LANDING BEHAVIOUR OF THE HOST-SEEKING MALARIA VECTOR, *ANOPHELES COLUZZII*, IN RESPONSE TO HOST-ASSOCIATED CUES

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DECLARATION

I certify that the work contained in this thesis, or any part of it, has not been accepted in substance for any previous degree awarded to me or any other person, and is not concurrently being submitted for any other degree other than that of Doctor of Philosophy which has been studied at the University of Greenwich, London, UK.

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Este trabajo esta' dedicado a todos los Nadie de Galeano,

y a todos los que trabajan dia a dia para colmar esta injusticia.

This work is dedicated to all the Nobodies by Galeano,

And to all those that work day after day trying to bridge this injustice.

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ABSTRACT

Female mosquitoes of the genus *Anopheles* transmit the parasites responsible for malaria, one of the most serious vector-borne diseases, which affects one-fifth of the world population. Current malaria prevention relies heavily on vector control interventions, which are most effective and sustainable when based on mosquito behaviour. Although much research has focussed on host-seeking behaviour, less is known about the close-range phase, including landing and post-landing behaviour. Thus, this research aimed to 1) quantify the relative effect that host-associated stimuli have on landing response, 2) quantify the effect that physical target characteristics have on flight behaviour and landing response, and 3) characterise pre- and post-landing behaviour and feeding success in relation to variations of target temperatures. Using behavioural assays, this project firstly quantified the relative role of three host-associated stimuli (thermal, visual, and olfactory) in driving landing and found that they act synergically to increase landing response, that host odour is an essential cue in this phase, and that the landing response is the result of a flexible yet accurate stimuli integration. Secondly, mosquitoes were offered targets with different physical characteristics, and it was found that more mosquitoes landed on the target when at least half of its surface was heated, which suggests a basis for more cost-effective trap designs. Furthermore, more mosquitoes landed on large targets, although small targets caught a greater density of mosquitoes per unit area; however, no effect on landing was observed when targets were oriented vertically or horizontally. Thirdly, it was demonstrated that the surface temperature of a target strongly influenced the feeding success, with mosquitoes being equally successful in feeding on blood at temperatures that ranged from normal human physiological to febrile conditions. Surface temperature also influenced post-landing behaviour, as mosquitoes displayed few foraging events and fed promptly on blood at 36 °C, whilst behaved radically different when presented with blood at 30 °C and 48 °C. Altogether, these results indicate that mosquitoes respond with distinct behaviours depending on the set of cues encountered during host-seeking. The results presented here could be used to improve vector surveillance and control tools by incorporating specific stimuli that are known to elicit specific behavioural responses. Thus, this research aids the cause of reducing the global burden of mosquito-transmitted diseases.

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LANGUAGE DISCLAIMER

For centuries, language has evolved and has been moulded to reflect human emotions, actions, and ways of thinking. There is no doubt that current language reflects an anthropomorphic idea of the world that surrounds humans. The author is aware that different organisms process information in radically different manners. However, the common terminology used to describe animal actions and reactions, in many cases, suggest a more anthropomorphic interpretation of the words. When considering terminology referred to animals, and in particular for this thesis, to mosquitoes, the author invites the readers to interpret the words as mere analytical and mechanical ways of processing the information, leaving the anthropomorphic concepts of the words behind. For example, words such as "finding", "choice", etc, when referred to mosquitoes, are to be considered in a mechanical way, excluding human emotions, feelings, intentionality, and way of thinking.

GLOSSARY

Term	Definition
Activator	A physical or chemical signal that initiates a process.
Anautogenous	In entomology: it refers to a reproductive strategy in which females must ingest blood in order to produce eggs.
Anemotaxis	The directed movement of an organism in response to a current of air.
Anthropophagic	Said of organisms that present an anthropophagy trait.
Anthropophagy	The preference of feeding on humans over other animals.
Anthropophilic	Said of organisms that present an anthropophily trait.
Anthropophily	The preference of humans over other animals.
Attractant	A physical or chemical signal which stimulates attraction.
Attraction	In this thesis: referred to the response elicited in an entity (e.g. a mosquito) that draws such entity towards the source of the stimulus.
Attractive	In this thesis: referred to something (e.g. cue, object) that has the capacity to attract.
Defibrinated	Blood that does not contain fibrin.
Ecdysis	The process of changing the cuticle.
Eclosion	The emergence of an adult insect from a previous life stage, i.e. pupae or nymphs.
Ectotherm	Refers to an animal that cannot regulate its body temperature and therefore depends on external sources for body heat. Also called "cold-blooded" animal.

Term	Definition
Endemic	Refers to a disease that is regularly found in a specific geographic area.
Endophagic	Said of organisms that present an endophagy trait.
Endophagy	The habit of feeding indoors.
Endophilic	Said of organisms that present an endophily trait.
Endophily	Tendency to inhabit or rest indoors.
Endotherm	Refers to an animal that can regulate its body temperature, and therefore relies on heat that is metabolically produced. Also called "warm-blooded" animal.
Entomological inoculation rate	The measure of the exposure per person to infectious mosquito bites in a given time.
Exophagic	Said of organisms that present an exophagy trait.
Exophagy	The habit of feeding outdoors.
Exophilic	Said of organisms that present an exophily trait.
Exophily	Tendency to inhabit or rest outdoors.
Exuvia	The exoskeleton no longer inhabited after a moult.
Fecundity	Indicates the potential ability to produce offspring.
Fertility	The number of offspring produced by an organism, i.e. actual reproduction output.
Fitness	The relative success of a genotype to be transmitted to the next generation. It depends on the survival and reproduction of the organisms carrying such genotype.
Gonotrophic cycle	In haematophagous insects: it refers to the reproductive cycle which begins with the ingestion of a blood meal and ends with the oviposition of the eggs.

Term	Definition
Gravid	A female bearing developed eggs or embryos.
Haematophagous	Said of organisms that present a haematophagy trait.
Haematophagy	The habit of feeding on blood.
Host odour	Blend of all chemical compounds (organic and inorganic) that emanate from a host.
Host-seeking	The process that brings a vector (e.g. a female mosquito) close to its host.
Imago	In insects: the adult form, in its final, sexually mature stage.
Instar	Insect larval stage between moults.
Klinokinesis	Undirected movement of an organism in which the rate of turning depends on the intensity of the external stimulus.
Odorant	Chemical compound that, alone or together with other odorants, stimulate an olfactory organ, i.e. it binds olfactory-binding proteins triggering a cascade of events which culminates in the signal of a distinctive "smell".
Odour	Often a mixture of different volatile chemical compounds that stimulates an olfactory organ.
Opportunistic behaviour	Referring to feeding habit: it applies to organisms that feed on whichever food source is available without displaying a marked preference for a particular source.
Oviposition	The deposition of eggs through an ovipositor.
Resolution angle	Also known as angular resolution, is the minimum angular distance that two small objects need to have in order to be distinguished by an optical instrument (e.g.

Term	Definition
	an eye) as two different entities. This angle describes the resolving power and precision of the optical instrument.
Retinal velocity	Speed of the image movement perceived by an insect eye.
Sensillum (plural sensilla)	A cuticular sensory organ found in arthropods' exoskeleton. Sensilla can either form protrusions or hollows in the cuticle. All sensilla host sensory receptors (i.e. sensory neurons) that can detect information about the environment, e.g. thermoreceptors, chemoreceptors, mechanoreceptors, hygroreceptors.
Tortuosity	In this thesis: the index that indicates the state of being tortuous (i.e. full of bends and twists).
Vector	In biology: an organism that carries and transmits an infectious agent from one organism to another.
Vectorial capacity	The daily number of future infectious bites that would arise from a single infective patient, if all the females biting that patient become infected.
Zoophagic	Said of organisms that present a zoophagy trait.
Zoophagy	The preference of feeding on animals over humans.
Zoophilic	Said of organisms that present a zoophily trait.
Zoophily	The preference of animals over humans.

ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ANOVA	In statistics: analysis of variance
CDC	Centers for Disease Control and Prevention
DDT	Dichlorodiphenyltrichloroethane
d.f.	In statistics: degrees of freedom
EIR	Entomological inoculation rate
EPF	Entomopathogenic fungi
GLM	In statistics: generalised linear model
H_0	Null hypothesis
H_1	Alternative hypothesis
HDT	Host decoy trap
HSP(s)	Heat Shock Protein(s)
IR	Infrared (radiation)
IRS	Indoor residual spraying
ITN	Insecticide-treated net
IVM	Integrated vector management
LD	Light : dark

LED	Light-emitting diode
LLINs	Long lasting insecticide-treated nets
MAP	The Malaria Atlas Project
N_a	Number of mosquitoes activated
N_c	Number of mosquitoes in the release cage
NRI	Natural Resources Institute
N_t	Number of mosquitoes recovered on the landing target
N_w	Number of mosquitoes recovered in the wind tunnel excluding those in the release cage and on the landing target
PC	In statistics: principal component
PCA	In statistics: principal component analysis
PCR	Polymerase chain reaction
RDA	In statistics: redundancy analysis
RH	Relative humidity
SEM	In statistics: standard error of the mean
US	Unites States of America
WHO	World Health Organization

1. INTRODUCTION

1.1.   Rationale

Due to their blood-sucking habit, mosquitoes acquire a vast range of pathogens from their hosts. They can incubate and transmit those agents to other hosts, spreading fatal diseases. Currently, malaria is the vector-borne disease with the highest annual mortality (Becker *et al.*, 2010; Riveron *et al.*, 2018). Yearly it affects more people, more persistently, and on a larger scale than any other vector-borne disease (Becker *et al.*, 2010; Gullan and Cranston, 2014), with 85 countries being classified as "malaria-endemic" (WHO, 2021). Even though a significant reduction of malaria incidence occurred since the beginning of the millennia, in 2020 about 241 million cases were reported worldwide (WHO, 2021). Despite the existence of malaria treatment, the disease was reported to be responsible for the deaths of 627000 people in that year, 12% more compared to the estimated for 2019, with this increase caused by service disruption due to the SARS-CoV-2 pandemic (WHO, 2021). As pregnant women and children under five years old are more vulnerable to malaria, the majority of these deaths are within these two groups of patients. The most affected continent is Africa (WHO, 2020, 2021). Within this continent, the burden falls mainly on the sub-Saharan region, as it accounts for approximately 95% of the global incidence (WHO, 2021).

The cost of malaria is not just limited to its high annual mortality, but it extends also to the loss of labour days, the loss of energy and wellbeing in affected individuals, and to the cost of treatments, control, and prevention programs, making malaria a significant social and economic burden (Feachem *et al.*, 2019). In fact, malaria can be held accountable for millions of dollars of economic losses every year (Sachs and Malaney, 2002; Smith *et al.*, 2020b), and it has been estimated that malaria has decreased the affected countries' Gross Domestic Product by up to 1.3% (European Alliance Against Malaria, 2007). On the social side, malaria negatively influences school attendance, cognitive development, learning abilities, and foetal development (Sachs and Malaney, 2002; Fernando

et al., 2010; Saito *et al.*, 2020). Furthermore, chronic malaria is associated with an increased vulnerability to other infections and medical conditions such as severe anaemia, chronic renal damage, nephrotic syndrome, Burkitt's lymphoma and hyper-reactive malarial splenomegaly (Sachs and Malaney, 2002). Thus, it is evident that malaria has a large impact on society, affecting its development and the general quality of life.

The burden is not equally distributed worldwide as it mainly affects tropical areas and reaches to a significant extent also the subtropical regions, adversely affecting the areas where most global poverty is concentrated (Chen *et al.*, 2018). In low-income countries, poverty itself could be counted as a cause of some of the most intense malaria outbreaks (Sachs and Malaney, 2002). It is hard to completely attribute poverty as a cause of malaria, as the severity of malaria outbreaks is mainly determined by biological and climate factors, and therefore it is difficult to discern whether the principal causative factor is the geographical position or the effect of poverty, or a combination of both. Nonetheless, there is enough evidence to prove that malaria is a contributing factor causing poverty (Gallup and Sachs, 2001; Sarma *et al.*, 2019).

