	6753-98-6	Hexane	Bokore *et al.*, 2021[a]
		+	P	Germacrene D	37839-63-7	Hexane	Bokore *et al.*, 2021[a]
		+	P	α-guaiene	3691-12-01	Hexane	Bokore *et al.*, 2021[a]
		+	P	α-muurolene	10208-80-7	Hexane	Bokore *et al.*, 2021[a]
		+	P	δ-cadinene	483-76-1	Hexane	Bokore *et al.*, 2021[a]

+ attraction/stimulation, - aversion, ± attraction/stimulation with aversion at high concerntration, ns no significant response, C compound, B blend, E extract, HS head space, SPME solid phase microextraction, [a]includes identification and behaviour, [b]only identification, [c]only behaviour

Table 8: Effects of oviposition cues from other families of vegetation on the response of gravid female mosquitoes

Source of oviposition cue		Species	Bioactivity	Tested	Compounds	Cas number	Extraction	References (a,b,c)
Source	**Strain/biotype/ stage**							
Myrtaceae								
Eucalyptus globules		*Ae.aegypti*	-	C	1,8 cineole	470-82-6	Natural product	Waliwitiya *et al.*, 2009[a]
Betulaceae								
Betula papyrifera	Paper birch infusions	*Ae. triseriatus*	+	C	4-methylphenol (p-cresol)	108-39-4	Pentane	Bentley *et al.*, 1979[a]
Polygonaceae								
Polygonum hydropiper	Oil from leaves	*Ae. albopictus*	-	C	Confertifolin	1811-23-0	Hexane / Ethyl acetate	Maheswaran and Ignacimuthu, 2014[a]
		Ae. aegypti	-	C	Confertifolin	1811-23-0	Hexane / Ethyl acetate	Maheswaran and Ignacimuthu, 2015[a]
		Cx. quinquefasciatus	-	C	Confertifolin	1811-23-0	Hexane / Ethyl acetate	Maheswaran and Ignacimuthu, 2013[a]
		An. stephensi	-	C	Confertifolin	1811-23-0	Hexane / Ethyl acetate	Maheswaran and Ignacimuthu, 2013[a]
Rutaceae								
Poncirus trifoliata	leaves and roots	*Ae.aegypti*	-	C	Poncirin	14941-08-3	Natural product	Rajkumar and Jebanesan, 2008[a]
			-	C	Rhoifolin	17306-46-6	Natural product	Rajkumar and Jebanesan, 2008[a]
			-	C	Naringin	10236-47-2	Natural product	Rajkumar and Jebanesan, 2008[a]
			-	C	Marmesin	13849-08-6	Natural product	Rajkumar and Jebanesan, 2008[a]
Asteraceae								
Parthenium	Root	*An. gambiae*			α-pinene	7785-70-8	Exudate	Milugo *et al.*, 2021
					β-pinene	127-91-3	Exudate	Milugo *et al.*, 2021
					α-phellandrene	99-83-2	Exudate	Milugo *et al.*, 2021
					β-phellandrene	555-10-2	Exudate	Milugo *et al.*, 2021
					(E)-caryophyllene	87-44-5	Exudate	Milugo *et al.*, 2021
Various								
Dried vegetation mix	Rabbit chow infusion	*Cx. quinquefasciatus*	+		Nonanal	124-19-6	HS-SPME	Leal *et al.*, 2008[a]
			+		Trimethylamine	75-50-3	HS-SPME	Leal *et al.*, 2008[a]
			+		3-methylindole	83-34-1	HS-SPME	Leal *et al.*, 2008[a]

+ attraction/stimulation, - aversion, ± attraction/stimulation with aversion at high concerntration, ns no significant response, C compound, B blend, E extract, HS head space, SPME solid phase microextraction, [a]includes identification and behaviour, [b]only identification, [c]only behaviour

Table 9: Effects of oviposition cues from microbes on the response of gravid females

Source of oviposition cue		Species	Bioactivity			Compounds	Cas number	Extraction	References (a,b,c)
Source	Strain/ stage		B/M	VOC	B/M				
Vibrio metschnikovii	E2.5[1]	*An. gambiae*	+		M	2-methyl-3-decanol	83909-79-9	HS-SPME	Lindh *et al.*, 2008a[a]
Proteus vulgaris	L2[1]	*An. gambiae*	+	-	M	3-methyl-1-butanol	123-51-3	HS-SPME	Lindh *et al.*, 2008a[a]; Eneh *et al.*, 2016a[c]
			+	ns	M	2-phenylethanol	1960-12-08	HS-SPME	
			+	-	M	Indole	120-72-9	HS-SPME	
Micrococcus isolate	L4[1]	*An. gambiae*	+	+	M	3-methyl-1-butanol	123-51-3	HS-SPME	Lindh *et al.*, 2008a[a]; Eneh *et al.*, 2016a[c]
Bacillus subtilis	L6[1]	*An. gambiae*	+		M	Diisopropylpyrazine	64931-21-1	HS-SPME	Lindh *et al.*, 2008a[a]
			+		M	2,5-diisopropylpyrazine	24294-83-5	HS-SPME	
			+		M	Isopropyl-secbutylpyrazine		HS-SPME	
			+		M	Isopropyl-isobutylpyrazine		HS-SPME	
Exiguobacterium aurantiacum	L9[1]	*An. gambiae*	+		M	3-methylbutanoic acid	503-74-2	HS-SPME	Lindh *et al.*, 2008a[a]
			+	+	M	3-methyl-1-butanol	123-51-3	HS-SPME	Lindh *et al.*, 2008a[a]; Eneh *et al.*, 2016a[c]
Comamonas aquatica	L11[1]	*An. gambiae*	+	ns	M	Phenylmethanol	100-51-6	HS-SPME	Lindh *et al.*, 2008a[a]; Eneh *et al.*, 2016a[c]
			+	ns	M	2-phenylethanol	1960-12-08	HS-SPME	
Pantoea stewartii	LB[2]	*An. gambiae*	+		M	2-tridecanone	593-08-8	HS-SPME	Lindh *et al.*, 2008b[a]
			+		M	2-undecanone	112-12-9	HS-SPME	
			+		M	1-nonanol	143-08-8	HS-SPME	
			+		M	1-decanol	112-30-1	HS-SPME	
			+		M	1-dodecanol	112-53-8	HS-SPME	
			+	ns	M	2-phenylethanol	1960-12-08	HS-SPME	Lindh *et al.*, 2008b[a]; Eneh *et al.*, 2016a[c]
			+	+	M	3-methyl-1-butanol	123-51-3	HS-SPME	
			+	-	M	Indole	120-72-9	HS-SPME	
			+	-	M	Dimethyltrisulfide	3658-80-8	HS-SPME	Lindh *et al.*, 2008b[a]; Schoelitsz *et al.*, 2020[c]
			+		M	Dimethyltetrasulfide	5756-24-1	HS-SPME	Lindh *et al.*, 2008b[a]
Elizabethkingia meningoseptica	LB[2]	*An. gambiae*	+		M	3-methylbutanoic acid	503-74-2	HS-SPME	Lindh *et al.*, 2008b[a]
			+		M	2-methylbutanoic acid	116-53-0	HS-SPME	
			+		M	Dimethyltetrasulfide	5756-24-1	HS-SPME	
			+		M	*S*-methyl 3-methylbutanethioate	23747-45-7	HS-SPME	
			+	+	M	3-methyl-1-butanol	123-51-3	HS-SPME	Lindh *et al.*, 2008b[a]; Eneh *et al.*, 2016a[c]
			+	ns	M	2-phenylethanol	1960-12-08	HS-SPME	
			+	-	M	Indole	120-72-9	HS-SPME	
			+	-	M	Dimethyldisulfide	624-92-0	HS-SPME	Lindh *et al.*, 2008b[a]; Schoelitsz *et al.*, 2020[c]
			+	-	M	Dimethyltrisulfide	3658-80-8	HS-SPME	
Cyanobacteria	Marshes	*A. albimanus*		+		n-pentadecanol	629-76-5	HS-SPME	Rejmankova *et al.*, 2000[a]
Mixed bacteria	Infusion	*Ae. aegypti*	+	+	B	Nonanoic acid	112-05-0	MeOH	Ponnusamy *et al.*, 2008a[a]
			+	+	B	Tetradecanoic acid	544-63-8	MeOH	
			+	+	B	Tetradecanoic acid, methyl ester	124-10-7	MeOH	
				-		Hexadecanoic acid, methyl ester	112-39-0	MeOH	
Kamptonema sp.	PCC 6505	*Ae. aegypti*		+		Geosmin	19700-21-1	HS-SPME	Melo *et al.*, 2020[a]
Pseudomonas aeruginosa	Field water	*Ae. aegypti*		+/ ns		7, 11-dimethyloctadecane	65431-89-2	Ether / synthesis	Ikeshoji *et al.*, 1979[a]; Hwang and Schultz, 1983[a]

[1]isolate from *An. arabiensis* midgut, [2]isolate from *An. gambiae* guts, + attraction/stimulation, - aversion, ns no significant response, B blend, M microbial isolate(s), VOC individual volatile organic compound, HS head space, SPME solid phase microextraction, MeOH methanol, [a]includes identification and behaviour, [b]only identification, [c]only behaviour

Table 10: Effects of synthetic alcohol/cyclics and carboxylic acids on the response of gravid females

Compounds	Cas number	OAI	Species	References
Alcohol/cyclics				
Phenol	108-95-2	-	*Ae. triseriatus*	Bentley *et al.*, 1981
4-ethylphenol	123-07-9	+	*Ae. triseriatus*	Bentley *et al.*, 1981
2-methylphenol	95-48-7	+	*Cx. quinquefasciatus*	Gjulin and Johnsen, 1965
		-	Cx. tarsalis	Gjulin and Johnsen, 1965
		+	*Tx. moctezuma / Tx. amboinensis*	Collins and Blackwell, 2002
		+	*Ae. triseriatus*	Bentley *et al.*, 1981
3-methylphenol	108-39-4	+	*Tx. moctezuma / Tx. amboinensis*	Collins and Blackwell, 2002
	108-39-4	+	*Ae. triseriatus*	Bentley *et al.*, 1981
4-methylphenol	106-44-5	+	*Tx. moctezuma / Tx. amboinensis*	Collins and Blackwell, 2002
4-methylanisole	104-93-8	-	*Ae. triseriatus*	Bentley *et al.*, 1981
4-methylcyclohexanol	589-91-3	+	*Ae. triseriatus*	Bentley *et al.*, 1981; 1982
			An. gambiae	Rinker *et al.*, 2013
		+	*Tx. moctezuma / Tx. amboinensis*	Collins and Blackwell, 2002
3-methylindole	83-34-1	+	*Tx. moctezuma / Tx. amboinensis*	Collins and Blackwell, 2002
2-propylphenol	644-35-9		*An. gambiae*	Rinker *et al.*, 2013
2,3-dimethylphenol	526-75-0	+	*Ae. triseriatus*	Bentley *et al.*, 1981
2,4-dimethylphenol	105-67-9	+	*Ae. triseriatus*	Bentley *et al.*, 1981
2,6-dimethylphenol	576-26-1	ns	*Ae. triseriatus*	Bentley *et al.*, 1981
α-ethyl-p-methoxybenzyl alcohol	5349-60-0	+	*Cx. quinquefasciatus*	Gjulin and Johnsen, 1965
		-	*Cx. tarsalis*	Gjulin and Johnsen, 1965
p-bromophenol	17878-44-3	ns	*Ae. triseriatus*	Bentley *et al.*, 1981
p-chlorophenol	106-48-9	ns	*Ae. triseriatus*	Bentley *et al.*, 1981
p-isopropylphenol	99-89-8	ns	*Ae. triseriatus*	Bentley *et al.*, 1981
p-tert-butylphenol	98-54-4	ns	*Ae. triseriatus*	Bentley *et al.*, 1981
2,6-dimethoxyphenol-ethylene oxide		+	*Cx. quinquefasciatus*	Gjulin and Johnsen, 1965
		-	Cx. tarsalis	Gjulin and Johnsen, 1965
Toluene	108-88-3	-	*Ae. triseriatus*	Bentley *et al.*, 1981
Benzene	71-43-2	-	*Ae. triseriatus*	Bentley *et al.*, 1981
Carboxylic acids				
Butyric acid	107-92-6	-	*Cx. quinquefasciatus*	Hwang *et al.*, 1980
(Z)-9-hexadecenoic acid	373-49-9	+	*Ae. aegypti*	Ganesan *et al.*, 2006
(Z)-9-octadecenoic acid	112-80-1	-	*Cx. quinquefasciatus*	Hwang *et al.*, 1984
Nonanoic acid	112-05-0	-	*Ae. aegypti*	Hwang *et al.*, 1982, Schultz *et al.*, 1982
		-	*Cx. quinquefasciatus*	Ikeshoji and Mulla, 1974b[c]; Hwang *et al.*, 1982[c]; Millar *et al.*, 1992[c]; Schultz *et al.*, 1982
		-	*Cx. tarsalis*	Schultz *et al.*, 1982
Octanoic acid	124-07-2	-	*Ae. aegypti*	Schultz *et al.*, 1982
		-	*Cx. quinquefasciatus*	Schultz *et al.*, 1982
		-	*Cx. tarsalis*	Schultz *et al.*, 1982
Decanoic acid	334-48-5	+	*Ae. aegypti*	Ganesan *et al.*, 2006
Tridecanoic acid	638-53-9	ns	*Cx. quinquefasciatus*	Hwang *et al.*, 1982
9-tetradecanoic acid	544-64-10	-	*Cx. quinquefasciatus*	Sivakumar *et al.*, 2011