In 2015, the World Health Organisation (WHO) and the Roll Back Malaria Partnership set an aim of reducing the incidence of malaria by 90% by 2030 (Roll Back Malaria Partnership, 2015). It was predicted that to achieve this goal, more than 101.8 billion US dollars of investment would be needed. In addition to this, a further 673 million US dollars would be necessary each year to support the required research. However, the economic gains from these activities are predicted to be 40:1, and even 60:1 in sub-Saharan Africa, meaning that for every US dollar spent on malaria control, up to 40 or 60 US dollars will be gained overall if the plan is achieved (Roll Back Malaria Partnership, 2015; Chen *et al.*, 2018). Thus, it is evident that the economic benefit will far outweigh the initial investment and effort. Nonetheless, despite the enormous benefits that could arise if the investment target set by WHO was met, the level of investment has repeatedly fallen short year after year (Sachs and Malaney, 2002; WHO, 2017, 2018, 2020, 2021). In the past few years, less than half of the estimated yearly

amount needed to reach the goal was invested in malaria control programs (WHO, 2017; Smith *et al.*, 2020b). In 2020 for example, of the estimated 6.8 billion US dollars required, only 3.3 billion US dollars were invested in malaria control programs (48% of the amount needed) (WHO, 2021). It is estimated that, if the targets set for the upcoming years are to be met, the yearly investment of 3.3 billion US dollars should triple in the upcoming ten years (WHO, 2021).

Current malaria prevention relies heavily on vector control techniques, which prevent the population at risk from being bitten by mosquitoes (WHO, 2017). Over the past three decades there was a significant decline in malaria incidence, which has been largely attributed to the use of insecticides (Kweka *et al.*, 2017). Even though the level of coverage of insecticide-treated nets (ITNs) and indoor residual spraying (IRS) has increased since 2000, in 2020 only 43% of the population at risk was sleeping under ITNs (compared to the 2% reported in 2000), and the total number of people protected with malaria prevention methods fell from 161 million in 2010 to 87 million in 2020 (WHO, 2021). Although great effort is being put into malaria eradication, the milestone that was set in 2015 for the year 2020 (i.e. a 40% reduction in morbidity and mortality) was not achieved (WHO, 2021). Instead the decline rate of malaria incidence and mortality was subjected to a reduction, and in some African regions, the decline rate appeared to have reversed since 2014 (WHO, 2017, 2020). In some countries in 2020 the burden of malaria was reported to be returning to 2010 levels (Smith *et al.*, 2020b; WHO, 2021). This is thought to be caused by the rapid spread of insecticide resistance (Riveron *et al.*, 2018) and behavioural resistance (Sokhna *et al.*, 2013; Thomsen *et al.*, 2017).

Behavioural resistance, which is defined as the changes in vector behaviour that enable the avoidance or the reduction of exposure to insecticides, represents a major obstacle in the fight against malaria (Sokhna *et al.*, 2013). Alarmingly, many studies show that after the introduction of indoor control strategies, various *Anopheles* vectors are already displaying sophisticated adaptive behaviour that reduces their contact with insecticides (Reddy *et al.*, 2011; Yohannes and Boelee, 2012; Thomsen *et al.*, 2017).

The emerging threat to the efficacy of current vector control programmes shows the need for developing new tools, along with improving old vector control techniques (Padonou *et al.*, 2012; Sokhna *et al.*, 2013; Degefa *et al.*, 2017; Thomsen *et al.*, 2017; Riveron *et al.*, 2018), which should also include strategies to reduce outdoor malaria transmission that are currently under-represented (Govella and Ferguson, 2012; Sougoufara *et al.*, 2020). Vector control interventions prove to be most efficient and cost-effective when they are developed using knowledge of vector behaviour and habits (Vale, 1993; Torr and Vale, 2015; Hawkes and Gibson, 2016). Therefore, it is essential to fill the gaps in the knowledge of mosquito behaviour. In particular, their response to visual, thermal, odour, and other host cues should be studied, as these cues could be key features in the development of surveillance and control tools that exploit vector behaviour (Hawkes *et al.*, 2017a). Although the last phases of host-seeking behaviour represent a crucial part of the vector-host interaction, as this is generally the moment immediately surrounding pathogen transmission, little research has been done to investigate the cues that induce landing and post-landing behaviour, such as foraging and feeding, in *Anopheles* mosquitoes. Given the behavioural changes exhibited by the vector and the impelling need of better understanding mosquito behaviour, this project explores mosquito responses to different host stimuli, both in the landing and post-landing phase of the host-seeking behaviour. To do so, a wide range of inter-disciplinary techniques were used, allowing to achieve a detailed and broad understanding of mosquito behaviour in response to environmental and host stimuli.

1.2.   Aims

The overall aim of the project was to investigate the role of different host-associated cues on the behaviour of *Anopheles gambiae s.l.* mosquitoes, specifically in the last phases of host-seeking behaviour. This was done by first focussing on the landing behaviour, and at a later stage, the post-landing behaviours were examined. The model organism chosen for this project was *Anopheles coluzzii* (Wilkerson & Coetzee, 2013), one of the major vectors of

malaria within the *An. gambiae* complex. The overall aim was achieved by addressing three aims:

- To devise new assays that allow for the direct observation and quantification of mosquito landing behaviour relative to a step-wise presentation of host-associated stimuli;

- To describe and quantify mosquito landing behaviour, and their behaviours immediately pre- and post-landing, according to the effects of host-associated stimuli;

- To identify behavioural responses to specific stimuli that may be incorporated into the design of future vector surveillance and control tools.

Understanding the factors that modulate host-seeking behaviour could have an impact on the effectiveness of possible vector control methods by providing evidence of exploitable behavioural traits. Thus, this research will contribute to improvements in strategies for malaria surveillance and disease control.

1.3. Objectives

In order to achieve the aims of the project, the following objectives were identified:

- Objective 1: Quantify the effect of distinct host-associated stimuli (specifically thermal, chemical, and visual stimuli) in driving the landing response of *An. coluzzii* females. This is explored in Chapter 4.

- Objective 2: Assess if, when presented in combination, host-associated stimuli interact in an additive or a synergistic manner in driving the landing response of *An. coluzzii* females. This is also investigated in Chapter 4.

- Objective 3: Quantify the landing response of host-seeking *An. coluzzii* mosquitoes in relation to different physical characteristics of a target. This is examined in Chapter 5.

- Objective 4: Characterise flight parameters of *An. coluzzii* mosquitoes when approaching targets with different physical characteristics and relate differences in the observed flight parameters to differences in the landing responses. This objective is explored in Chapter 5.

- Objective 5: Characterise the sequence of behaviours that occur in the pre- and post-landing phases when host-seeking *An. coluzzii* mosquitoes are exposed to artificial feeders set at different temperatures. This objective is the subject of Chapter 6.

- Objective 6: Determine the temperature ranges at which females successfully take a blood meal from an artificial feeder and quantify the resulting bloodmeal according to blood temperature. This objective is investigated in Chapter 6.

1.4. Overview of thesis

The following chapter (Chapter 2) will begin with a general overview of the systematics of *Anopheles* mosquitoes, which will be followed by a broad description of their morphology and biology. The next section will focus on an in-depth review of their host-seeking behaviour and the importance of these insects as vectors of diseases. Chapter 3 will describe the main protocols used to maintain the mosquito colonies and carry out the experiments. A description of the methods used for data analysis is also presented here. Chapters 4-6 will present the research conducted to address the objectives outlined above. Finally, Chapter 7 will describe the main findings of this project, its limitations, and will provide suggestions for future work.

2. LITERATURE REVIEW

Mosquitoes are well known not only for causing severe annoyance to humans but also for being the primary vectors that transmit several dangerous infectious pathogens to people, causing millions of deaths every year (World Health Organization (WHO)). Once the morbidity and mortality of the diseases that they transmit are taken into account, mosquitoes are considered the most dangerous animals to humans (Becker *et al.*, 2010; The malaria atlas project, 2018). According to the Centers for Disease Control and Prevention (CDC, 2021), the many pathogens that mosquitoes are responsible for transmitting to humans include the agents responsible for malaria, filariasis and several viruses including yellow fever, dengue, Zika, West Nile and Chikungunya.

Malaria is one of the most serious vector-borne diseases, affecting one-fifth of the world's population (Allossogbe *et al.*, 2017). According to the WHO, over 200 million cases of malaria are reported each year worldwide, and the vast majority of the burden falls on sub-Saharan countries (WHO, 2014, 2017, 2018, 2020, 2021). Human malaria is caused by five species of the protozoan parasite *Plasmodium* spp. (Marchiafava and Celli, 1885), which are transmitted solely by anopheline mosquitoes (Verra *et al.*, 2018; CDC, 2021). Given that the ability of a vector to pass parasites from one host to another depends on its ecology and physiology, the importance of studying such aspects in mosquitoes is therefore evident. Having a deep knowledge of the biology, ecology, and behaviour of the target organism is required in order to develop appropriate and successful control methods (Becker *et al.*, 2010).

The following chapter will introduce the main characteristics of *Anopheles* mosquitoes. Specifically, it will include an overview of this genera's systematics, morphology, biology, life cycle, and the mechanisms that are used by this vector during host-seeking behaviour. Special focus will be given to reviewing the current state of knowledge pertaining to the last phases of this behaviour, as it will be especially relevant for this project. An outline of the malaria parasite life

cycle will follow, along with a summary of current vector control methods. Finally, the importance of behavioural studies will also be discussed.

2.1. Systematics

Mosquitoes are haematophagous insects belonging to the family Culicidae, which forms part of the Nematocera suborder within the order Diptera. The Culicidae family has historically been divided into three subfamilies: Toxorhynchitinae, Anophelinae, and Culicinae, although recent publications suggest the reorganisation of these groups into only two subfamilies, Anophelinae and Culicinae (Foster *et al.*, 2017). The Culicinae subfamily is subsequently divided into several tribes such as Aedini, Culicini, Mansoniini, and so forth. (Clements, 1992). The genus *Anopheles* is entirely included in the Anophelinae subfamily (Fig. 1).

ORDER		Diptera	
SUBORDER	Brachycera (horse flies)	Nematocera	Cyclorrhapha (house flies)
FAMILY	Tipulidae (crane flies)	Culicidae (mosquitoes)	Ceratopogonidae (biting midges)
SUBFAMILY	Anophelinae	Culicinae	Toxorhynchitinae (elephant mosquito)
GENERA	*Anopheles*	*Culex* *Aedes*	
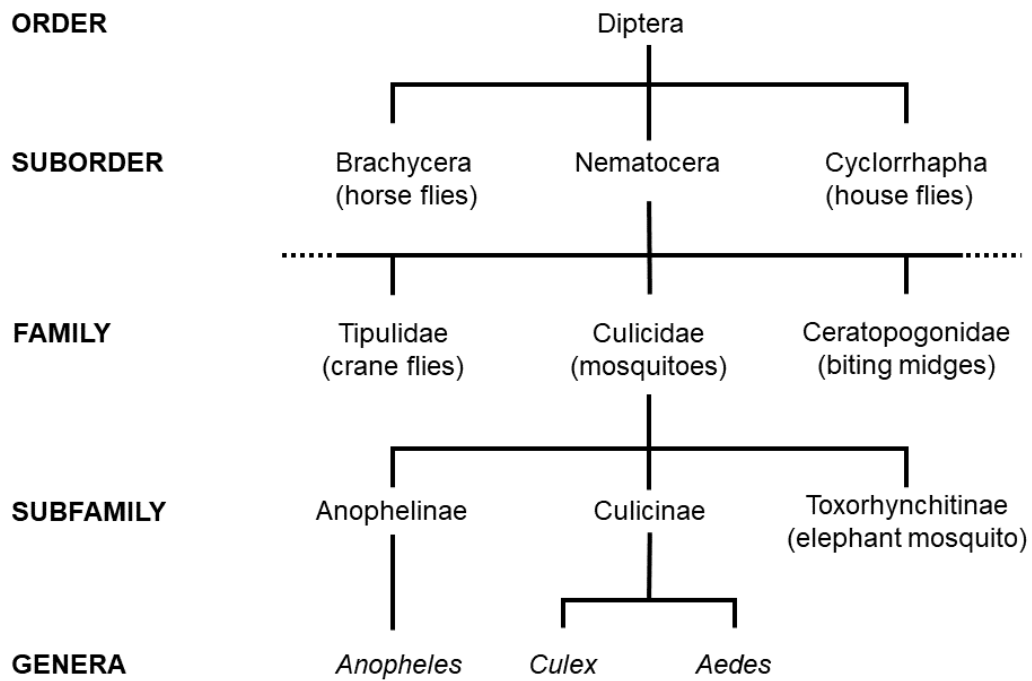
Figure 1: Taxonomic classification of mosquitoes. The three main vector genera are outlined at the bottom of the figure (drawn by author from information in Snow, 1990).

Forming part of the Diptera order, also known as the "true flies" order, mosquitoes have only one pair of functional front wings while the hindwings are reduced to halteres, which are specialised organs used to maintain balance and

flight control (Foster and Walker, 2019; Singh *et al.*, 2021). Furthermore, like other true flies, mosquitoes are holometabolous as they exhibit a complete metamorphosis, and they undergo four life stages (egg, larval, pupal and adult) (Clements, 1992; Foster and Walker, 2019). Dipteran insects are also identifiable by the lack of legs during the larval phase (Gullan and Cranston, 2014). Nematoceran flies are characterised by their elongated bodies, filamentous and multi-segmented antennae, slender legs, an abdomen with ten distinct segments, and aquatic juvenile stages (Gullan and Cranston, 2014; Foster and Walker, 2019).

Despite the medical relevance of mosquitoes, the knowledge on the taxonomy of these organisms remains limited and there are still many unsolved issues with the classification of mosquitoes at the level of genera and species (Besansky *et al.*, 2003). Their classification had become more problematic since the discovery of cryptic species complexes (Harbach, 2007; Becker *et al.*, 2010), i.e. the grouping of two or more different species that are morphologically identical to each other and therefore are erroneously classified as a single species. Owing to the variability of their external morphological features (colour, body size, etc.), different authors have named the same species differently, resulting in the existence of several synonyms for some species (Harbach, 2004). The application of genetic and molecular techniques has facilitated the work in this field, especially for the anopheline clade (Harbach, 2004, 2007) and since their introduction, new species have been separated successfully from sibling complex species (Hunt *et al.*, 1998; Kengne *et al.*, 2003; Wilkerson *et al.*, 2004; Coetzee *et al.*, 2013). Accurate differentiation of species into their correct taxonomic groups is essential for epidemiological studies. Each species has unique characteristics as a vector, which impacts their vectorial capacity; therefore the design and development of control and monitoring strategies needs to take into account the unambiguous identification of different species (Besansky, 1999; Harbach, 2007; Akogbéto *et al.*, 2018).

In total, there are about 3500 described species of mosquitoes and this number could increase severalfold with the separation of isomorphic species (Clements,

1992; Besansky *et al.*, 2003; Harbach, 2007). The Anophelinae subfamily comprises around 476 species, the majority being within the genus *Anopheles* (Harbach, 2007; Foster *et al.*, 2017). However, only about 30-40 species of *Anopheles* can transmit *Plasmodium* species well enough to be considered vectors of malaria in humans (Foster *et al.*, 2017; CDC, 2021). To transmit the parasite between hosts, the mosquito needs to have a number of different characteristics (Kamareddine, 2012; The malaria atlas project, 2018) which will be covered later in this chapter (see section 2.5.2). Of major relevance for malaria transmission is the *Anopheles gambiae* species complex (*An. gambiae sensu lato*) as some of its members are well known to be important vectors of *Plasmodium* in Africa (Léong Pock Tsy *et al.*, 2003; Bass *et al.*, 2007; Coetzee *et al.*, 2013). In fact, *Anopheles arabiensis* (Patton, 1905) and the two former molecular forms of *An. gambiae* species, *Anopheles gambiae sensu stricto* (Giles, 1902) and *An. coluzzii*, are considered to be the principal malaria vectors in Africa (The malaria atlas project, 2018).

However, not all species included in the *An. gambiae* complex are able to efficiently transmit the parasite. It is notable that the vectorial capacity varies enormously between species of the complex (Léong Pock Tsy *et al.*, 2003; Akogbéto *et al.*, 2018) and having knowledge of the biology and ecology of each species is essential to understand its role in malaria transmission. For this reason, the *An. gambiae* complex has received much attention for a long time and the first attempt to catalogue the different components of the complex and list their characteristics dates back to 1987 (Gillies and Coetzee, 1987). The main characteristics of each of the eight known species of the *An. gambiae* complex are briefly described below and the African distribution of some of the species is presented in Fig. 2.

Anopheles arabiensis   Anopheles coluzzii   Anopheles gambiae s.s.

Anopheles melas   Anopheles merus

LEGEND
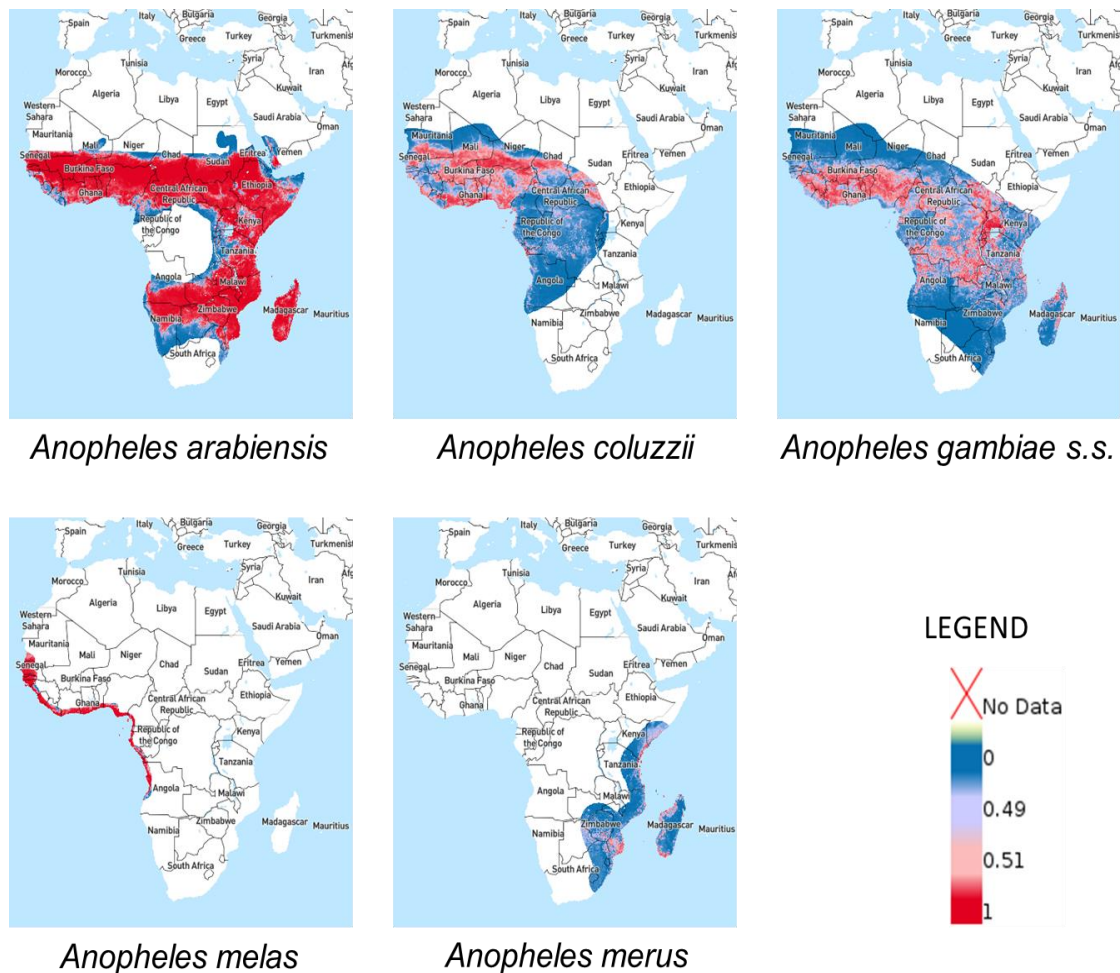
Figure 2: Predicted distribution of some **Anopheles gambiae s.l.** *species with a focus on the African continent. These maps show the prediction of the probability of finding a given species. Note that blue indicates a low probability while red indicates a high probability. The top row displays the distributions of the species which are considered the principal malaria vectors in Africa (figure created using extracts of MAP* (The malaria atlas project, 2022) *available under the Creative Commons Attribution 3.0 Unported License).*

- *Anopheles amharicus* (Hunt, Wilkerson & Coetzee, 2013): formerly considered part of *Anopheles quadriannulatus* (Theobald, 1911) species, it presents an Ethiopian distribution, strongly associated with cattle or animal shelters. Rarely found in human-only shelters and is not known to transmit malaria (Coetzee *et al.*, 2013).

- *Anopheles arabiensis*: is considered a species of arid or savannah environments, although can also be found in recently disturbed forests (Léong Pock Tsy *et al.*, 2003; Sinka *et al.*, 2010). Its larvae inhabit

temporary freshwater pools and are abundantly found in irrigated rice fields. It displays a flexible behaviour, being mainly zoophilic, exophagic, and exophilic but showing the ability to adapt to an anthropophilic, endophilic, and endophagic behaviour (Sinka *et al.*, 2010).

- *Anopheles bwambae* (White, 1985): is only found in the proximities of the geothermal hot springs in the Semliki forest of Uganda's Bwamba county (Coetzee *et al.*, 2013). It is reported to be a local malaria vector (White, 1985).

- *Anopheles coluzzii*: until recently, it was considered the M molecular form of *An. gambiae s.s.* (Coetzee *et al.*, 2013; Giraldo-Calderón *et al.*, 2015). It is prevalent in West Africa and its larvae can inhabit temporary breeding sites, therefore this species is responsible for a high percentage of malaria transmission during the dry season (Akogbéto *et al.*, 2018). It is considered a major malaria vector being strongly anthropophilic, endophagic, and endophilic.

- *Anopheles gambiae sensu stricto*: formerly considered the S molecular form of *An. gambiae s.s.*, it is widely distributed in sub-Saharan Africa. It is considered to be one of the principal vectors for malaria. It is highly anthropophilic, endophilic, and endophagic and displays a very adaptable behaviour to various environments (Dekker *et al.*, 1998; Bass *et al.*, 2007; Akogbéto *et al.*, 2018).

- *Anopheles melas* (Theobald, 1903): found in West Africa, is normally associated with saltwater breeding sites (coastal habitats) although it is not restricted to brackish water for larval development (Sinka *et al.*, 2010; Coetzee *et al.*, 2013). It is not considered a major malaria vector as human biting appears to be opportunistic and it rests outdoors. However, in coastal areas, its contribution to malaria transmission is notable (Sinka *et al.*, 2010).

- *Anopheles merus* (Dönitz, 1902): found in East Africa, in association with saltwater breeding sites (coastal habitats) but it can also occur further inland. It is considered as opportunistic, with a tendency to display an endophilic and endophagic behaviour. It was mostly considered as a minor vector for malaria transmission, although recent information proved its role as an important vector in coastal areas in Tanzania and Mozambique (Léong Pock Tsy *et al.*, 2003; Sinka *et al.*, 2010; Coetzee *et al.*, 2013).