Table 11: Effects of synthetic aldehydes and fatty acid esters on the response of gravid females

Compounds	Cas number	OAI	Species	References
Aldehydes				
nonanal	124-19-6	-	*An. gambiae*	Eneh *et al.*, 2016a
		+	*Cx. quinquefasciatus*	Du and Millar, 1999
		+	*Cx. tarsalis*	Du and Millar, 1999
Fatty acid esters				
Ethyl 2-(phenyldiazenyl)-3-oxobutanoate		ns	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Methyl 2-((3-chlorophenyl)diazenyl)-3-oxobutanoate		-	*Ae. aegypti*	Guha *et al.*, 2012
		-	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl 4,4,4-trifluoro-2-((4-methoxyphenyl)diazenyl)-3-oxobutanoate		+	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl 2-((1-hydroxynaphthalen-2-yl)diazenyl)-3-oxobutanoate		+	*Ae. aegypti*	Guha *et al.*, 2012
		+	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl 4-((1-ethoxy-1,3-dioxobutan-2-yl)diazenyl)benzoate		ns	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl 2-((4-nitrophenyl)diazenyl)-3-oxobutanoate		+	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl -2-(p-tolyldiazenyl) 3-oxobutanoate		ns	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl 2-((4-hydroxyphenyl)diazenyl)-3-oxobutanoate		ns	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl 2-((2-acetylphenyl)diazenyl)-3-oxobutanoate		ns	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl 2-((2-(hydroxymethyl)phenyl)diazenyl)-3-oxobutanoate		ns	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl 2-((4-acetylphenyl)diazenyl)-3-oxobutanoate		ns	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Ethyl 2-((4-fluorophenyl)diazenyl)-3-oxobutanoate		-	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Isobutyl 2-((4-fluorophenyl)diazenyl)-3-oxobutanoate		-	*Ae. aegypti*	Guha *et al.*, 2012
		ns	*Ae. albopictus*	Bandyopadhyay *et al.*, 2011
Butyl benzoate	136-60-7	+	*Cx. quinquefasciatus*	George *et al.*, 1986
Butyl heptadecanoate	42232-36-0	-	*Ae. aegypti*	Sharma *et al.*, 2008
		-	*Ae. albopictus*	Sharma *et al.*, 2008
		-	*An. stephensi*	Sharma *et al.*, 2009
Butyl-2,4-dihydroxybenzoate	37622-42-7	+	*Cx. quinquefasciatus*	George *et al.*, 1986
Butyl-3,5-dinitrobenzoate	10478-02-1	+	*Cx. quinquefasciatus*	George *et al.*, 1986
Buyl-2-ethylhexanoate	68443-63-0	+	*Cx. quinquefasciatus*	George *et al.*, 1986
Decyl undecanoate	42231-60-7	-	*Ae. aegypti*	Sharma *et al.*, 2008
		-	*Ae. albopictus*	Sharma *et al.*, 2008
		+	*An. stephensi*	Sharma *et al.*, 2009
Dodecyl nonanoate	17671-26-0	-	*Ae. aegypti*	Sharma *et al.*, 2008
		ns	*Ae. albopictus*	Sharma *et al.*, 2008
		-	*An. stephensi*	Sharma *et al.*, 2009

Table 12: Effects of synthetic fatty acid esters on the response of gravid females

Compounds	Cas number	OAI	Species	References
Fatty acid esters				
Ethyl acetate	141-78-6	+	*Ae. aegypti*	Perry and Fay, 1967
Ethylcrotonate (Ethyl 2-butenoate)	10544-63-5	+	*Cx. quinquefasciatus*	George *et al.,* 1986
Heptadecyl butanoate	84869-41-0	-	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		-	*An. stephensi*	Sharma *et al.,* 2009
Heptyl tetradecanoate	42232-00-8	ns	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		ns	*An. stephensi*	Sharma *et al.,* 2009
Hexadecyl pentanoate		-	*Ae. aegypti*	Sharma *et al.,* 2008; Seenivasagan *et al.,* 2010
		-	*Ae. albopictus*	Sharma *et al.,* 2008; Seenivasagan *et al.,* 2010
		-	*An. stephensi*	Sharma *et al.,* 2008; Seenivasagan *et al.,* 2010
Hexyl pentadecanoate	1002-84-2	ns	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		-	*An. stephensi*	Sharma *et al.,* 2009
Methyl butyrate (Butanoic acid, methyl ester)	623-42-7	+	*Ae. aegypti*	Perry and Fay, 1967
Methyl phenoxyacetate	2065-23-8	+	*Cx. quinquefasciatus*	George *et al.,* 1986
Methyl propionate	554-12-1	+	*Ae. aegypti*	Perry and Fay, 1967
Methyl-3-aminobenzoate	4518-10-09	+	*Cx. quinquefasciatus*	George *et al.,* 1986
Methyl-3,5-dinitrobenzoate	2702-58-1	+	*Cx. quinquefasciatus*	George *et al.,* 1986
Nonyl dodecanoate	42231-74-3	-	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		+	*An. stephensi*	Sharma *et al.,* 2009
Octadecyl propanoate	52663-48-6	+	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		-	*An. stephensi*	Sharma *et al.,* 2009
Octyl tridecanoate	42231-85-6	-	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		ns	*An. stephensi*	Sharma *et al.,* 2009
Pentadecyl hexanoate		-	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		-	*An. stephensi*	Sharma *et al.,* 2009
Pentyl hexadecanoate		ns	*Ae. aegypti*	Sharma *et al.,* 2008
		ns	*Ae. albopictus*	Sharma *et al.,* 2008
		+	*An. stephensi*	Sharma *et al.,* 2009
Propyl octadecanoate	3634-92-2	+	*Ae. aegypti*	Sharma *et al.,* 2008, Seenivasagan *et al.,* 2012
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		+	*An. stephensi*	Sharma *et al.,* 2009, Seenivasagan *et al.,* 2012
Tetradecyl heptanoate	29710-33-6	-	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		-	*An. stephensi*	Sharma *et al.,* 2009
Tridecyl octanoate	42231-41-4	-	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		-	*An. stephensi*	Sharma *et al.,* 2009
Undecyl decanoate		-	*Ae. aegypti*	Sharma *et al.,* 2008
		-	*Ae. albopictus*	Sharma *et al.,* 2008
		-	*An. stephensi*	Sharma *et al.,* 2009
α-conidendrol tetraacetate		+	*Cx. quinquefasciatus*	Gjullin and Johnsen, 1965
		-	Cx. tarsalis	Gjullin and Johnsen, 1965

Table 13: Effects of synthetic carbamate esters, amines and other chemicals on the response of gravid females

Compounds	Cas number	OAI	Species	References
Carbamate esters				
Ethyl N-methylcarbamate (Ethyl methylcarbamate)	105-40-8	+	*Cx. quinquefasciatus*	Gjullin and Johnsen, 1965
		-	*Cx. tarsalis*	Gjullin and Johnsen, 1965
Phenethyl methylcarbamate		+	*Cx. quinquefasciatus*	Gjullin and Johnsen, 1965
		-	*Cx. tarsalis*	Gjullin and Johnsen, 1965
Amines				
N-butyl-N-ethyl-overatrylamine		+	*Cx. pipiens.quinquefasciatus*	Gjullin, 1961
N-ethyl-o-veratrylamine		+	*Cx. quinquefasciatus*	Gjullin and Johnsen, 1965
		-	Cx. tarsalis	Gjullin and Johnsen, 1965
1-myristoyl pyrrolidine		-	*Cx. quinquefasciatus*	Gjullin and Johnsen, 1965
		-	*Cx. tarsalis*	Gjullin and Johnsen, 1965
Other chemicals				
dimethyltrisulfide	3658-80-8	ns	*Ae. albopictus*	Trexler *et al.,* 2003a
beechwood creosote	8021-39-4	+	*Cx. pipiens.quinquefasciatus*	Gjullin, 1961

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Parasites & Vectors

RESEARCH

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Odour-mediated oviposition site selection in *Aedes aegypti* depends on aquatic stage and density

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Abstract

Background Olfaction plays an important role in the selection and assessment of oviposition sites by mosquitoes. Volatile organic compounds (VOCs) associated with potential breeding sites affect the behaviour of gravid mosquitoes, with VOCs from aquatic stages of conspecific mosquitoes influencing and regulating oviposition. The purpose of this study was to conduct a systematic analysis of the behavioural response of gravid *Aedes aegypti* to conspecific aquatic stage-conditioned water, to identify the associated bioactive VOCs and to determine how blends of these VOCs regulate oviposition site selection and stimulate egg-laying.

Methods Using a multi-choice olfactory oviposition assay, controlling for other sensory modalities, the responses of individual females to water conditioned with different densities of conspecific aquatic stages were assessed. The conditioned water samples from the most preferred density of each aquatic stage were subsequently compared to each other using the same oviposition assay and analysed using an analysis of variance (ANOVA) followed by a Tukey post-hoc test. Using combined gas chromatography and electroantennographic detection or mass spectrometry, bioactive VOCs from the preferred density of each aquatic stage were identified. Synthetic blends were prepared based on the identified ratios of bioactive VOCs in the aquatic stages, and then tested to determine the oviposition choice of *Ae. aegypti* in a dose-dependent manner, against a solvent control, using a dual-choice assay. This dataset was analysed using nominal logistic regression followed by an odds ratio comparison.

Results Gravid *Ae. aegypti* responded stage- and density-dependently to water conditioned with eggs, second- and fourth-instar larvae, and pupal exuviae, but not to water conditioned with pupae alone. Multi-choice assays demonstrated that gravid mosquitoes preferred to oviposit in water conditioned with fourth-instar larvae, over the other aquatic stage-conditioned water. Gravid *Ae. aegypti* were attracted, and generally stimulated, to oviposit in a dose-dependent manner to the individual identified synthetic odour blends for the different aquatic stages.