- *Anopheles quadriannulatus*: was first recorded in Ethiopia, Zanzibar, and South Africa (Coetzee *et al.*, 2013), now only refers to the southern African populations (Giraldo-Calderón *et al.*, 2015). It is a zoophilic species, normally rests outdoors and is not considered to be a vector for human malaria (Coetzee, 1987; Cardé and Gibson, 2010; Sinka *et al.*, 2010).

2.2. Morphology of adult *Anopheles* mosquitoes

Adult mosquitoes have three well-divided body parts which are richly covered with setae: the head, the thorax, and the abdomen (Service, 2008). The head comprises a pair of antennae, two compound eyes, two maxillary palps, and the proboscis (Fig. 3).
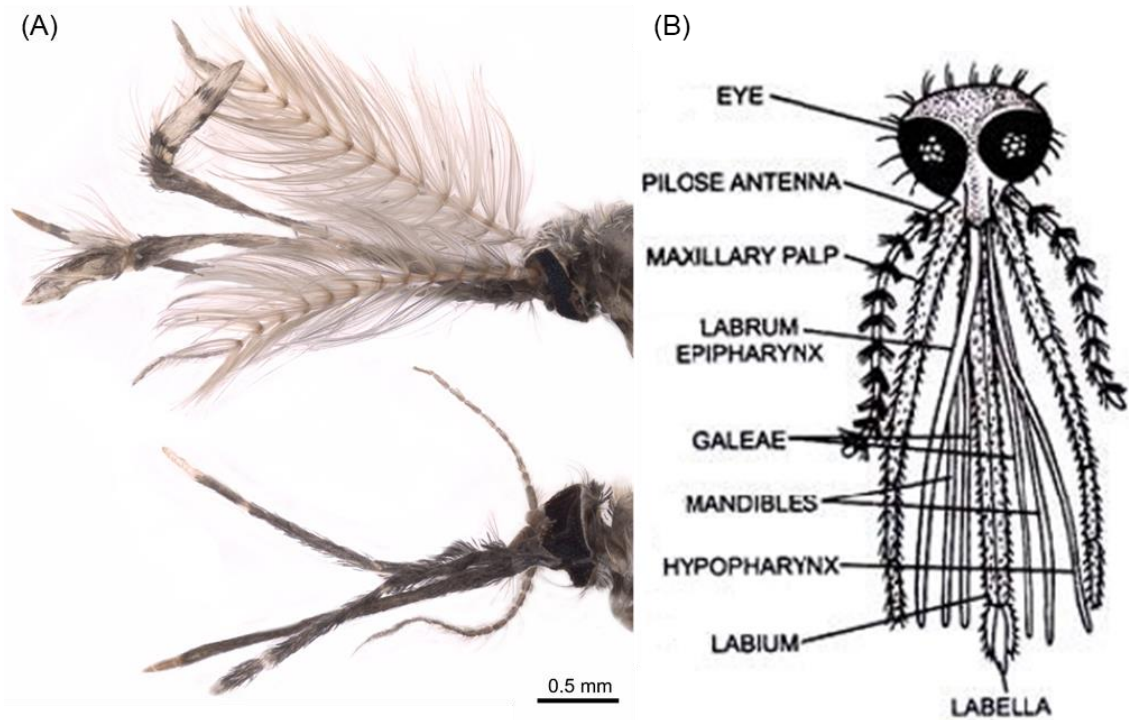
Figure 3: Head of **Anopheles** **mosquitoes.** *Panel (A) shows the head of a male (top) and female (bottom)* Anopheles *mosquitoes. It is possible to see a clear sexual dimorphism in the antennae of the two specimens. Panel (B) shows the mouthparts of a female mosquito. The mouthparts are artificially separated in order to show all the components, whilst normally all mouthparts would be enclosed in the labium, which acts as a sheath (photographs courtesy of Charlie Woodrow for panel (A), while diagram in panel (B) is after* http://www.notesonzoology.com*).*

2.2.1. Maxillary palps

The maxillary palps are important organs that detect chemical and physical cues. They are divided into five segments and contain chemical receptors and mechanosensory setae (Lu *et al.*, 2007; Bohbot *et al.*, 2014). Males of most mosquito species and anopheline females present maxillary palps approximately equal in length to the proboscis, while for culicine females, the maxillary palps are normally shorter than the proboscis (Service, 2008).

2.2.2. Antennae

Mosquito antennae are divided into three segments: the scape, the pedicel (which is enlarged as it also includes the Johnston's organ), and the flagellum. The antennae accommodate a large number of different receptors and are

therefore sensitive to a variety of chemical and physical stimuli (Steward and Atwood, 1963; Boo, 1980). About 90% of these antennal receptors have an olfactory function and many receptors have been shown to respond to compounds related to oviposition sites, nectar sources, host animals, and repellents (Amer and Mehlhorn, 2006). Furthermore, gustatory receptors and two types of thermoreceptors, one warm-sensitive and another cold-sensitive can also be found on the antennae (Davis and Sokolove, 1975; Gingl *et al.*, 2005; Montell and Zwiebel, 2016). Another crucial function of the antennae is the detection of air movement through mechanosensory cells. This sense is important both for stabilising flight and for the detection of sound waves (Steward and Atwood, 1963; Clements, 1999; Montell and Zwiebel, 2016). It has been shown that for mating, male specimens detect and locate conspecific flying females using the female flight tone and that both sexes actively respond to the flight tone of the opposite sex by altering their own tone (Gibson *et al.*, 2010; Pennetier *et al.*, 2010). The Johnston's organ, which is found in the second antennal segment of all adults, is a key part of the auditory system in mosquitoes (Lapshin and Vorontsov, 2017), and is also sensitive to Coriolis forces (Dekker and Carde, 2011).

2.2.3. Proboscis

Adult mosquitoes, as primitive Diptera, are characterised by having mouthparts that form a composite pierce-sucking proboscis, which is used by both adult males and females to feed on plant-based sugar sources, including nectar (Barredo and DeGennaro, 2020). In males, the mandibles and the maxillae are too weak to be used for piercing, therefore the uptake of sugary solution occurs mainly through suction (Service, 2008; Becker *et al.*, 2010). Both sexes can survive purely on plant sugars as an energy source (Esposito and Habluetzel, 1997), but females of the anopheline and culicinae subfamily typically require a blood meal to obtain the necessary proteins for the development of eggs. Therefore, in females, the proboscis is also used to pierce the host's skin during the blood meal. Within the proboscis, there can be found gustatory chemoreceptors that sense the presence of a blood vessel (Montell and Zwiebel,

2016), stylets that pierce the skin, a food channel for blood suction (the labrum), and a salivary channel (hypopharynx), which injects saliva that contains several substances, among which are anticoagulants and anaesthetics that prevent clotting from the natural aggregation of the hosts' platelets and reduce the pain inflicted to the host (Service, 2008; Choumet *et al.*, 2012; Foster and Walker, 2019) (Fig. 3). It is through the injection of saliva that mosquitoes transmit most of the pathogens they carry to the vertebrate host (Choumet *et al.*, 2012).

2.2.4. Eyes

Mosquitoes possess large compound eyes, which occupy the front and lateral portion of the head (Foster and Walker, 2019). Each eye is composed of approximately 200-300 independent photoreceptor units, called ommatidia (Fig. 4). Each ommatidium comprises a corneal lens and eight photoreceptors cells that form the retinula. Light is detected in the distal part of the photoreceptors cells, which present a microvillar portion (called the rhabdomeres) that contains the visual pigments (Clements, 1999; Montell and Zwiebel, 2016). Although most species of mosquitoes are active during dusk or night-time, their eye structure falls within the category of apposition eyes. This type of eye is more commonly found in diurnal insects, in contrast to the superposition eye structure which is more common in nocturnal insects (Land *et al.*, 1999). In apposition eyes, each rhabdom collects the light only from its own corneal lens and therefore the gathering of light is limited (Land *et al.*, 1997). Even so, mosquitoes' ommatidial structure has evolved to enhance their light-capturing ability as the rhabdomeres of each ommatidium are fused together and are organized in a conical shape which increases imagine brightness at the expense of resolution (Land *et al.*, 1997; Cardé and Gibson, 2010; Montell and Zwiebel, 2016).
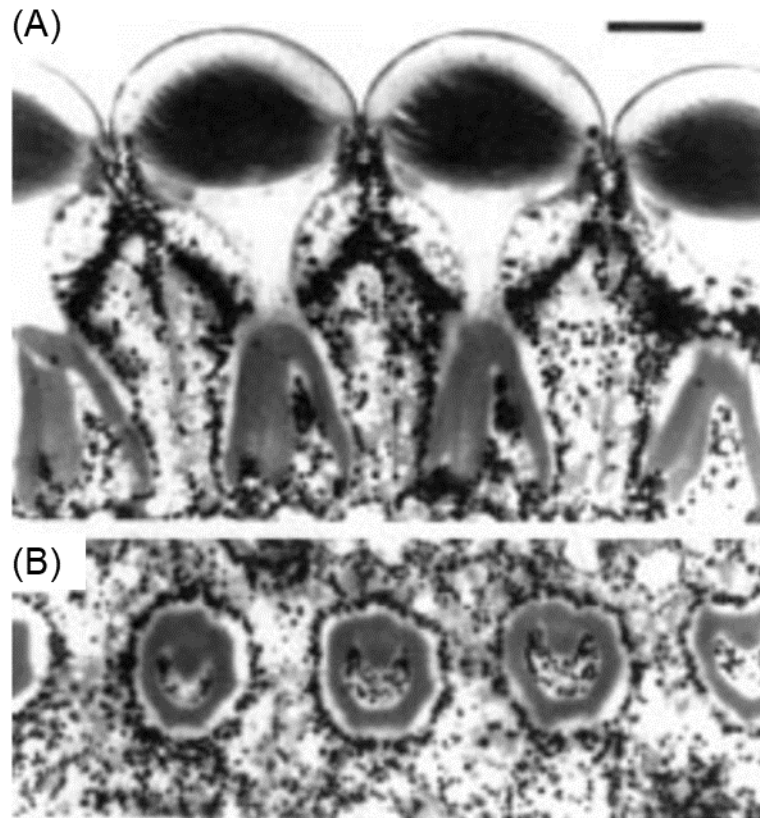
Figure 4: Radial (A) and transverse (B) sections of the lateral retina of a male **An. gambiae***.* *Each photoreceptor unit comprises a hemispherical cornea lens and the photoreceptors cells. The rhabdomeres are fused together and present a conical structure. Scale bar 10 µm (adapted after Land et al., 1997).*

The role of visual perception during host-seeking behaviour is discussed in more detail later in this chapter (see section 2.4).

2.2.5. Thorax and abdomen

The thorax comprises a pair of wings, two halteres, three pairs of legs and the majority of the muscles used for locomotion (Fig. 5). The distal part of each leg, called tarsus, carries a large number of gustatory sensilla which are used during oviposition, mating, and feeding (Sparks *et al.*, 2013; Montell and Zwiebel, 2016).

The third part of the body, the abdomen, is composed of ten abdominal segments and contains the main organs used in digestion, excretion, and reproduction.
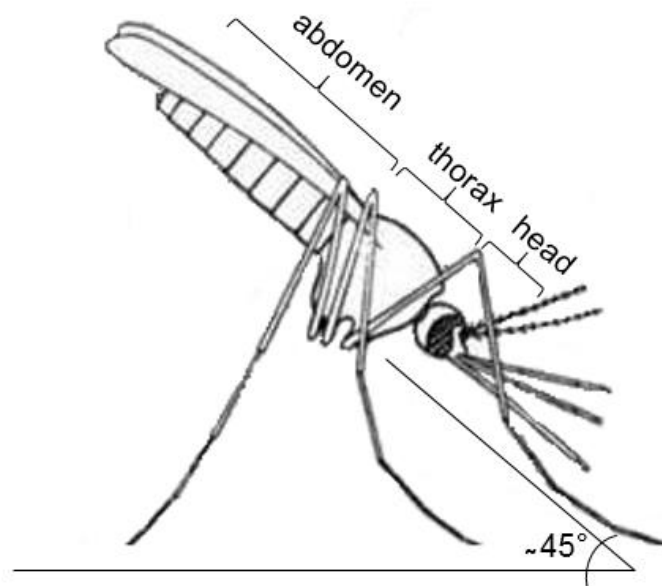
Figure 5: Adult female of the genera Anopheles. *Three body parts can be distinguished: the head, the thorax and the abdomen. The typical resting position that* Anopheles *adopt when resting is displayed (adapted after Snow, 1990).*

Anopheles adult mosquitoes generally present a smaller body size compared to culicines, with long and slender legs and narrow wings (Becker *et al.*, 2010). Adult anophelines can be quickly recognized as they adopt a typical resting position with the three body parts forming a 45⁰ angle with respect to the surface (Fig. 5). This position is different from the parallel position with respect to the surface that culicines mosquitoes adopt while resting.

2.2.6. Sexual dimorphism

Mosquitoes generally present an evident morphological and behavioural sexual dimorphism as males do not display classic host-seeking behaviour, possess hairier maxillary palps, and their antennae are more plumose, i.e. are more heavily coated with long hair-like setae, which are used to detect the sound produced by flying females (Snow, 1990; Opfert *et al.*, 1999; Montell and Zwiebel, 2016).

2.3. Biology and life cycle

Mosquitoes are extremely adaptable organisms and can be found throughout the world in all habitats except for permanently frozen areas and a few islands (Clements, 1999; Day, 2005; Service, 2008). Some species have adapted to survive in difficult environments like temporary, polluted, brackish breeding sites and habitats with tough climatic conditions (Becker *et al.*, 2010). Like all Dipteran insects, mosquitoes use an aquatic habitat for the first phases of their life cycle (Foster and Walker, 2019) (Fig. 6).

2.3.1. Eggs

Female mosquitoes typically lay between 50 to 500 eggs per gonotrophic cycle on either a moist surface near a water source or directly onto the water surface. Both the external morphology of the eggs and the way in which they are laid varies between genera. Anophelines lay single eggs on the water's surface, which are vulnerable to desiccation and are adapted for floating, having two lateral floats filled with air (Snow, 1990; Esposito and Habluetzel, 1997).
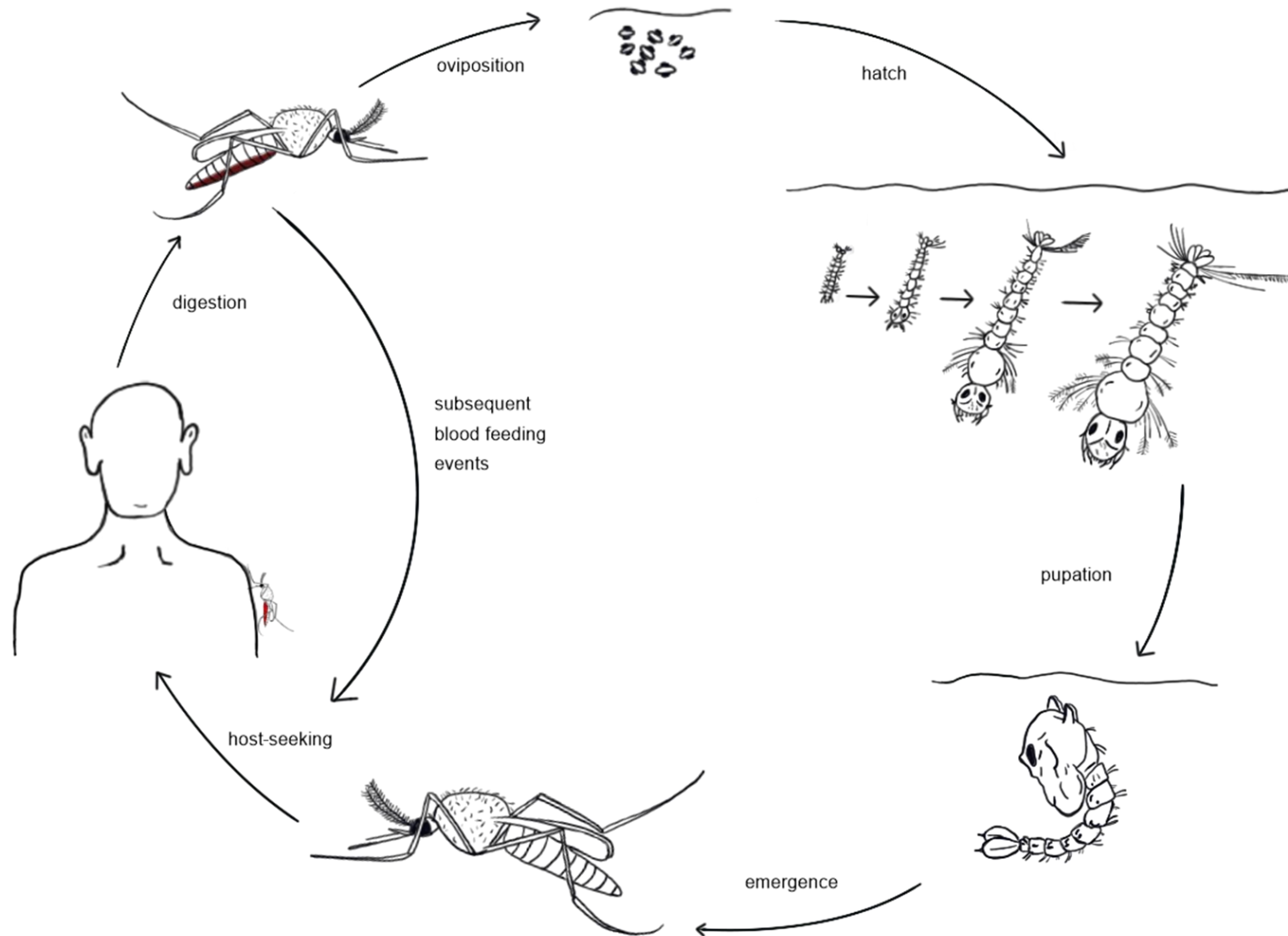
Figure 6: Mosquito life cycle. *The female adults need to acquire blood meals in order to permit the development of their eggs. Anophelines eggs are laid individually on the water's surface, larvae and pupae develop in aquatic habitat while the adults are terrestrial (drawn by author).*

2.3.2. Larval stage

The larva hatches from the egg and presents a distinct head, thorax, and abdomen, where the spiracles, which are the breathing openings that allow air intake, are located. While culicine mosquitoes have siphons connected to their tracheal trunks, anopheline larvae lack such structures, having instead the tracheal trunks directly connected to the spiracles (Clements, 1992; Esposito and Habluetzel, 1997). The head carries many "mouth brushes", thin hair-like appendages that are used to create a current of water around the mouth so that small particulate matter, such as bacteria, algae, protozoa, pollen, and detritus, which are the primary larval food source, is transported to the larva's mouth (Christophers and Puri, 1927; Merritt *et al.*, 1992; Esposito and Habluetzel, 1997; Ye-Ebiyo *et al.*, 2000). The lack of siphons forces anopheline larvae to lie horizontally at the water's surface, therefore they can only feed from a thin water column below the water surface (Clements, 1992; Esposito and Habluetzel, 1997; Briegel, 2003; Becker *et al.*, 2010). The larva undergoes four moults, with larger larval instars emerging sequentially, casting its cuticle each time as the body size grows. The final size of the larva and consequently, of the pupa and then adult, depends principally on the dietary conditions and larval population densities (Briegel, 2003).

2.3.3. Pupal stage

The pupa emerges from the ecdysis of the fourth larval instar. The pupa is also an aquatic organism, and the head and thorax are fused together forming a cephalothorax, where two respiratory trumpets are located (Esposito and Habluetzel, 1997; Ha *et al.*, 2017; Foster and Walker, 2019). Unlike the larva, the pupa does not feed, although it remains motile. Buoyancy is maintained by an air bubble that is enclosed in the ventral air space, a cavity formed by the developing legs, wings, and mouthparts. During this stage, the insect undergoes a holometabolous (complete) metamorphosis and the adult body is formed (Margam *et al.*, 2006; Foster and Walker, 2019).

2.3.4. Adult stage

The adult emerges from the pupal exuvia and remains over the water's surface for several minutes until the legs and wings are stretched, and the cuticle sclerotized. During this short period of time, the mosquito is highly susceptible to predation or falling into the water (Becker *et al.*, 2010). After eclosion, the mosquito imago undergoes a maturation period, which normally lasts 24 h and leads to a series of major anatomical, physiological, and behavioural changes (Briegel, 2003). As newly emerged male mosquitoes are not sexually mature, they emerge one or two days before conspecific females to achieve sexual maturity at the same time (Becker *et al.*, 2010; Foster and Walker, 2019).

After the maturation period, both male and female adults are ready to mate. Mating in *An. gambiae s.l.* species occurs at the beginning of the dark phase (dusk) and initiate during flight (Charlwood and Jones, 1979). Males aggregate and form swarms above a high contrast object or pattern called swarm marker. Virgin females are also attracted to the marker, and after entering the swarm are rapidly intercepted by a male with whom they copulate. The sperm is held in a storage organ called spermatheca and it will be used every time it will be required to fertilize a new batch of eggs, as the male along with the sperm also injects accessory gland secretions, which makes the female unreceptive to further copulation (Shutt *et al.*, 2010). Males, on the other hand, may mate many times (Foster and Walker, 2019).

In order to obtain the necessary proteins for egg development, most females of the anopheline and culicinae subfamilies need to acquire a blood meal. Anopheline species are considered crepuscular, as they predominantly display host-seeking behaviour shortly after sunset and during dawn (Service, 2008). However, biting and other flight activity can occur throughout the night. Jones *et al.* (1967) reported moderate flight activity throughout the dark period, when individuals of *An. gambiae* were kept in a constant 12:12 h light : dark (LD) photocycle. Furthermore, female mosquitoes belonging to the *Anopheles* genus often require two or three blood meals to develop eggs, since they emerge with smaller body size and less stored lipids than culicine mosquitoes (Briegel and

Hörler, 1993; Briegel, 2003; Fernandes and Briegel, 2005). Accordingly, they have adapted well to successfully feed on hosts: they can feed on blood within 12 h of eclosion, they can take multiple blood meals within a short period of time (including across a single night), and they are able to ingest double their own weight in blood (Briegel and Hörler, 1993; Esposito and Habluetzel, 1997; Briegel, 2003). After ingesting a blood meal, the female mosquito finds a place to rest while the development of eggs takes place. During their resting period, adult mosquitoes are most commonly found in places with high humidity and cooler temperatures (Harbach, 2007). At an average temperature of 23 °C, *Anopheles* mosquitoes require about 48 h to complete a gonotrophic cycle, i.e. from the moment the insect takes the blood meal to oviposition (Esposito and Habluetzel, 1997). Once the eggs' development is completed, the female searches for a suitable water body, where it will oviposit the eggs.

The duration of all life stages and therefore the entire duration of the complete life cycle from egg to adult is strictly dependent on biotic and abiotic factors, of which temperature is the most important (Esposito and Habluetzel, 1997; Dhiman *et al.*, 2008; Chandrasegaran *et al.*, 2020; Ranasinghe and Amarasinghe, 2020). For *Anopheles* mosquitoes, the whole cycle requires a minimum of seven days if it is carried out at elevated intertropical temperatures (Esposito and Habluetzel, 1997), although it is more typically achieved in 15 to 20 days.