Conclusions Intraspecific VOCs regulate oviposition site selection in *Ae. aegypti* in a stage- and density-dependent manner. We discuss the need for further studies to evaluate the identified synthetic blends to modulate the odour-mediated oviposition of *Ae. aegypti* under field conditions.

Keywords Mosquito behaviour, Immature stages, Volatile organic compounds, Synthetic odour blends

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Background

For mosquitoes, oviposition site selection is essential, as this decision directly regulates the growth, development and survival of the next generation, as well as population dynamics [1–5]. The aquatic stages of mosquitoes are limited in their movement, and thus the fate of the

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offspring is largely dependent on the maternal selection of oviposition sites [2, 6, 7]. While seeking oviposition sites, gravid mosquitoes must search for, and distinguish between, potential oviposition sites over multiple spatial scales to ensure the availability of nutrients for larval development and survival, and to reduce competition and offspring mortality [2, 8–10]. For this purpose, mosquitoes rely predominantly on olfactory cues emanating from potential oviposition sites and their surroundings [6, 9, 11]. Emanates from conspecific immature stages associated with breeding sites can act as reliable signals for females to assess the quality of an oviposition site, in terms of overcrowding and competition from con- and heterospecific aquatic stages [11]. An increased understanding of the signals regulating conspecific oviposition site selection may lead to the development of species- and/or genera-specific attractants for vector control.

Oviposition site selection by gravid mosquitoes can be modulated by cues associated with the aquatic stages, in a species-, stage- and density-dependent manner [11]. Gravid mosquitoes generally avoid ovipositing in breeding sites in which the risk of con- and heterospecific competition and cannibalism/predation is high [12–19]. The manner by which individual species assess oviposition sites differs in accordance with species-specific breeding site requirements [2, 6, 11, 20]. This is, therefore, dependent on the ability of gravid females to evaluate cues emanating from distinct conspecific aquatic stages, as these provide reliable signals of breeding site conditions [11, 12, 15, 18, 19]. Limitation in nutrient resources, regulated by, e.g., competition and the dynamic nature of mosquito-associated microbial communities, differentially affects oviposition site choice in a taxon-dependent manner [5, 6, 19, 21, 22]. In addition, the persistence of individual breeding sites affects mosquitoes species-specifically, often depending on the drought tolerance of the aquatic stages, with conspecific stage-associated cues providing reliable temporal information concerning, e.g., ephemeral and cyclically flooded sites [2, 6, 21]. For example, gravid yellow fever and Asian tiger mosquitoes *Aedes aegypti* and *Aedes albopictus*, respectively, preferentially select breeding sites that contain or have previously contained, late aquatic stages, which is believed to indicate the availability of larval food resources [20, 22]. The density of the conspecific aquatic stages in the breeding sites also modulates mosquito oviposition site selection, as overcrowding leads to competition while low conspecific densities increase the risk of predation, with optimal densities being species-dependent [12, 15, 17, 18, 23–25]. While a limited number of behaviourally active volatile organic compounds (VOCs) have been identified associated with conspecific aquatic stages, there is a further need for a systematic cross-disciplinary chemical ecological analysis to identify intraspecific signals regulating oviposition site selection and egg laying in mosquitoes [11].

This study aimed at exploring the stage- and density-dependent behavioural response of the yellow fever mosquito *Ae. aegypti* during oviposition site selection and egg-laying, as well as identifying the natural bioactive VOCs associated with water conditioned with aquatic stages, regulating this choice. Synthetic blends of these bioactive VOCs were evaluated for their ability to manipulate the oviposition response of gravid *Ae. aegypti*. Understanding oviposition in mosquitoes, and the bioactive VOCs involved, will assist in enhancing existing vector surveillance and control programmes by targeting gravid mosquitoes to reduce vector populations and the burden of disease transmission.

Methods

Mosquito rearing

Aedes aegypti (Rockefeller) eggs, laid on filter paper (90 mm diameter; Ahlstrom, Munksjö, Finland), were placed in 3-l plastic rearing trays (L: 24.5 × W: 18.5 × H: 7.5 cm; Emballator Lagan AB, Ljungby, Sweden) filled with 1 l distilled water. Larvae were fed daily with TetraMin® fish food (Tetra GmbH, Melle, Germany). The pupae were collected in 30-ml containers (Essentra Components, Malmö, Sweden) kept in a BugDorm-1 cage (L: 30 × W: 30 × H: 30 cm; MegaView Science, Taichung, Taiwan) for adult emergence. Adults were maintained at 27 ± 1 °C, 65 ± 5% relative humidity, and at a 12:12 h light–dark cycle, with ad libitum access to a 10% sucrose solution. For colony maintenance and experiments, adults 5–7 days post-emergence (dpe) were provided with sheep blood (Håtunalab, Bro, Sweden) for 2 h using a collagen membrane through a Hemotek membrane feeding system (Hemotek Ltd., Blackburn, UK) at 37 °C. Fully engorged females were used in the oviposition experiments, five days after blood meal ingestion (10–12 dpe).

Conditioning water with aquatic stages of *Aedes aegypti*

Water was conditioned with eggs, second-instar larvae, fourth-instar larvae, pupae or pupal casings of *Ae. aegypti*. To produce the egg-conditioned water (ECW), filter papers containing c. 250, 500, 1000 and 2000 eggs were collected and incubated at −20 °C for 30 min to prevent hatching. This treatment is unlikely to have damaged the chorion or outer egg casing [26], and thus unlikely to affect the VOCs released. Individual filter papers, with or without eggs, were then placed in rearing trays containing 1 l distilled water for 22 ± 2 h. To obtain the larvae-conditioned water (LCW), first-instar larvae were transferred to rearing trays (see above) containing one of four densities (c. 50, 150, 300 and 600 larvae l^{-1}), and reared to

second instar or fourth instar without changing the water prior to subsequent assays. Throughout its development, each larva was fed c. 10 mg of TetraMin fish food, with first- to early third-instar larvae provided with 0.6 mg of food larva per day, and late third- and fourth-instar larvae provided with 2 mg of food larva per day. As a control, distilled water was treated with equivalent amounts of fish food and under the same conditions, with food particles sieved out daily prior to the next allotment of fish food to reflect the consumption of food by the larvae. To generate the water conditioned with pupae and their exuviae, two groups of pupae were collected. The pupae, at densities of either c. 50, 100, 150 and 300 or 6, 12, 24 and 48, were rinsed with distilled water to remove any extraneous particles and then transferred to rearing trays containing 1 l of distilled water. The first group of pupae were kept for 22 ± 2 h to produce the pupae-conditioned water (PCW). The second group of pupae were retained until all adults emerged (3 nights), and only the exuviae remained in the distilled water, after which the exuviae-conditioned water (XCW) was obtained. As controls, 1 l of distilled water was kept without pupae or pupal exuviae over the same time period under the same conditions. The conditioned water was then strained through nylon mesh and a folded filter paper (18.5 cm; Whatman International Ltd., Maidstone, England), and used immediately in subsequent assays.

Multi-choice oviposition assay

To characterize the density-dependent effect of odours emanating from water conditioned with aquatic stages on oviposition site selection and egg laying by gravid *Ae. aegypti*, multi-choice oviposition assays were performed (Additional file 1: Fig. S1a). The conditioned water (40 ml of test or control) was added into the bottom section of an artificial oviposition site consisting of a blue plastic cup (250 ml; Duni, Malmö, Sweden), within which a second transparent cup (120 ml; ÖoB, Lund, Sweden), with six 2.5-mm-diameter perforations in the bottom, was placed in order to exclude input from sensory modalities other than olfaction (Additional file 1: Fig. S1b). A third cup (30 ml), containing distilled water and a filter paper, was placed inside the second cup (Additional file 1: Fig. S1b). The filter paper served as the oviposition substrate. Artificial oviposition sites containing the water conditioned with different densities of each aquatic stage and a distilled water control were placed in a BugDorm-1 cage. The treatments and control were randomly designated among the five artificial oviposition sites within the cage, one artificial oviposition site in each corner and one in the middle of the cage (Additional file 1: Fig. S1a). Five days after blood-feeding, an individual gravid mosquito (10–12 dpe) was released into each cage 2 h prior to scotophase, and provided ad libitum access to 10% sucrose. The placement of the sucrose dispensers alternated between the left and right sides of the cages, and had no effect on oviposition choice. The bioassays were kept under similar climate conditions for 48 h, as described above, after which the eggs laid in each artificial oviposition site were counted.

Solid-phase microextraction headspace collections

Solid-phase microextraction (SPME) divinylbenzene/carboxen/polydimethylsiloxane Supelco StableFlex™ fibres (50/30 µm, 24 ga, 2 cm; Sigma-Aldrich, Stockholm, Sweden) were conditioned at 225 °C for 30 min using a gas chromatograph (GC; Agilent Technologies 6890, Santa Clara, CA, USA) prior to headspace collections. The glassware used for headspace collections was cleaned and placed at 250 °C for 8 h prior to use. Either the conspecific aquatic stage-conditioned water eliciting the highest oviposition response in the multi-choice assays (400 ml) or the control water (400 ml) was poured into 1 l glass bottles (VWR, Stockholm, Sweden). The water used for these odour collections was conditioned to match that of the conditioned water used in the behavioural experiments. Thereafter, sodium chloride (NaCl; ≥ 99%, Sigma-Aldrich), was dissolved into each to enhance the emission of volatiles following modified protocols of Lindh et al. [27] and Mozūraitis et al. [28]. Three concentrations of NaCl (150, 225 and 255 mg ml^{-1}) were tested, from which it was determined that 255 mg ml^{-1} elicited the highest abundance of VOCs trapped on the SPME fibre from the conditioned water. The water was then incubated at room temperature for c. 5 min. The PCW was omitted due to the lack of a density-dependent oviposition response (Additional file 1: Fig. S2). The clean SPME fibre was introduced into a small hole (1.4 mm diameter) drilled in the polypropylene lid of the glass bottle containing the samples to collect the headspace for 17 h.

Combined gas chromatography and electroantennographic detection

Antennal responses of 5-day post-blood-fed (10–12 dpe) *Ae. aegypti* to VOCs contained within the headspace collected on the SPME fibre of each of the aquatic stage-conditioned water were determined using combined gas chromatography (GC), flame ionization detection (FID) and electroantennographic detection (EAD) analyses. The GC (Agilent Technologies 6890) was equipped with an HP-5 column (30 m length × 0.25 mm inner diameter [i.d.] × 0.25 µm film thickness) and an effluent splitter between the column and the detectors. Hydrogen was used as the carrier gas at a linear flow rate of 45 cm s^{-1}. The VOCs adsorbed onto the SPME fibres were injected into the GC in splitless mode for 1 min at 225 °C and

thermally desorbed in the inlet. The GC oven temperature was programmed from 50 °C (hold for 1 min), increased at a rate of 8 °C min^{-1} to 275 °C (10 min hold). At the GC effluent splitter, nitrogen was added and the gas flow split between the FID and the EAD in a 1:1 volume four-way cross-splitter (Gerstel, Mülheim, Germany). The GC effluent moving towards the EAD passed through a Gerstel Olfactory Detector Port (ODP)-2 transfer line (Gerstel) that tracked the GC oven temperature before it was delivered into a glass tube (10 cm × 8 mm), where it was mixed with charcoal-filtered humidified air (1.5 l min^{-1}). The antennal preparation was positioned 0.5 cm away from the outlet of the glass tube.