2.4.   Host-seeking behaviour

Anopheline females must find a host to acquire a blood meal. Therefore, they have evolved a sensory system that allows them to detect and orient towards a potential host from many metres away. They have a repertoire of behavioural responses that maximise the likelihood of encountering host stimuli, locating a host, and ultimately, acquiring a blood meal.

Host-seeking is defined as the specific behaviour that mosquitoes display when they are tracking a host organism. This behaviour is generally divided into four main stages: activation, long-range orientation, pre-attack resting, and

short-range approach. Nevertheless, it is important to point out that these stages are not to be considered as singular separate events but rather as a continuous package where small stimuli changes take the mosquito's response from one stage to another, creating a "behavioural momentum" (Sutcliffe, 1987). Each stage is modulated and guided by a specific set of cues that are detectable by the mosquito over different spatial scales (Fig. 7) (Cardé, 2015). The three main sensory stimuli that are thought to influence mosquito behaviour during host-seeking are: chemical cues sensed through the olfactory system, visual features, and thermal stimuli, which are normally also associated with a change in humidity (Clements, 1999; Becker *et al.*, 2010; Cardé and Gibson, 2010; Cardé, 2015). The stimuli have been divided into two main groups: those that are initially sensed at a distance from the host, which can be detected over 5 m away from the host and are mostly activators and attractants, and those that are perceived near the host (< 1 m), which elicit landing and biting behaviour (Dekker *et al.*, 2005).
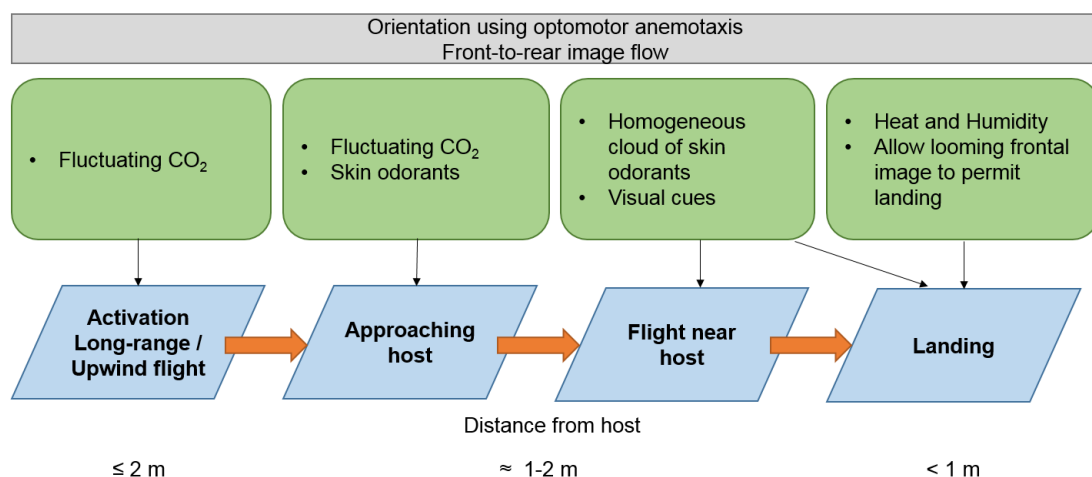
Figure 7: Sequence of cues used by the mosquito when searching for a host. *Olfactory stimuli (carbon dioxide and body odour) are the key cues that trigger activation and guide long-range orientation (over one – two metres from the host). In proximity (one metre away) visual cues also contribute to guiding orientation. At a short-range distance (less than one metre) heat, and possibly humidity, lead the mosquito to land on the host (adapted after Cardé, 2015).*

As a generalisation, it is thought that activation and subsequent long-range orientation can occur several metres away from the host and are mainly

mediated by host odour (Cardé and Gibson, 2010; Cardé, 2015), where host odour refers to the blend of all chemical compounds (organic and inorganic) that emanate from hosts. When the mosquito arrives closer to the host, at a medium to short-range distance, visual stimuli become important for orientation, while according to some authors, heat and humidity play a role in landing behaviour only when the mosquito is within centimetres from the host (Cardé and Gibson, 2010; Cardé, 2015).

It is important to note that although different sensory cues might act at different distances, the perception of each cue should not be considered as a separate system working in parallel to other sensory channels, as different cues (i.e. inputs) are integrated and the information is elaborated together, producing a single response. For example, a study conducted on mutated mosquitoes that were unable to sense carbon dioxide proposed that different sensory cues (temperature, host odour, and carbon dioxide) are integrated at a central level. The simultaneous presence of at least two such cues allowed for host localisation with a high degree of fidelity (McMeniman *et al.*, 2014). Similarly, Duistermars and Frye (2010) proposed that the fruit fly, *Drosophila melanogaster* (Meigen, 1830), requires the integration of both mechanosensory and visual inputs to track and follow an odour plume.

Understanding host-seeking behaviour in mosquitoes has been the focus of a considerable amount of research, as this is the key interspecies interaction by which mosquitoes transmit pathogens to humans. Moreover, the majority of current vector control methods exploit this behaviour to reduce vector-host contact. In the following section, a brief review will outline the main characteristics of each phase that takes a female mosquito from resting to landing and probing on a host. Special attention will be given to describing the sensory cues that guide mosquito behaviour in each phase, focussing on anophelines behaviour when results are available.

2.4.1. Activation

The first step of host-seeking behaviour corresponds to the activation phase. Activation is broadly considered as the induction of flight activity (Gillies, 1980). This phase includes both the activation mediated by host cues (e.g. host odour) and the spontaneous nocturnal activity, which is guided by the internal clock and drives the insect to periodically engage in searching flight. Short bursts of flight activity were recorded every half-hour during the dark phase in specimens of *An. gambiae* when kept individually in chambers under a 12:12 h LD cycle (Jones *et al.*, 1967). In general, both types of activation increase mosquito's chance of encountering a host (Hawkes *et al.*, 2012). For the purpose of this thesis, hereafter activation will be used to describe the first response of a mosquito to host cues, which takes the mosquito to transition from either a non-oriented (ranging) flight or resting behaviour, to an oriented flight toward the host, following the definition of activation given by Sutcliffe (1987). In order to actively respond to detected host cues the mosquito needs to be receptive. The state of receptivity varies over time and is mainly modulated by environmental conditions, the physiological state of the female mosquito, and the endogenous rhythm that regulates when the mosquito is in the "active" phase (Sutcliffe, 1987; Barrozo *et al.*, 2004; Hawkes *et al.*, 2012). Many studies have proven how physiological factors such as the reproductive state of the female (e.g. whether virgin or mated), the hunger level, and egg maturation stage can profoundly affect when a female mosquito will be in the active phase (Jones *et al.*, 1972; Jones and Gubbins, 1978; Jones, 1982; Rowland, 1989).

Carbon dioxide has been proven to be the most common activator compound, eliciting a response in virtually all biting flies (Sutcliffe, 1987). This is thought to be because carbon dioxide is ubiquitously emitted by all vertebrate animals through exhalation, therefore its presence in specific concentrations is a reliable signal of the presence of a nearby host (Takken and Knols, 1999; Dekker *et al.*, 2005). Many different studies have demonstrated an activation response from different mosquito species when exposed to carbon dioxide (Sutcliffe, 1987;

Eiras and Jepson, 1991; Healy and Copland, 1995; Dekker *et al.*, 2005; Dekker and Carde, 2011).

In *Aedes aegypti* (Linnaeus, 1762), receptors for carbon dioxide are located on the maxillary palps and were shown to respond to increments in gas concentration of as little as 0.01% above the background level (Kellogg, 1970). This sensory physiology finding is supported by a behavioural test conducted on *An. gambiae* by Healy and Copland (1995), who reported an activation of 60% of the female mosquitoes when exposed to an increase of 0.01% or more of carbon dioxide concentration. They also recorded similar activation levels when presenting whole human breath containing the equivalent concentration of carbon dioxide, thus indicating that secondary chemical components in the exhaled air do not have a major impact on activating *An. gambiae*. These findings suggest that even a small increase in the concentration of carbon dioxide is sufficient to induce activation from either ranging flight or resting behaviour (Gillies, 1980).

It is important to note that only pulsed streams of the gas produce a substantial prolonged response (Gillies, 1980). In fact, it has been demonstrated that exposure to a homogeneous plume of carbon dioxide inhibited the flight of *Ae. aegypti* (Geier *et al.*, 1999; Dekker *et al.*, 2001; Dekker and Carde, 2011) and *An. gambiae* (Dekker *et al.*, 2001) towards the emission source. Two other species of mosquitoes, *An. arabiensis* and *Culex pipiens fatigans* (Wiedemann, 1828) had a similar dispersion in a flight chamber when exposed to a constant carbon dioxide emission and when exposed to clean air, thus showing no significant response to exposure to constant carbon dioxide (Omer, 1979). This could be explained because carbon dioxide receptors have a phasic performance, i.e. respond rapidly to minimal concentration changes but also habituate quickly to continuous stimulation, therefore inhibiting a response under prolonged exposure to carbon dioxide (Geier *et al.*, 1999). This theory is coherent with the idea that carbon dioxide is only exhaled periodically by the host and that in the field, due to turbulence in air movement, the gas is presented downwind as an intermittent stimulus (Gillies, 1980; Geier *et al.*, 1999). A field

study demonstrated that even if released in a constant manner, the odour plume that reached 100 m downwind from the odour source appeared to be recovered as separated air puffs (Barynin, 1970). Zollner *et al.* (2004) demonstrated that in some African habitats, carbon dioxide emitted by natural hosts (cattle) is detectable tens of metres downwind, thus indicating that bursts of carbon dioxide could play a role as a host cue at great distances (Dekker *et al.*, 2005). It is important to note that a pulsed plume deriving from the natural breathing of an animal would have a much lower frequency of puffs compared to the frequency of intermittent puffs that result from the scattering of a constant odour source due to air turbulence. Nevertheless, independently from the source of intermittence (either breathing or air turbulence), the pulsed streams are more attractive to mosquitoes than constant streams of carbon dioxide.

Another point highlighted by Gillies (1980) is the fact that mosquitoes do not respond to specific concentrations of carbon dioxide, but rather they respond to increases in its concentration above the background level, regardless of the baseline concentration. This was further proved in a study conducted by Geier *et al.* (1999) where the responses in *Ae. aegypti* increased with the increment of the fluctuation of carbon dioxide stimulus, regardless of the concentration of the gas. This implies that mosquitoes are not activated by a defined concentration of carbon dioxide but, instead, constantly compare the concentration of carbon dioxide relative to the background level and display a response depending on the signal to noise ratio (Bowen, 1991; Gibson and Torr, 1999). However, different concentrations of carbon dioxide might elicit different levels of response, as demonstrated by a recent laboratory study conducted by Reinhold *et al.* (2022), where two *Culex* species landed more on a warm object when this was presented together with high concentrations of carbon dioxide (30000 ppm) compared to when the object was presented with low concentrations of carbon dioxide (2100 ppm).