For the EAD analysis, a female mosquito was cold anesthetized prior to mounting the excised head on a reference electrode, which was inserted through the foramen. The cut, distal flagellomere of the antenna was connected to the recording electrode. Both electrodes were made from pulled glass microcapillaries, filled with Beadle–Ephrussi Ringer solution [29] and placed over chlorinated silver wires in the electrode holders. The recording electrode was attached to a pre-amplifier (1×), then to a high-impedance direct current (DC) amplifier interface (IDAC-2 Ockenfels Syntech GmbH, Buchenbach, Germany) and finally to a personal computer (PC) for visualization and storage. Three to five stable recordings were performed for the headspace collected from the water conditioned with each aquatic stage. The data were analysed using GC-EAD software (v.1.2.3, Ockenfels Syntech GmbH).

Chemical analysis

The SPME samples from each of the aquatic stage-conditioned water and the corresponding controls were injected into a combined GC (6890, Agilent Technologies) and mass spectrometer (MS, 5975, Agilent Technologies) operated in the electron ionization mode at 70 eV. The GC–MS unit was equipped with the same type of fused silica capillary HP-5 column (60 m length × 0.25 mm i.d. × 0.25 µm film thickness) as the GC–EAD. Helium was used as the carrier gas at a linear flow rate of 34 cm s^{-1}. The same temperature programme was used for both the GC–MS and the GC–EAD analyses. A total of three to five SPME samples, collected from the water conditioned with each of the aquatic stages and control, were injected into the GC–MS. All physiologically active VOCs, which were determined using GC–EAD analysis, were identified using linear retention times (Kovats index) and mass spectra in comparison with the National Institute of Standards and Technology (NIST) 17 library, and subsequently confirmed with authentic standards (Table 1). The relative abundance of the bioactive VOCs in each of the extracts was approximated by comparing the areas of each total ion chromatogram. The approximate relative abundance of each compound was then used to formulate the synthetic blends. The composition and ratio of, as well as the physiological response to, the compounds used in the synthetic blends were confirmed using GC–MS and GC–EAD, respectively. The purity of the commercial compounds was confirmed by injection on the GC–MS (Table 1).

Table 1 Physiologically bioactive volatile organic compounds identified from conspecific stage-conditioned water through GC–EAD and GC–MS analyses, and used for bioassays

Retention time	Retention indices	Compound	Purity (%)
6.56	839	2,4-Dimethylhept-1-ene	99.4
8.76	957	(*E*)-2-Heptenal	95.3
10.04	1023	4-Cyanocyclohexene	97.9
10.74	1059	(*E*)-2-Octenal	95
10.89	1072	2,6-Dimethyl-7-octen-2-ol	100
11.64	1105	Nonanal	99.4
12.50	1156	Camphor	99
12.70	1162	(*E*)-2-Nonenal	97.6
13.54	1207	Decanal	99.5
14.57	1264	(*E*)-2-Decenal	96.4
14.88	1281	Unknown	
16.93	1402	4-(2-Methylbutan-2-yl) phenol	99.8

Dual-choice oviposition bioassay with synthetic blends

To determine the behavioural preference of gravid *Ae. aegypti* to the identified synthetic blends from the water conditioned with the different conspecific aquatic stages, a dose-dependent analysis was performed in a dual-choice oviposition assay. The artificial oviposition sites, containing either the treatment or the control, were placed in opposite corners of a BugDorm-1 cage, c. 8 cm from each cage wall. Each dilution of an individual synthetic blend (6 ml) was tested against a solvent control (6 ml hexane). Both the blend and the control were delivered from wick dispensers [30], constructed of a 12-ml vial (Genetec, Stockholm, Sweden) with a perforated lid (2-mm diameter hole) and a wick. The wick was made from Teflon tubing (75 mm length × 1.68 mm i.d. × 0.30 mm wall thickness), with a piece of unbleached cotton string inserted [30]. The wick dispensers allowed for the control of the release rate and ratio of the VOCs in the blend during the bioassay [30]. Each wick dispenser was then placed in a 250 ml glass wash bottle (VWR). Charcoal-filtered air was passed through the glass wash bottles, via Teflon tubing (6 mm outer diameter [o.d.]), using an air pump (model V-20; Guangdong Hailea

Group Co., Ltd., Guangdong, China), into two 12-channel flow meters (Kytola Instruments, Muurame, Finland), in which the flow rate was adjusted to 0.1 l min^{-1}. Teflon tubing connected the flow meters to the artificial oviposition sites (Additional file 1: Fig. S3) [31]. Individual 5-day post-blood-fed *Ae. aegypti* were introduced into each of the 12 BugDorm-1 cages containing the artificial oviposition sites, 2 h prior to scotophase. Females were offered ad libitum access to 10% sucrose during the bioassay (19 ± 1 h). Subsequently, the number of eggs laid in the treatment and the control was counted and oviposition choice indices were calculated: C/(C + T) and T/(T + C), in which C is the number of eggs laid in the control and T is the number of eggs laid in the treatment. Dual-choice assays with solvent-filled wick dispensers on both sides determined that there was no positional bias in the egg-laying choice of the gravid mosquitoes (Additional file 1: Fig. S4) [31]. Three or four replicates, each containing 12 mosquitoes, were performed.

Statistical analysis

The Shapiro–Wilk test was performed to test for a normal distribution of eggs laid by each female, which determined that all the datasets failed the assumption of normality (JMP Pro version 16, SAS Institute Inc., Cary, NC, 1989–2021). The datasets for the multi-choice assays across different densities of each aquatic stage, and across the most preferred densities of a conspecific aquatic stage, were compared using the average total number of eggs laid per female by analysis of variance (ANOVA) followed by a Tukey post-hoc test. For the multi-choice assays, the artificial oviposition sites were rotated through each of the five positions, to minimize any location bias. Following the ECW and XCW experiments, the data were analysed using a general linear model (JMP Pro version 16), determining that there was no positional bias in the egg-laying response of gravid *Ae. aegypti* to these treatments, ECW ($\chi^2 = 12.84$, $df = 8$, $P = 0.12$) and XCW ($\chi^2 = 9.71$, $df = 8$, $P = 0.29$). For dual-choice assays, a binary logistic regression followed by odds ratio comparison was used to test for oviposition site preference, while, an ANOVA followed by a Tukey post-hoc test was used to assess egg stimulation in response to both treatments and controls (JMP Pro version 16). The egg-laying response was the dependent variable determined by the number of gravid females in the oviposition bioassay, and dose was the independent fixed effect.

Results

Water conditioned with aquatic stages affects oviposition

To assess the effect of water conditioned with different densities of aquatic stages of *Ae. aegypti* on the behavioural response of gravid mosquitoes, a series of multi-choice oviposition assays were conducted (Fig. 1a–d; Additional file 1: Figs. S1 and S2). Gravid mosquitoes demonstrated an overall density-dependent response to water conditioned with each aquatic stage compared to the control, as assessed using an ANOVA followed by a Tukey post-hoc test, for ECW ($F = 2.72$, $P = 0.029$); second-instar LCW ($F = 3.89$, $P = 0.0042$); fourth-instar LCW ($F = 2.90$, $P = 0.021$); and XCW ($F = 2.76$, $P = 0.027$); but not for PCW ($F = 0.61$, $P = 0.66$; Fig. 1a–d; Additional file 1: Fig. S2). In all experiments, gravid mosquitoes demonstrated a preference for conditioned water that had contained an intermediate density of the aquatic stages (Fig. 1a–d), with the exception of PCW, for which there was no preference for any density (Additional file 1: Fig. S2). When gravid mosquitoes were given the choice of the most preferred density of ECW, second-instar and fourth-instar LCW, PCW, and XCW in a subsequent multi-choice oviposition assay, individual gravid mosquitoes preferred to lay significantly more eggs in water conditioned with fourth-instar larvae ($F = 7.46$, $P < 0.0001$; Fig. 2).

Bioactive compounds identified in water conditioned with aquatic stages

While combined GC–EAD and GC–MS analyses identified nine bioactive VOCs associated with the ECW extracts, only four (2,4-dimethylhept-1-ene, 2,6-dimethyl-7-octen-2-ol, camphor and decanal) were present in this treatment and not in the control, and thus are considered egg-associated compounds (Fig. 1e). Similarly, of the six and 11 bioactive VOCs from the second- and fourth-instar LCW, five (4-cyanocyclohexene, (*E*)-2-octenal, nonanal, decanal and 4-(2-methylbutan-2-yl) phenol) and eight (2,4-dimethylhept-1-ene, (*E*)-2-heptanal, nonanal, camphor, (*E*)-2-nonenal, (*E*)-2-decenal and 4-(2-methylbutan-2-yl)phenol, and an unidentified branched C12-alkane) were found in these treatments and not in their controls (Fig. 1f, g). From the XCW samples, nine VOCs elicited a response from the antennae, three of which (4-cyanocyclohexene, 2,6-dimethyl-7-octen-2-ol and nonanal) were present in this treatment and not in the associated control (Fig. 1h). While *n*-heneicosane, a previously identified putative oviposition pheromone component in *Ae. aegypti* larvae [32, 33] was specifically sought for in each of the SPME collections, none was identified in the GC–MS analysis.

Synthetic odour blends elicit oviposition in *Ae. aegypti*

To assess whether synthetic odour blends, designed based on the bioactive VOCs identified to be associated with the aquatic stages of *Ae. aegypti*, elicit attraction and stimulation of oviposition, these were evaluated in a dual-choice assay and compared with a solvent

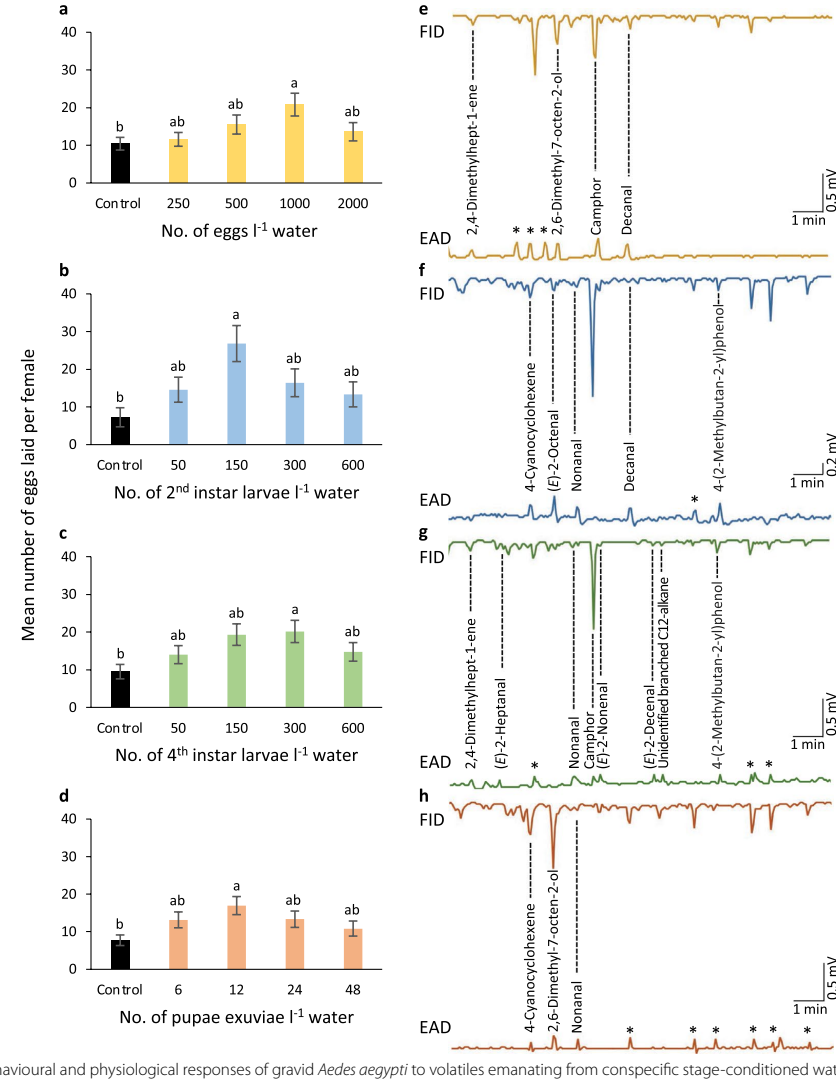

Fig. 1 Behavioural and physiological responses of gravid *Aedes aegypti* to volatiles emanating from conspecific stage-conditioned water. The number of eggs laid by female mosquitoes in response to water conditioned with different densities of **a** eggs, **b** second-instar larvae, **c** fourth-instar larvae and **d** pupal exuviae. The different lowercase letters indicate significant differences ($P < 0.05$), as determined by an ANOVA followed by a Tukey post-hoc test. Error bars represent the standard error of the mean. In **e–h**, the combined gas chromatograph, flame ionization detection and electroantennographic detection (EAD) analyses demonstrate antennal responses of *Ae. aegypti* (mV) in response to bioactive volatile organic compounds in the headspace of conspecific immature-conditioned water eluting over time (min) from the gas chromatograph, and detected by the flame ionization detector (FID). Asterisks in the EAD traces represent compounds that were also present in the control SPME headspace

control (hexane) (Additional file 1: Fig. S3). The synthetic blends were prepared to mimic the identified ratio of bioactive VOCs: eggs (2,4-dimethylhept-1-ene: 2,6-dimethyl-7-octen-2-ol: camphor: decanal, 6:1:17:1); second instar (4-cyanocyclohexene: (*E*)-2-octenal:

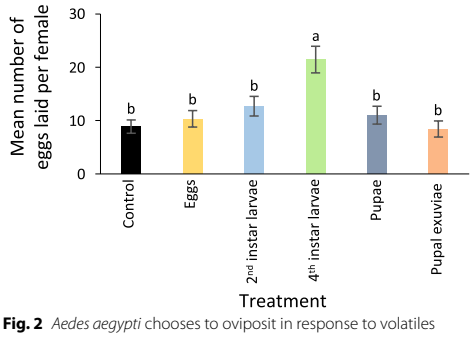

Fig. 2 *Aedes aegypti* chooses to oviposit in response to volatiles from late instar-conditioned water. The different lowercase letters denote significant differences ($P < 0.05$), as determined by ANOVA followed by a Tukey post-hoc test. Error bars represent standard error of the mean

nonanal: decanal: 4-(2-methylbutan-2-yl)phenol, 1:7:9:5:17); fourth instar (2,4-dimethylhept-1-ene: (*E*)-2-heptanal: nonanal: camphor: (*E*)-2-nonenal: (*E*)-2-decenal: 4-(2-methylbutan-2-yl)phenol, 11:9:3:48:1:9:20); and pupal exuviae (4-cyanocyclohexene: 2,6-dimethyl-7-octen-2-ol: nonanal, 1:6:4), respectively. The four blends were diluted in hexane and assayed at different doses against a solvent control (hexane) (Fig. 3a–d). Gravid *Ae. aegypti* were attracted to oviposit in a dose-dependent manner in response to each of the four synthetic blends: eggs ($\chi^2 = 12.09$, $df = 3$, $P = 0.0071$); second instars ($\chi^2 = 15.22$, $df = 3$, $P = 0.0016$); fourth instars ($\chi^2 = 6.79$, $df = 3$, $P = 0.079$); and pupal exuviae ($\chi^2 = 11.11$, $df = 3$, $P = 0.011$) (Fig. 3a–d). Moreover, the synthetic blends significantly stimulated the gravid *Ae. aegypti* to lay eggs dose-dependently, except in response to that of the second instars: eggs ($F = 3.39$, $P = 0.020$); second instar ($F = 1.22$, $P = 0.30$); fourth instar ($F = 4.90$, $P = 0.0030$); and pupae exuviae ($F = 3.13$, $P = 0.027$) (Fig. 3e–h). While a higher overall number of eggs were laid in response to the synthetic odour blends based on the VOCs identified associated with eggs and fourth-instar LCW (Fig. 3e, g), the eggs were laid differentially, with those laid in response to the egg-based odour blend being predominantly placed in the treatment site, and those laid in response to the fourth-instar-based odour blend being predominantly laid in the control site (Fig. 3e, g).

Discussion

Volatiles associated with conspecific aquatic stages differentially attract mosquitoes to oviposition sites and stimulate egg laying [this study, 11, 12, 15, 17, 18, 20, 24, 34–40]. In this study, gravid *Ae. aegypti* were preferentially attracted to oviposit in response to the VOCs emanating from water conditioned with late-stage larvae [11, 20, 22], which likely signals a productive breeding site and reduced competition for resources between the existing, soon to pupate, larvae and the new generation [6, 20, 41, 42]. Furthermore, the density of the conspecific aquatic stages affected oviposition site choice, with gravid mosquitoes laying fewer eggs, and even avoiding ovipositing, on the treated sites when the odours of these sites indicated high densities of conspecific competitors [this study, 12, 15, 17, 18, 24, 25, 41, 43]. Both the aquatic stage and odour release rate significantly affected the manner in which females were stimulated to oviposit in either the treated or controlled sites. Our findings indicate that gravid mosquitoes rely on the detection of aquatic stage-specific VOC blends for the identification and discrimination among potential oviposition sites to provide a reliable signal of the suitability of a potential breeding site for their offspring [24, 39]. This differential preference for a specific conspecific aquatic stage, and its volatiles, may have a direct effect on the population dynamics of *Ae. aegypti*, and provide a potential route by which to manipulate vector behaviour.

The presence of conspecific aquatic stages in a breeding site, currently or in the recent past, influences and mediates the oviposition site selection and egg-laying decision of gravid mosquitoes [this study, 11, 12, 15, 17, 18, 20, 24, 34–40, 44]. The behavioural responses of gravid mosquitoes to these sites are species- and taxon-specific [34, 36, 38, 41, 44]. For example, gravid *Ae. aegypti* preferentially oviposit in water conditioned with late-stage larvae [this study, 20], whereas *Anopheles coluzzii* preferentially lay eggs in breeding sites containing first-instar larvae [15]. This indicates that the odour profile of breeding sites changes depending on the developmental stage of conspecifics, as demonstrated in this study for *Ae. aegypti*, and that of Schoelitsz et al. [39] for *An. coluzzii*.

The ecological rationale for the observed taxon-specific strategies of gravid mosquitoes to use cues from particular conspecific aquatic stages as indicators of the suitability of a potential breeding site for their offspring is based on species-related differences in the ability of their offspring to withstand the changing biotic (e.g., food resources, competition) and abiotic (e.g., water availability, dissolved oxygen, salinity) conditions in the breeding sites [2, 5, 19, 20, 34, 36, 38, 41, 44–46]. *Aedes aegypti* selects breeding sites which contain/contained late-stage larvae for oviposition, which may at first seem counter-intuitive, as by the time that larvae reach the fourth instar there is the risk that these will have consumed the bulk of the resources in the restricted, local environment, and may compete with newly hatched larvae for the limited

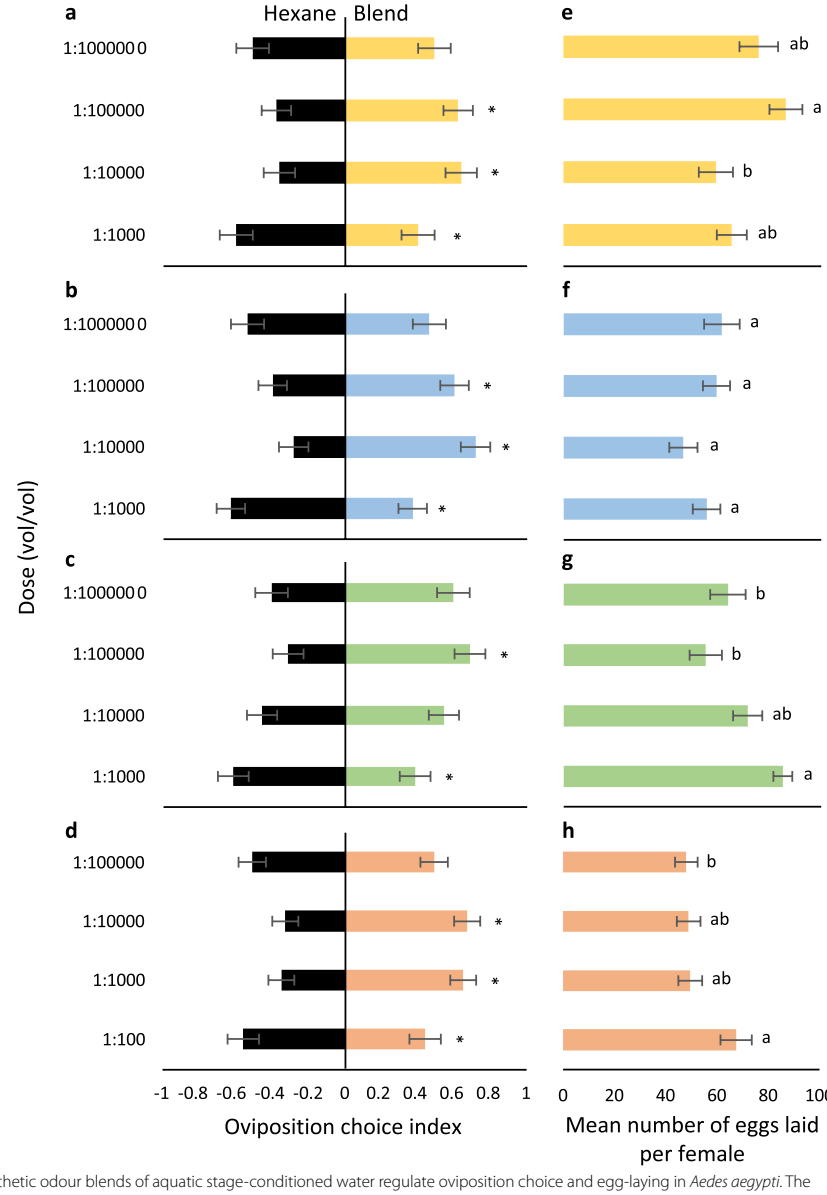

Fig. 3 Synthetic odour blends of aquatic stage-conditioned water regulate oviposition choice and egg-laying in *Aedes aegypti*. The dose-dependent oviposition choice by gravid mosquitoes to individual synthetic odour blends identified as associated with water conditioned with **a** eggs, **b** second-instar larvae, **c** fourth-instar larvae and **d** pupal exuviae, in comparison with a solvent control (hexane). Asterisks indicate significant differences ($P < 0.05$), as determined by a nominal logistic general regression analysis followed by an odds ratio comparison. Gravid *Ae. aegypti* were differentially stimulated to lay eggs in response to these treatments (**e**–**h**). The different lowercase letters represent significant differences ($P < 0.05$), as determined by an ANOVA followed by a Tukey post-hoc test. Error bars represent the standard error of the mean. The sample size is greater than 30 for all comparisons

resources contained within [20, 47]. However, late-stage larvae will soon transition to the non-feeding pupal stage, thereby reducing the risk of conspecific competition with newly hatched larvae, reflecting the oviposition preference of *Ae. aegypti* under natural and laboratory conditions [this study, 20]. The lack of attraction of the gravid mosquitoes to the conditioned water associated with the pupal stage, as the pupae do not produce the volatile signals attracting the gravid females [this study], reflects the inability of the pupae to excrete and thereby to affect the microbiota and uric acid in the environment [48, 49]. Upon adult emergence, on the other hand, the meconium harboured within the pupa is egested, releasing the gut microbiota and other urate-based wastes into the breeding site [49]. Moreover, infested breeding containers typically contain single cohorts of aquatic stages developing in synchrony [50], demonstrating that gravid mosquitoes only recolonize breeding sites in the presence of conspecific eggs, to which gravid mosquitoes are stimulated to lay larger clutches of eggs, or late aquatic stages [this study, 20], to further reduce competition for their offspring. Indirect effects related to the development of the aquatic stages of *Ae. aegypti* may also contribute to oviposition site preference. Breeding sites showing signs of high levels of microbial growth, specifically microbes inoculated during oviposition [51–54], and associated detritus [55–57] that have accumulated during conspecific larval development, provide a rich, abundant larval food source, which reduces the potential competition between established and newly hatched larvae [58]. Gravid mosquitoes ovipositing in temporary waters, such as *Ae. aegypti*, may also indirectly use abiotic factors to assess potential oviposition sites, as cannibalism increases with the increasing numbers of interactions between stages as density increases due to spatial limitation, rather than due to food restriction [14, 59].

Gravid mosquitoes respond in a density-dependent manner to water that contains, or has contained, conspecific aquatic stages [this study, 12, 15, 17, 18, 24, 60]. In our study, gravid *Ae. aegypti* laid fewer eggs in response to water conditioned with high densities of conspecific eggs, larvae and pupal exuviae, supporting the hypothesis that laying the majority of eggs in water with low densities of conspecific aquatic stages will enhance progeny growth, development and the probability of survival, as a result of reduced competition [23, 25, 61]. In contrast, high larval densities generate competition for food resources, which affects the hatching rate and larval development, as well as adult size and survival rate [23, 25, 61, 62]. While in other mosquito species, larvae that are overcrowded, and/or starved, emit deterrent VOCs, which negatively affect the oviposition behavioural response [15, 24, 43], such VOCs have yet to be demonstrated for *Ae. aegypti*. Together, these density-related factors affect the fitness of gravid mosquitoes, which in turn affects the vectorial capacity [61, 63–65]. The assessment of the factors associated with the stage and density of immature conspecifics by gravid mosquitoes can be in large part attributed to the quality and quantity of the VOCs emanating from the potential breeding sites.

Available data indicate that mosquito taxa use distinct blends of VOCs to mediate oviposition site selection and egg-laying behaviour in a species-dependent manner [this study, 11, 31]. While mosquitoes mainly respond to species-specific VOCs, gravid females make use of signature VOCs from breeding sites, particularly select straight-chain aldehydes, monoterpenes, and straight-chain fatty acids and esters, that are commonly detected across mosquito taxa [this study, 11 and references therein, 34, 35, 66–68]. The straight-chain aldehydes [(*E*)-2-heptanal, (*E*)-2-octenal, nonanal, (*E*)-2-nonenal, decanal, (*E*)-2-decenal] and monoterpenes (2,6-dimethyl-7-octen-2-ol, camphor) identified in this study have previously been demonstrated to be emitted by multiple resources used by mosquitoes to locate floral nectar [69–71], host [71, 72] and oviposition site sources [11, 73], reflecting chemical parsimony, suggesting that mosquitoes are under a strong adaptative pressure to make use of the same VOCs as signals to identify various resources [74]. In addition, these and other parsimonious VOCs, detected and used by gravid mosquitoes to locate species-dependent oviposition sites [6, 11], emanate from diverse resources, including conspecific stages [11], food resources [6, 75, 76], fermenting vegetation [77] and living vegetation associated with breeding sites [78–81]. For example, nonanal, decanal and camphor have been identified from several of the conspecific developmental stages of *Ae. aegypti* [this study, 45], but also from infusions of vegetation and grass pollen used by *Culex quinquefasciatus* and *Anopheles arabiensis* larvae as food sources, respectively [75–77, 82]. In addition, (*E*)-2-decenal has been reported from chicken faeces, which attracts *Culex* mosquitoes to preferred oviposition sites [73, 83]. Moreover, straight-chain fatty acids and esters associated with the aquatic stages of mosquitoes appear to be detected across the culicines, and play a role in oviposition site selection [34, 35, 66–68, 84]. While several straight-chain fatty acids and esters have been previously identified from the conspecific aquatic stages of *Ae. aegypti*, none were identified in this study. Moreover, the putative larval pheromone component, *n*-heneicosane [32, 33], was not identified from the larvae-conditioned water in this study, possibly due to the mostly insoluble nature of alkanes in water. The other compounds identified in this study are structurally diverse and have not previously been associated with oviposition sites.

Evidence strongly suggests that gravid *Ae. aegypti* do not detect these VOCs singly, but rather as blends, or chemical codes, which enable them to discriminate among potential sites to lay their eggs.

The combined chemical and electrophysiological analyses of the stage-specific conditioned water extracts demonstrated partially overlapping, yet distinct, VOC blends that provide the basis for gravid *Ae. aegypti* to discriminate and select among potential breeding sites. In previous studies, single VOCs, identified from the chemical analyses of breeding sites, were tested in behavioural assays without prior experiments supporting their physiological activity. Here, we present a workflow that demonstrates the importance of combinatorial coding of VOCs by mosquitoes for oviposition site selection. This coding strategy is not restricted to conspecific signalling, but has also been demonstrated in *An. arabiensis* in relation to oviposition site selection among vegetative resources [75, 76, 79, 85]. An increased understanding of the chemical codes underlying oviposition site selection may lead to the development of novel odour lures used for vector control and surveillance [11, 73].

Conclusions

Gravid *Ae. aegypti* use conspecific stage- and density-dependent cues to identify potential oviposition sites. The volatile profile of oviposition sites conditioned with conspecific aquatic stages changes throughout developmental time, in terms of the quality of the VOCs emitted, providing gravid mosquitoes a means by which to discriminate among oviposition sites, as well as cues for egg stimulation. The identified stage-specific synthetic odour blends regulating oviposition site selection in *Ae. aegypti* may be good candidates for the development of lures to be used in integrated vector control programmes. Future research will optimize and formulate synthetic lures, and subsequently investigate the combining of select blends with, e.g., the In2Care mosquito traps [86], to assess whether these will improve the efficacy of attract-and-kill mosquito control devices.

Abbreviations

EAD	Electroantennographic detection
ECW	Egg-conditioned water
FID	Flame ionization detector
GC–MS	Gas chromatography–mass spectrophotometry
LCW	Larvae-conditioned water
PCW	Pupae-conditioned water
SPME	Solid-phase microextraction
VOCs	Volatile organic compounds
XCW	Exuviae-conditioned water

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-023-05867-1.

Additional file 1: Figure S1. Multi-choice assay used to assess oviposition preference of *Aedes aegypti* to conspecific-conditioned aquatic stage water. **a.** The placement of the artificial oviposition sites (triple cups) within a BugDorm-1 cage. **b.** The construction of the triple cups, allowing olfactory cues, but no other sensory stimuli, to perfuse the assay. **Figure S2.** Oviposition site selection by gravid *Aedes aegypti* in response to pupae-conditioned water. The lowercase letters indicate no significant differences ($P > 0.05$), as determined by an ANOVA followed by a Tukey post-hoc test. Errors bars represent the standard error of the mean. **Figure S3.** Dual-choice oviposition assay used to evaluate choice and egg-laying of *Aedes aegypti* to synthetic blends. **Figure S4.** Oviposition choice of gravid *Aedes aegypti* to solvent controls (hexane) in a dual-choice assay. Gravid *Ae. aegypti* demonstrated no behavioural preference for either side of the oviposition assay (ANOVA followed by a Tukey post-hoc test). Error bars represent the standard error of the mean.

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Author contributions
ZK, RI, BB and SRH contributed to the conception and design of the study. BB contributed to chemical analyses. ZK performed the experiments, conducted data analysis and wrote the original draft of the manuscript. RI and SRH critically reviewed the manuscript. RI acquired funding from the Swedish University of Agricultural Sciences (SLU). All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

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Not applicable.

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The authors declare that they have no competing interests.

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Odor-mediated response of gravid *Aedes aegypti* to mosquito-associated symbiotic bacteria

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ABSTRACT

Complex oviposition decisions allow gravid *Aedes aegypti* mosquitoes to select suitable sites for egg-laying to increase the probability that their progeny will thrive. The bacterial communities present in larval niches influence mosquito oviposition behavior, and gravid mosquitoes transmit key microbial associates to breeding sites during oviposition. Our study evaluated whether symbiotic *Klebsiella* sp., which are strongly associated with mosquitoes, emit volatiles that affect mosquito oviposition decisions. Dual-choice behavioral assays demonstrated that volatile organic compounds emitted by *Klebsiella* sp. induce a preference in oviposition decisions by *Ae. aegypti*. Bacterial headspace volatiles were sampled by solid-phase microextraction, and subsequent combined gas chromatography and electroantennogram detection analysis, revealed that the antennae of gravid females detect two compounds present in the *Klebsiella* sp. headspace. These compounds were identified by gas chromatography and mass spectrometry as 2-ethyl hexanol and 2,4-di-tert-butylphenol. The binary blend of these compounds elicited a dose-dependent egg-laying preference by gravid mosquitoes. We propose that bacterial symbionts, which are associated with gravid mosquitoes and may be transferred to aquatic habitats during egg-laying, together with their volatiles act as oviposition cues indicating the suitability of active breeding sites to conspecific females.

1. Introduction

Microbial symbiosis is widespread among virtually all insects, including mosquitoes, which harbor a diverse panel of mutualistic, commensal, and/or pathogenic symbiotic microorganisms that have important implications for their hosts (Gao et al., 2020; Hosokawa and Fukatsu, 2020). The symbiotic interactions established between different microorganisms and mosquitoes have probably contributed to their evolutionary success, adaptability to different environments, and broad geographic distribution (Ricci et al., 2012). Mosquitoes are holometabolous insects, *i.e.*, undergo complete metamorphosis, with immature forms and adults inhabiting markedly different niches. Strong selection pressure acts on oviposition site-seeking females because progeny growth, survival, and reproductive potential rely on egg-laying decisions, as immature stages are unable to move from poor-quality habitats (Ponnusamy et al., 2008; Mwingira et al., 2021). Mosquito larvae develop in aquatic habitats and acquire a significant proportion of their bacterial symbionts during this stage through feeding (Dada et al., 2014; Dickson et al., 2017; Wang et al., 2018; Scolari et al., 2021). Bacteria are considered a fundamental component of breeding sites, contributing a major nutritional component to larval development by assisting energy storage and biosynthesis of vitamin B_9 (Romoli et al., 2021). Moreover, the bacterial microbiota found in breeding sites mediate gravid mosquito attraction and elicit oviposition (Sumba et al., 2004; Lindh et al., 2008; Ponnusamy et al., 2015). Although there is an increasing understanding of which bacteria play important roles in mosquito breeding sites, there continues to be a lack of information concerning the microbially-derived volatile compounds involved in

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mosquito oviposition site selection and egg-laying. Symbiotic relations support the spreading of mosquitoes and their associated microbiota through the urban landscape. In this context, the extent to which mosquitoes can spread bacteria and other potentially pathogenic microorganisms deserves attention, and the physiological mechanisms underlying these interactions need clarification.

While current approaches to curb pathogen transmission focus on the vector, using chemical and biological strategies to reduce mosquito populations and manage their breeding sites (WHO, 2017), there is growing evidence showing that a flawed use of insecticides has caused prevalent resistance (Dusfour et al., 2019), and even that mosquitoes can avoid pesticide compounds after a single sublethal exposure (Sougoufara et al., 2022). Tools that target gravid females using odor-based lures will likely reduce the reliance of mosquito control strategies on insecticides. In this context, bacteria promoting egg-laying represent a rich source of potentially attractive volatiles that could lead to the development of lures targeting gravid mosquitoes.