Host skin odour alone can also activate mosquitoes (Clements, 1999; Dekker *et al.*, 2005). Geier *et al.* (1999) reported that activation and upwind flight of *Ae. aegypti* was elicited by exposing the mosquitoes to a homogenous plume of skin

odour alone. Similar results were obtained with *An. gambiae* mosquitoes, where homogeneous skin odour induced a significant activation and upwind flight into a trap entrance (Dekker *et al.*, 2001). As mentioned before, carbon dioxide is thought to be a universal activator for all haematophagous mosquitoes as it is present in all vertebrates exhalations and therefore it may be a more important cue for non-host specific mosquitoes (Takken and Knols, 1999). On the other hand, host-specific odours may be of major importance in the attraction and host-species recognition for mosquitoes with a more limited host range (Takken, 1991; Dekker *et al.*, 2001, 2005; Pates *et al.*, 2001; Takken and Verhulst, 2013). It has been proposed that host-recognition is likely to be caused by a complex of many different volatiles and not by a singular compound (Omer, 1979; Takken, 1991; Dekker and Takken, 1998), and that host odour is crucial to initiate host-species recognition at long range distance (Gillies and Wilkes, 1969). A more detailed overview of host-species recognition is found in section 2.4.2. Further studies demonstrated that skin odour only activates *Ae. aegypti* mosquitoes when presented at high concentrations, equivalent to those found near a human host (Dekker *et al.*, 2005). Skin odour receptors are thought to have a tonic response, i.e. they do not rapidly adapt to a continuous stimulus and a stable stimulation of these receptors is needed to evoke a prolonged upwind flight (Geier *et al.*, 1999). This is consistent with the fact that skin odour is emanated continuously from the host body, rather than intermittently as is the case with exhaled carbon dioxide (Geier *et al.*, 1999). Incomplete odour blends or blends that do not contain the natural ratio of host odour components may not elicit a natural behavioural response, therefore extra caution has to be used when analysing studies that presented only a partial or a non-natural odour blend (Cardé and Willis, 2008).

Even if single chemical compounds can act as activators, the most efficient blend of activator compounds is achieved when both carbon dioxide and skin odours are included, activating the highest proportion of mosquitoes and demonstrating a synergistic interaction between the different components of host odour (Omer, 1979; Geier *et al.*, 1999). In addition, it was demonstrated that exposure to a brief filament of carbon dioxide increased five times the sensitivity of *Ae. aegypti*

mosquito to skin odour (Dekker *et al.*, 2005). This rapid sensitisation could have an important impact on host-seeking, as although the presence of carbon dioxide could indicate the vicinity of a vertebrate, by becoming more receptive to skin volatiles the mosquito could then detect the host sooner, which could then lead to a quick orientation toward the vertebrate, or not, depending if the vertebrate fall into its host range.

It has also been speculated that when a mosquito is nearby a host, visual cues alone can too induce activation (Clements, 1999). Many experiments have explored the effect of visual cues as attractants (see following sections). However, none of these studies had considered the role of visual cues merely as activators, thus further research is needed to clarify this.

2.4.2. Long-range orientation

Once activated, a mosquito will detect and follow host cues to arrive closer to the potential host. To do so, it has to navigate in a three-dimensional (3D) space and orient itself towards the cue source. As it has been well described that mosquitoes use host odour as the main set of long-range attractants, long-range orientation is normally described as motor responses to host emanated compounds (Costantini *et al.*, 1998; Gibson and Torr, 1999). Bidlingmayer (1994) defined long-range orientation as the set of responses that reduce the distance between the mosquitoes and the host. However, this definition is rather general and would encompass even movements within centimetres from the host, as long as the space between the insect and the host is reduced. Thus, for the purpose of this thesis, long-range orientation will be used to refer to the set of responses that take mosquitoes that are many metres away from the host to navigate toward the host (Cardé and Gibson, 2010). This stage is mainly regulated by chemical and visual cues (Takken, 1991). A brief overview of the orientation toward these two types of cues is presented below.

Chemical cues

To understand how mosquitoes orient themselves towards an odour source, it is necessary to understand how odour is distributed in the field. An odour source

generally creates an odour plume that diffuses along the wind axis and odour filaments within the plume can be transported several metres downwind without dilution (Gillies, 1980; Murlis *et al.*, 1992). A plume of odour is defined as the volume of air that contains an odour at concentrations equal to or higher than the concentration that will elicit a behavioural response (Cardé and Willis, 2008). In the field, vegetation and changes in wind speed and direction will create turbulence, which disrupts the odour plume and creates gaps of odour-free air within the plume (Brady *et al.*, 1989; Murlis *et al.*, 1992; Cardé and Willis, 2008; Cardé and Gibson, 2010). Consequently, the plume does not present a continuous concentration gradient of the odour and by itself cannot give exact information on the location of the source (Gillies, 1980; Cardé and Willis, 2008). Thus, the strategy used by mosquitoes to locate their host combines the information deriving from the chemical stimuli and the direction of the wind. As mosquitoes present a delicate body and a low flight speed, odour plume following is possible only at relatively low wind speed (Gibson and Torr, 1999). Following the chemical stimuli by remaining in the odour plume trajectory and heading upwind, the mosquito travels to the odour source (David *et al.*, 1982; Cardé and Willis, 2008; Cardé and Gibson, 2010). The two parts of this navigational system are analysed separately below.

<u>Following the chemical stimuli and host selection</u>

Host odour, comprising body odours and carbon dioxide, apart from acting as an activator, functions also as an attractant and governs long-range orientation (Clements, 1999; Gibson and Torr, 1999; Cardé and Willis, 2008). The attractiveness of a stimulus is commonly measured using the "source finding" percentage (Dekker *et al.*, 2005), intended as the percentage of mosquitoes that arrive at the source of the odour. This parameter also includes trap catch percentages as a proxy for mosquito attraction to the stimulus emanating from the trap (Dekker *et al.*, 2002). As reported in several laboratory and field studies, high source finding rates were observed in mosquitoes exposed to either one or a combination of the following chemical stimuli: whole human body odour, acetone, lactic acid, 1-octen-3-ol, and carbon dioxide (Clements, 1999; Geier *et al.*, 1999; Takken and Knols, 1999; Dekker *et al.*, 2001, 2005; Dekker and Carde,

2011). Some studies have found that combining different compounds (e.g. lactic acid and carbon dioxide, body odour and carbon dioxide) had a synergistic effect on the number of mosquitoes attracted to the odour source, while in other studies the absence of one compound did not affect overall source finding (Clements, 1999; Takken and Knols, 1999).

The interaction and synergism of components of host odour is very complex and involves a number of different factors. The response to different odour stimuli is species-specific, it begins at a long-range stage, and plays a central role in host selection (Costantini *et al.*, 1998; Cardé and Gibson, 2010). It can be expected that more generalist species rely more strongly on carbon dioxide as an indicator of a suitable host nearby because it is ubiquitously present in the expired breath of all vertebrates, while species with a more narrow host range rely more on host-specific body odours to recognize their preferred host (Cardé and Willis, 2008). However, the host preference of many mosquito species is not strictly divided, as some species will feed on different hosts depending on the prevailing host availability, i.e. opportunistic species (Costantini *et al.*, 1998). For species with a host preference, specific body odours play an important role in activation and orientation during host-seeking behaviour.

Gillies and Wilkes (1969) reported that *An. melas* was significantly more attracted to traps that were baited with calf emanations compared with traps where only carbon dioxide was offered. Furthermore, host odour attracted some species from a greater distance compared to carbon dioxide only baited traps. They concluded that for *An. melas* host odour played an essential role in orientation and suggested that host-species recognition starts at long-range distance with detection of these odours. Similarly, a field study conducted in South Africa showed that *An. quadriannulatus* displays a highly zoophilic behaviour, being preferentially attracted to traps baited with a calf or high levels of carbon dioxide (comparable with the equivalent volume of carbon dioxide emitted by a cow) compared with traps baited with a human (Dekker and Takken, 1998). In the same study, *An. arabiensis* showed a preference for traps baited with a man (Dekker and Takken, 1998). In a laboratory experiment it was shown

that the antennal olfactory cells of *An. gambiae s.s.*, a highly anthropophilic species, responded significantly more when stimulated with fatty acids that are found in human sweat, compared with the response given when stimulated with 1-octen-3-ol (van den Broek and den Otter, 1999), which is a generic compound present in breath and sweat of mammals and readily emitted by bovine ruminants (Takken *et al.*, 1997a). Further laboratory studies reported that *An. gambiae s.s.* was more attracted to human skin odour rather than to carbon dioxide (Costantini *et al.*, 1998; Mukabana *et al.*, 2002; Day, 2005). In a semi-field trial, significantly more females of *An. gambiae s.s.* where recovered in traps baited with human odour, compared with traps baited with animals (cows and chickens) (Busula *et al.*, 2015). The mechanism underpinning this behaviour seems to be linked to skin microflora; *An. gambiae* is attracted to specific odours produced by the microflora associated with human skin, and skin odour was found to represent over 90% of the attractiveness of humans (Knols *et al.*, 1997; Mboera *et al.*, 1997; Day, 2005). Host selection has been widely explored using many different mosquito species and many different odour cues (Takken *et al.*, 1997a; Costantini *et al.*, 1998; Pates *et al.*, 2001; Zwiebel and Takken, 2004), therefore the studies described above are only a few examples of the enormous amount of research that has been done on this topic.

In addition to species-specific host-selection, even within the same host species there is variability in the attractiveness of host individuals to mosquitoes. This is caused by the variation in volumes, concentrations, and ratios of attractants that each individual emanates. As a result, mosquitoes bite certain individuals more often than others (Knols *et al.*, 1995; Mukabana *et al.*, 2002). The selection of human hosts is thought to be based on: compounds produced by certain skin microbial populations (Verhulst *et al.*, 2011b), the size, surface area, and weight of the host (Port *et al.*, 1980), the different rates of heat production, skin pH, and potentially, the metabolic rate of each person (Ellwanger *et al.*, 2021), as mosquitoes are more attracted to people that release more metabolic products.

Host-seeking behaviour has also an important temporal component. Even if the mosquito is in the "active" phase of the circadian rhythm, different compounds

could elicit responses at different times from exposure (i.e. one compound could induce little to no response initially but then provoke a strong behavioural response after a period of time). Thus an immediate response, either positive or negative, does not indicate the final overall behavioural response to specific stimuli and it is therefore essential to take into account the duration of the exposure to the compounds when testing their attractiveness (Cardé and Gibson, 2010).

Another aspect that needs to be considered when describing long-range orientation is the spatial distribution of the plume and how the mosquito responds to it. Plume-following behaviour has been the focus of much research. However, knowledge in this field has progressed slowly, partly due to the complexity of observing this behaviour, especially without altering the environmental conditions through the presence of the researcher (Gibson and Torr, 1999). With the development of technology that allowed two-dimensional (2D) and, later, 3D flight tracking, it finally became possible to observe and quantify mosquitoes flight without disturbing their natural behaviour.

In the field, wind direction also fluctuates, hence following the upwind direction may routinely lead the mosquito out of the plume boundaries (Cardé and Willis, 2008). Thus, it can be expected that mosquitoes have evolved an efficient strategy to re-contact a lost plume during upwind flight. A few authors have suggested that mosquitoes could use "casting behaviour", which has been well described in pheromone-following moths (Cardé and Willis, 2008; Cardé and Gibson, 2010). Male moths display two types of behaviour when flying towards a stationary pheromone source (a female moth): a straight upwind flight when in contact with the pheromone plume and a casting behaviour after losing the plume, which consists of a crosswind flight, rich in counterturning, that enhances their chances of re-encountering the plume (Cardé and Willis, 2008; Cardé and Gibson, 2010). This same behaviour has also been observed in the mosquito *Ae. aegypti* after the loss of a carbon dioxide plume, which induced a highly regular flight of counterturning across both vertical and horizontal planes. Specifically, the number of counterturning was 1.6 – 1.9 times higher in the

vertical plane compared with the horizontal one, which took the mosquito to counterturn without nearly displacing upwind. This behaviour led the mosquito to transect the area where the compound was previously detected (Dekker and Carde, 2011).

As mentioned previously, vertical barriers (e.g. vegetation or dwellings) and variations in wind speed affect the plume structure and create considerable odour-free gaps within the plume, presenting difficulties for locating an upwind source of odour (Brady *et al.*, 1989; Murlis *et al.*, 1992). A study conducted on *An. gambiae* showed that upon contacting a host odour plume, mosquitoes reduced their flight speed and course angle, and as a consequence, the mosquitoes remained within the plume (Beeuwkes *et al.*, 2008).