Several bacteria are consistently detected in association with mosquitoes and their breeding sites (Guégan et al., 2018; Scolari et al., 2019). This is the case for *Klebsiella*, a genus of bacteria associated with many mosquito species among the *Aedes, Anopheles,* and *Culex* genera (Chandel et al., 2013; Yadav et al., 2015; Rocha et al., 2021) but also a potential human pathogen (Rocha et al., 2021). The present study aimed to evaluate whether the volatiles emitted by *Klebsiella* sp. elicit oviposition by the arboviral vector *Aedes aegypti*. Moreover, we sought to identify the bioactive volatile organic compounds (VOCs) emitted by *Klebsiella* sp. and to determine the olfactory pathway by which these VOCs are detected. Finally, we demonstrated that a synthetic blend of these VOCs is capable of inducing egg-laying in a dose-response manner. The applications of microbial VOCs toward pathogen and mosquito control strategies are discussed.

2. Methodology

2.1. Mosquito rearing

Aedes aegypti were reared at 27 ± 1 °C, 65 ± 5% relative humidity, and under a 12:12 LL/DD photoperiod. Larvae were reared in plastic trays containing distilled water and fed Supervit-8 mix tropical flakes (VPG Sweden AB, Ängelholm, Sweden) daily. Pupae were transferred from rearing trays to Bugdorm-1 cages (Megaview Science, Taichung, Taiwan) in plastic cups for adult emergence. Adults were offered 10% sucrose solution *ad libitum*. Females were granted access to many males from adult emergence until experimental activities were initiated to ensure a high rate of insemination. Females were blood-fed 5 days post-emergence (dpe) using a Hemotek membrane feeding system (Hemotek Ltd, Blackburn, UK) with defibrinated sheep blood (Håtunalab, Bro, Sweden) for 30 min. Once blood-fed, females were provided with a 10% sucrose solution *ad libitum*. Only fully engorged females were used in the experiments 5 days after blood-meal ingestion (10 dpe).

2.2. Klebsiella sp. cultures

Klebsiella sp. (Genbank accession: JAGTYC000000000) cultures were plated on tryptic soy agar (TSA; VWR, Stockholm, Sweden) plates using the streak plate technique, and incubated overnight. A single colony was transferred from the TSA plates to tubes filled with 6 ml of 0.01%, 0.1%, 1%, or 10% tryptic soy broth (TSB; VWR, Stockholm, Sweden) and incubated for 24 h at 25 °C on a shaker at 200 rpm. After incubation, tubes were centrifuged at 4 °C and 3000 rpm for 15 min to collect a bacterial pellet. The supernatant was removed by decantation and the bacterial cells were resuspended in sterile 0.085% NaCl. This step was repeated twice, and resuspended in TSB (0.01%, 0.1%, 1%, or 10%) to obtain a stock suspension. Then, 15 µl of the stock suspension were transferred to 30 ml of TSB and incubated at 25 °C on a shaker at 200 rpm. Once the culture reached the stationary phase, an aliquot was serially diluted, and plated to count colony forming units (CFUs) to determine the bacterial load to be tested under each experimental condition.

2.3. Behavioral responses to klebsiella sp. volatiles

The oviposition choice of *Ae. aegypti* (Rockefeller strain) was evaluated in dual-choice assays. For this purpose, Bugdorm-1 cages were adapted with two holes (0.6 cm diameter) on opposite sides of the cage. This allowed Teflon tubing (o.d. 0.6 cm) to enter the cage and reach the artificial oviposition sites (Fig. 1a). The oviposition sites consisted of three plastic cups positioned one inside the other: at the bottom, a cup (250 ml) fitted with a hole (0.6 cm diameter) in the wall that was connected to the air inlet tube (test or control); a cup (120 ml) with eight perforations in the bottom (1 mm diameter) stacked into the first cup; and the top cup (30 ml) containing a cone-shaped piece of filter paper and *ca.* 20 ml of distilled water, which served as the oviposition substrate (Supplementary figure 1).

Tryptic soy broth (30 ml) with (test) and without (control) bacteria inoculation were placed in individual 100 ml glass bottles (Simax, VWR) adapted with an air inlet and an outlet. An airflow, generated using an air pump (Model V-20, Hailea, China), was pumped through silicone tubing (0.5 cm) that passed through an activated charcoal filter and a polyethersulfone sterile filter (pore size 0.22 µm, Thermo Fisher, Sweden), which was connected to the individual glass bottles containing bacterial cultures or sterile media. The air exiting the bottles through Teflon tubing (6 mm diameter) passed through a second sterile filter to avoid the spread of *Klebsiella* sp. (a potential human pathogen). The flow rate of the volatile-enriched air leaving the bottles was adjusted to 0.1 L min^{-1} using two 12-channel flow meters (Kytola Instruments, Finland), and then introduced into the artificial oviposition sites (test or control). A single gravid mosquito was then released in the center of the cage 2 h prior to the onset of the scotophase (ZT12) using an aspirator. Females were allowed to oviposit for 24 h. Experiments were conducted inside a room with controlled temperature (27 ± 1 °C) and humidity (65 ± 5%). For counting the number of eggs laid, the oviposition substrates, from both the test and control, were recovered from the cage and the eggs were manually counted on the laboratory bench with the help of a needle when eggs were glued together (N>30 females *per* bacterial load). Four bacterial loads were tested ($10^{6.3}$ CFU ml^{-1}, $10^{7.3}$ CFU ml^{-1}, $10^{8.3}$ CFU ml^{-1}, and $10^{9.2}$ CFU ml^{-1}) against their controls (0.01%, 0.1%, 1%, and 10% TSB, respectively). Due to the complex experimental design required to avoid the spread of *Klebsiella* sp., no positional alternation procedures for the control and test cups were used for these experiments. Before starting the assays, the experimental setup was verified to be unbiased by evaluating the oviposition choices of gravid mosquitoes when presented with two airstreams passed over the headspace of distilled water or of culture media, respectively. These tests were carried out for 24 h using the same experimental conditions previously described.

2.4. Electrophysiological analysis

Antennal responses of gravid *Ae. aegypti* to *Klebsiella* sp. headspace were recorded using combined gas chromatography and electroantennographic detection (GC-EAD). Bacterial cultures growing in 1% TSB were prepared as described above. The headspace of *Klebsiella* sp. cultures (2 ml culture in 10 ml sterile vials fitted with a PTF E-lined septum) was sampled for 4 h at 25 °C using solid-phase microextraction (SPME) fibers coated with DVB/CAR/PDMS (Supelco, VWR). The SPME fibers were conditioned in splitless mode at 250 °C for 30 min before exposure to the bacterial culture headspace.

The GC (Agilent Technologies 6890, Agilent Technologies, Santa Clara, USA) was fitted with an HP-5 column (30 m x 0.25 mm id and 0.25 µm film thickness, Agilent Technologies). The carrier gas was hydrogen at a linear flow rate of 45 m s^{-1}. The VOCs adsorbed on the

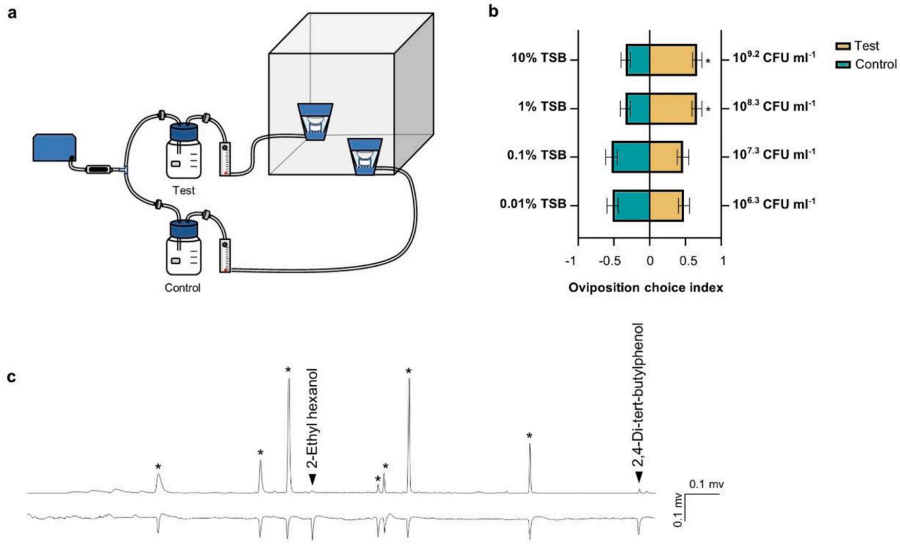

Fig. 1. *Aedes aegypti* gravid females respond to *Klebsiella* sp. volatiles. **(a)** Schematic representation of the dual-choice assay testing volatiles from bacterial cultures. **(b)** Oviposition choice of mosquitoes to *Klebsiella* sp. volatiles (test) compared to the culture medium (control). Volatiles emitted by bacteria cultured in 1% TSB and 10% TSB elicited significantly increased egg-laying by gravid females compared to the controls (*, $P < 0.05$). Error bars represent the standard error of the mean. **(c)** Combined gas chromatography (GC) and electroantennal detection (EAD) traces display voltage changes (mV) in the antennal electric potential as a response to the bioactive compounds in the headspace of *Klebsiella* sp. cultures, eluting from the GC and detected by the flame ionization detector (FID). Two biologically active compounds were unique to *Klebsiella* sp.: 2-ethyl hexanol and 2,4-di-tert-butylphenol. Volatile organic compounds (VOCs) associated with *Klebsiella* sp. are highlighted with ▼, while VOCs present in TSB are represented by asterisks.

SPME fibers were thermally desorbed in the inlet of the GC, in the splitless mode, for 30 s at 225 °C. The GC oven temperature was programmed from 35 °C (3 min hold) at 10 °C min^{-1} to 290 °C (10 min hold). At the GC effluent, nitrogen (4 psi) was added and split 1:1 in a 3D/2 low dead volume four-way-cross splitter (Gerstel, Mülheim, Germany) between the flame ionization detector (FID) and the EAD. The GC effluent for the EAD passed through a transfer line (ODP-2, Gerstel), which tracked the GC oven temperature, into a glass tube (10 cm x 8 mm), where it was diluted with a charcoal-filtered, humidified airstream (1.5 l min^{-1}). The antenna was placed 0.5 cm from the outlet of the effluent tube.

To mount the mosquito antenna for EAD analysis, a female mosquito was cold-anesthetized, the head excised, and the distal flagellomere of the antenna was cut. The reference electrode, filled with Beadle–Ephrussi solution, was inserted into the foramen and grounded. A recording glass electrode filled with Beadle–Ephrussi solution was then connected to the distal end of the antenna. The recording electrode was connected to a pre-amplifier probe (1X) and then to a high impedance DC amplifier interface box (IDAC-2, Ockenfels Syntech GmbH, Buchenbach, Germany). Three stable recordings were performed for each treatment.

2.5. Chemical analysis

The *Klebsiella* sp. headspace collected by SPME was analyzed using a combined gas chromatograph and mass spectrometer (GC–MS; GC: Agilent Technologies 6890 or 7890 model, MS: Agilent Technologies 5975) operated in the electron impact ionization mode at 70 eV. The samples were analyzed on either of two fused silica capillary columns (HP-5 or DB-Wax, 60 m x 250 μm x 0.25 μm film thickness, Agilent Technologies) and compared with control headspace collections. The temperature program of the GC oven was the same as described above. Helium was used as the carrier gas at a linear flow rate of 35 cm s^{-1}. Five independent injections of headspace collections from both bacterial cultures and sterile culture medium were made. The compounds were identified by matching their mass spectra and retention times (Kovat's indices) in comparison with custom-made and the reference National Institute of Standards and Technology (NIST) libraries (NIST14, Agilent Technologies). The identified compounds were confirmed by co-injection with synthetic standards: 2-ethyl hexanol (CAS no. 104–76–7; ≥ 99.6%; Sigma-Aldrich, Stockholm, Sweden) and 2,4-di-tert-butylphenol (CAS no. 96–76–4; 99%; Sigma-Aldrich). The relative ratio of these VOCs was determined by their abundance (area under the peak) in the headspace samples.

2.6. Bioassays with a synthetic blend

The preference of two wildtype strains of *Ae. aegypti*, Rockefeller and Orlando, and an *orco* mutant strain lacking the odorant receptor (OR) co-receptor (*orco*$^{5/16}$; DeGennaro et al., 2013), for a binary synthetic blend derived from the bioactive compounds identified in the headspace of *Klebsiella* sp. was tested in the dual-choice oviposition assay described above. The synthetic blend was prepared by dissolving 2-ethyl hexanol and 2,4-di-tert-butylphenol at a ratio of 3:7 in hexane (CAS no. 110–54–3; 95%; Sigma-Aldrich). Three doses of the synthetic blend were tested (0.001, 0.01, and 0.1 volume: volume) against the control. For this series of experiments, the bottom cup was filled with 30 ml of distilled water conditioned with 10 μl of hexane (control) or 10 μl of the synthetic blend (test). Thus, all assays testing synthetic compounds were performed using passive volatilization with no airflows associated, allowing alternating control and test cups to avoid positional bias.

In order to assess the antennal response to the synthetic blend of gravid mosquitoes from the three *Ae. aegypti* strains, GC-EAD analysis were performed, as described above. Three stable recordings were performed for each mosquito strain.

2.7. Statistical analysis

The oviposition choice for the behavioral assays was determined by calculating choice indices generated using the formula CI test=$T/(T + C)$ and CI control= $C/(T + C)$, where T is the number of eggs laid in the test cup (corresponding to volatiles from bacteria or the synthetic blend in Figs. 1 or 2, respectively) and C the number of eggs laid in the control cup (corresponding to volatiles from broth or solvent in Figs. 1 or 2, respectively). The Shapiro-Wilk test was used to evaluate whether datasets had a normal distribution. As none of these datasets passed the assumption of normality, the oviposition choice indices of gravid *Ae. aegypti* were analyzed using the Wilcoxon-matched pairs test (Prism v. 8.0.1, GraphPad, San Diego, California, USA). All raw data corresponding to behavioral experiments are reported in Supplementary material 1.

3. Results

3.1. Klebsiella sp. volatiles elicit oviposition

The VOCs emitted by *Klebsiella* sp. cultured in TSB elicited a bacterial load-dependent selection of oviposition sites by gravid *Ae. aegypti* compared with the VOCs emitted by sterile broth in a two-choice assay (Fig. 1b). No statistically significant preferences were detected in the corresponding control experiments, testing either distilled water or broth volatiles (Supplementary material 2).

3.2. Bioactive compounds identified in Klebsiella sp. headspace

Considering that volatiles present in the headspace of *Klebsiella* sp. triggered a bacterial load-dependent oviposition response by gravid mosquitoes, GC-EAD and GC–MS analyses were used to identify the bioactive compounds. While nine VOCs eluting from the GC consistently elicited responses from the antenna, only two, 2-ethyl hexanol and 2,4-di-tert-butylphenol, were found to be unique to *Klebsiella* sp. headspace, *i.e.*, were not present in the headspace of TSB (Fig. 1c).

3.3. Synthetic Klebsiella sp. odor blend induces oviposition choice

The synthetic binary blend of 2-ethyl hexanol and 2,4-di-tert-butylphenol induced a significant oviposition choice by females of the Rockefeller and Orlando strains at the highest dose tested (Fig. 2a and b). In contrast, *orco* mutants were indifferent to the binary blend (Fig. 2c).

3.4. Different electrophysiological responses to the synthetic Klebsiella sp. odor blend

The binary synthetic odor blend elicited responses in the antennae of gravid mosquitoes from the Rockefeller and Orlando strains (Fig. 2d). In contrast, the binary blend failed to elicit antennal responses in *orco* mutants (Fig. 2d).

4. Discussion

Infochemicals influencing mosquito oviposition behavior are produced by microorganisms present in soil, water, plants, and fermenting organic matter, which attract mosquitoes to lay eggs (Lindh et al., 2008; Ponnusamy et al., 2008; Mwingira et al., 2020). Despite increasing evidence indicating that bacteria promote behavioral responses by gravid mosquitoes, the mechanisms by which microorganisms are detected and affect female oviposition decisions have not been fully elucidated (Girard et al., 2021). In this study, the headspace volatiles of *Klebsiella* sp. cultures elicited egg-laying in gravid *Ae. aegypti*. A binary blend of bioactive compounds identified in the *Klebsiella* sp. headspace, detected by the odorant receptor pathway, were able to recapitulate the oviposition response of gravid mosquitoes. The binary blend identified here has the potential to be used as a lure together with existing tools for mosquito monitoring and control.

Klebsiella sp. can be vertically transferred in insect systems (Lauzon et al., 2009; Hassan et al., 2020), and it has been proposed that gravid mosquitoes could transmit this bacterium to breeding sites during egg-laying to nurture recently emerged larvae and accelerate larval molting into a more resilient instar (Díaz-Nieto et al., 2016). In addition, *Klebsiella* is a mosquito symbiont reported to colonize eggs, larvae,

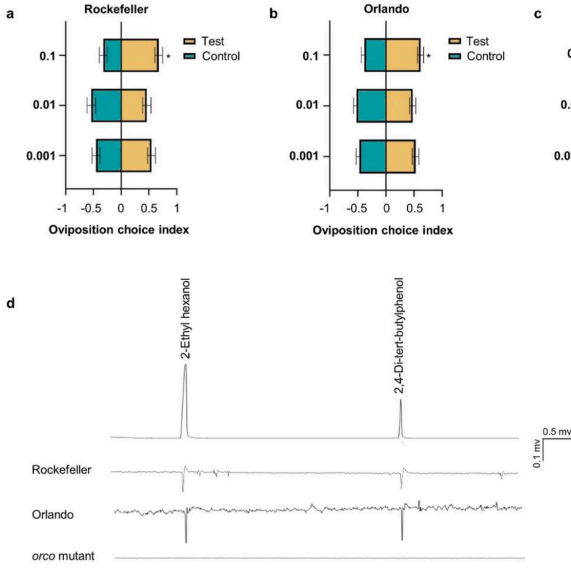

Fig. 2. Behavioral and physiological response to the synthetic *Klebsiella*-based binary odor blend. The binary blend (test) significantly increased egg-laying by **(a)** Rockefeller and **(b)** Orlando gravid *Aedes aegypti* at the highest dose tested, compared to the solvent control (*, $P < 0.05$). **(c)** No significant preference was observed in *orco* mutants. Error bars represent the standard error of the mean (a-c). **(d)** Combined gas chromatography (GC) and electroantennal detection (EAD) traces display voltage changes in the antennal electric potential of Rockefeller and Orlando gravid females as a response to the synthetic *Klebsiella*-based binary blend. No antennal responses were observed in *orco* mutant females.

pupae, and adults (Gusmão et al., 2010; Dada et al., 2014; Alvarado et al., 2021; Rocha et al., 2021). This association may be a strategy evolved by the bacteria to complete their life cycle and/or increase dispersal (Girard et al., 2021). Considering the reciprocal fitness advantage gained, there is an evolutionary pressure for the mosquito to develop the sensory systems to detect the cues that identify these symbiotic bacteria.

Volatile organic compounds of bacterial origin have the potential to act as reliable cues for oviposition site-seeking females when evaluating the quality of the breeding site (Lindh et al., 2008; Ponnusamy et al., 2015; Girard et al., 2021). An analysis of a *Klebsiella* sp. genome and metabolome from a strain associated with *Ae. aegypti* identified gene clusters that are implicated in primary metabolic pathways that synthesize VOCs which may influence the assessment of potential oviposition sites by gravid mosquitoes (Mosquera et al., 2021). *Klebsiella* sp. metabolites were reported in *Ae. aegypti* breeding water where a single gravid female had laid eggs and larvae developed (Mosquera et al., 2021). In our study, the VOCs present in the headspace of *Klebsiella* were shown to promote oviposition choice in *Ae. aegypti*, in line with that which was shown previously for *Culex pipiens* (Díaz-Nieto et al., 2016). While the metabolic pathways to produce 2-ethyl hexanol and 2, 4-di-tert-butylphenol were not highlighted in the study by Mosquera et al. (2021), it is interesting to note that both 2-ethyl hexanol and 2, 4-di-tert-butylphenol appear to be ubiquitous compounds of bacterial origin (Zhao et al., 2020; National Center for Biotechnology Information, 2022). A binary blend of these compounds is sufficient to drive oviposition site preference in *Ae. aegypti* indicating that this blend may act as a reliable and specific oviposition signal for this species. The detection of these VOCs relies on the OR pathway, as indicated by the lack of behavioral and physiological response in *orco* mutants, similar to that reported by Melo et al. (2020). Whether these observations extend to all microbial VOCs impacting mosquito oviposition behavior deserves attention.

Our findings demonstrate a link between bacteria and mosquito breeding sites, which could contribute to the flow of this potential human bacterial pathogen among the rain water containers, the airborne environment in which adult mosquitoes spend their lifetime, and, fundamentally, the urban anthroposphere where they interact with humans. *Aedes aegypti* is the main vector of dengue, Zika, yellow fever and chikungunya viruses, and it has spread to most tropical and subtropical regions in close association with urban environments. The ability of this species to transmit arboviruses threatens the health of millions, thus representing a major public health concern (WHO, 2017). Symbiotic bacteria have been shown to impact arboviral transmission and demonstrated to have potential for use in mosquito control. One novel vector control tool exploiting a mosquito-microbe interaction with tangible success in dengue control, has been the release of *Wolbachia*-infected *Ae. aegypti* for population replacement (Gesto et al., 2021; Pinto et al., 2021). *Wolbachia* (wMel strain) has emerged as an efficient pathogen-blocking and self-dispersing agent that reduces *Ae. aegypti* vector competence and impairs arbovirus transmission (Gesto et al., 2021). Moreover, volatile organic compounds emanating from symbiotic bacteria, such as *Klebsiella* sp., promote the choice of breeding sites by *Ae. aegypti*. Similarly, a recent study showed that geosmin, a volatile compound that mosquitoes likely associate with microbes in the aquatic habitats, promotes oviposition site preference in laboratory and field studies (Melo et al., 2020). These symbiotic bacteria-associated VOCs could be used to exploit mosquito behavior for population surveillance and control purposes, by using oviposition-inducing VOCs together with insecticide-containing devices to develop attract-and-kill mosquito management tools.

Author contribution

KDM, BA, SHR, RI, and MGL contributed to the conception of the study and the design. KDM wrote the original draft. SHR, RI, and MGL critically reviewed the manuscript. KDM, ZK, and BW performed the experiments. KDM, ZK, and BW conducted data analysis. BA, RI, and MGL acquired funding. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2022.106730.

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Mosquitoes detect and discriminate among potential oviposition sites, predominately using olfaction. This thesis investigated the odour-mediated oviposition site selection and preference of two disease vector mosquitoes, *Aedes aegypti and Culex quinquefasciatus,* in response to intra-, and interspecific aquatic stages, as well as a commensal bacterium. Gravid mosquitoes differentially respond to these biotic factors to minimise competition and secure food availability for the offspring.

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