Lastly, when considering the spatial distribution of an odour plume, it is necessary to take into account the vertical component. In an moving air environment, it can be assumed that unless the wind blows in the vertical plane, with distance from the source, the plume will gradually expand in the vertical plane as it does on the horizontal axis (Murlis *et al.*, 1992). In contrast, in still air, convection currents created by a warm body may significantly increase the displacement of the odour plume in the vertical plane (Cardé and Willis, 2008). However, these currents only persist until the air within them cools to ambient temperature, which occurs less than 2 m away from the host (Wright, 1968; Bowen, 1991). Thus, over long-distances, their behavioural effect is likely to be minimal.

Detection of wind direction

During host-seeking, a mosquito must detect the direction of the wind and navigate in an upwind direction. To do so, mosquitoes use optomotor-guided anemotaxis (Clements, 1999; Cardé and Gibson, 2010). This mechanism was first described in *Ae. aegypti* by Kennedy (1940), who reported the ability of mosquitoes to calibrate their groundspeed using visual references. In his experiments, he proved that upwind movement could be induced in the mosquitoes by moving a patterned floor underneath them, so that the image

moved from behind them forwards, thus providing visual stimuli that would suggest the insect was being transported downwind (Fig. 8).
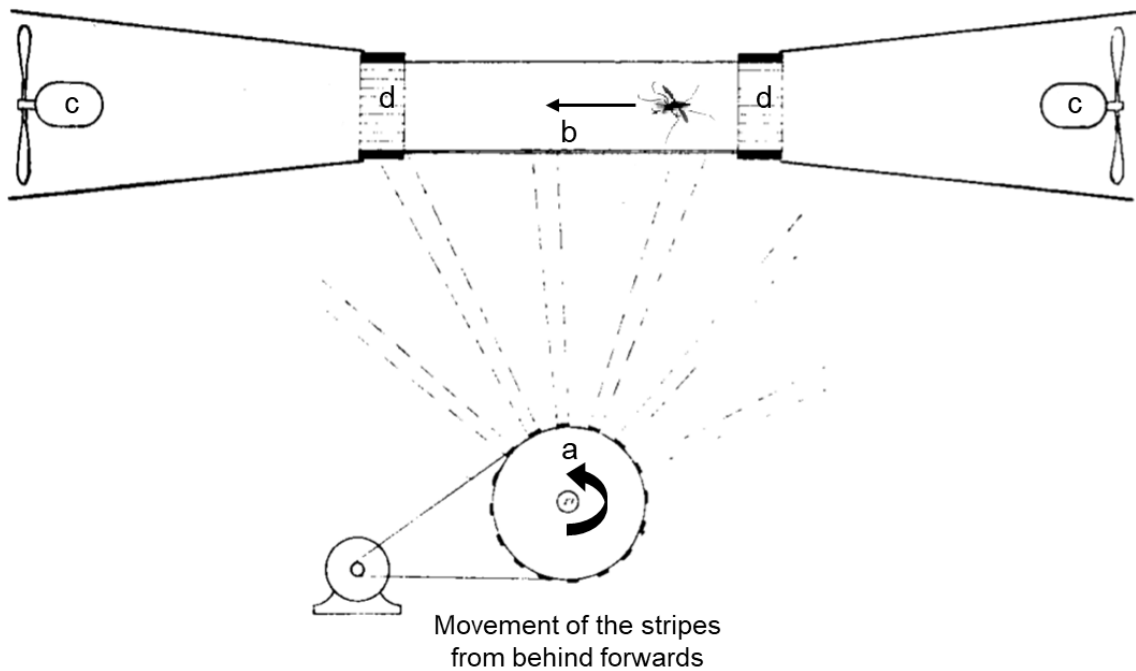
Figure 8: Diagrammatic side view of a mosquito in Kennedy (1940) wind tunnel. *The moving stripes are projected by the projector (a) on the wind tunnel floor (b), and the mosquito orients itself with the direction of the movement, that is from the right to the left (see thin arrow). Fans (c) are on both sides of the wind tunnel to allow drawing air from either way. Air turbulence is reduced on both sides using a honeycomb structure (d). Note: figure not to scale (adapted after Kennedy, 1940).*

Thus, mosquitoes modulate their speed using a fixed visual point as a reference to establish their movement relative to it. In variable wind conditions, the mosquito adjusts its speed to maintain an approximate constant retinal velocity (Budick *et al.*, 2007). Further studies have also suggested that the mosquito uses this guiding system to detect visual sideslip and therefore, wind direction. That is, if the insect is not navigating parallel to the wind, its eyes will record a transversal image flow because of wind-induced drift. Thus, in order to fly upwind, it has to minimise this drift (Fig. 9) (David, 1986; Cardé and Willis, 2008).
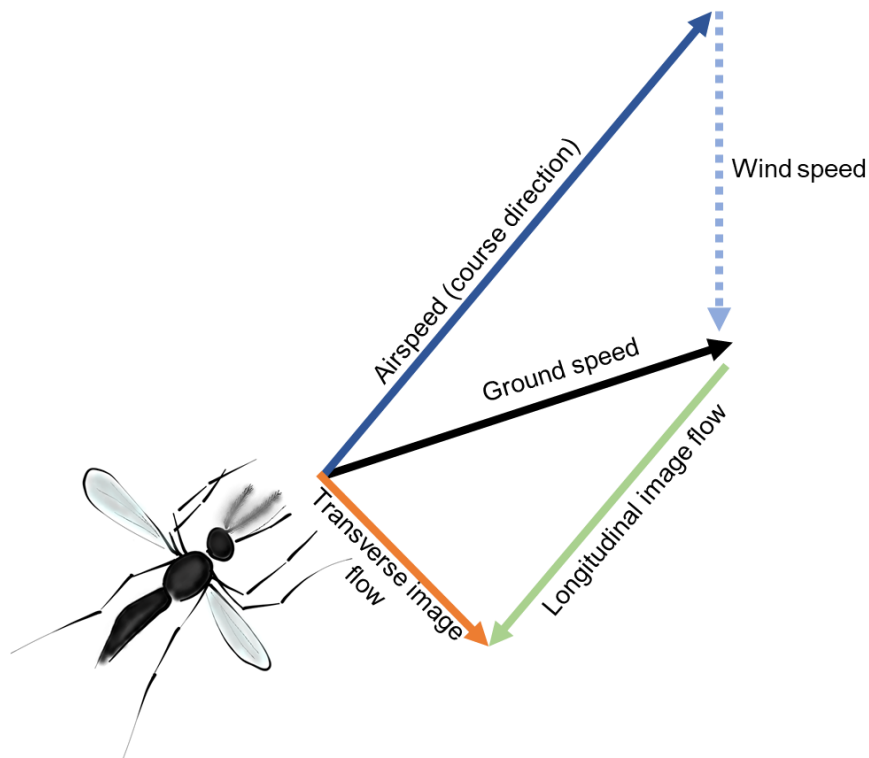
Figure 9: Spatial displacement of a mosquito under wind conditions. *Ground speed: the effective speed at which the mosquito moves in space (black arrow), airspeed: speed and trajectory at which the mosquito fixes its flight (blue arrow), wind speed: the wind, if not exactly aligned with the mosquito airspeed induces a drift in the course direction (light blue dotted arrow), longitudinal image flow: images in the ventral region of the visual field perceived moving with an antero-posterior direction (green arrow), transverse image flow: the drift from the original course direction is perceived by the mosquito in the ventral region of the visual field as a lateral image flow (orange arrow) (drawn by author).*

The optomotor-guided mechanism requires the mosquito to distinguish visual references (high contrast visual cues) even at extremely low light intensities (Gillies and Wilkes, 1982; Gibson and Torr, 1999). It has been demonstrated that mosquitoes are able to see at very low light intensities (as low as one log unit of starlight, 10^{-6} W/m^2) and that, under laboratory conditions, *An. gambiae* is able to orient itself and respond with an optomotor guided flight to a moving floor pattern in low light conditions (10^{-5} W/m^2) (Gibson, 1995). Further laboratory and field studies with *An. gambiae* and *An. coluzzii* have corroborated these results, reporting a visual response to high contrast objects in natural starlight conditions (Hawkes and Gibson, 2016; Hawkes *et al.*, 2017a). In addition, it has also been shown for several mosquito species that the presence of moonlight increases

flight activity severalfold, suggesting that light is indeed important for their flight (Bidlingmayer, 1964; Charlwood *et al.*, 1986). However, other studies have proven that there is no association between moon phases and biting activity (Davies, 1975; Rubio-Palis, 1992), thus additional studies are required to clarify the effect of moonlight on mosquito host-seeking behaviour.

Apart from optomotor-guided anemotaxis, other strategies for guiding upwind flight in mosquitoes have been proposed, one of which is based on mechanosensory feedback (Cardé and Gibson, 2010). For other insects, it was hypothesised that mechanosensory cues could be used to determine deviations from the pre-set trajectory and to detect wind direction. This was successfully demonstrated in *D. melanogaster* where it was found that the detection of wind orientation improved when the flies were allowed to use mechanosensory inputs from the antennae (Budick *et al.*, 2007). In their work with *An. coluzzii*, Hawkes and Gibson (2016) reported a clear repeated pattern of "dipping flight", which consisted of highly stereotyped vertical oscillations. These oscillations could stimulate mechanoreceptors, which convey information related to surface detection (Nakata *et al.*, 2020). However, no studies have examined whether mechanoreceptors in mosquitoes could also be used to convey information regarding wind orientation, thus further research is needed to confirm this.

Visual cues

Under certain conditions, visual cues can also play a role in orientation towards a host at a considerable distance. A study conducted at night time reported that nocturnal mosquitoes of different species could be attracted to dark visual cues and that host-seeking females respond more to those visible objects than engorged or gravid females (Bidlingmayer and Hem, 1979). Here, the authors found that different species approached the traps in different manners: woodland species flew very closely (<30 cm) to the objects while grassland species remained at greater distances. Thus, it is evident that different habitats have put specific selection pressure on the species inhabiting them, which resulted in distinct adaptations in the response that different species have when exposed to a visually conspicuous object. Given that trees can disrupt odour plumes, it can

be expected that plumes disperse differently depending on the habitat type. Thus, it is not surprising that woodland species may have evolved a particular plume following behaviour, which differs from the strategy used by grassland species (Cardé and Gibson, 2010). A subsequent study noted that in visually unobstructed fields, visually conspicuous objects could be used as long-range attractants (Bidlingmayer and Hem, 1980). Adults of different species responded to unpainted plywood traps of 1.5 m height from over 15 m of distance. Notably, in both studies conducted by Bidlingmayer and Hem (1979, 1980) there was no addition of host odour cues to the trap, therefore the trapped mosquitoes were assumed to respond to the visual stimulus only. However, considering that *An. gambiae s.s.* has an angle of visual resolution of approximately 8º, Cardé and Gibson (2010) calculated that this mosquito species would have difficulty distinguishing the image of the width of a human being from a distance of 5 m or more.

2.4.3. Pre-attack resting

Females of several anopheline species have been reported to rest near the host before making the final approach. Bringing the mosquito close to a host habitat and then having it rest until a host passes nearby or until the mosquito is spontaneously activated could be a valuable strategy to balance the efficacy of host-seeking and the energy spent in this activity (Cardé and Gibson, 2010). The duration of this behaviour varies between species, some studies describe a resting time of tens of minutes while for other species this period was extended for several hours (Clements, 1999). A study in Tanzania recorded a pre-attack resting time of four hours for *An. gambiae s.l.* species (Smith, 1958). The mechanisms that induce and modulate this behaviour are not well understood and very few studies have investigated this behaviour.

2.4.4. Short-range orientation and landing

According to Clements (1999), short-range orientation starts when host cues other than just host odour are first detected. However, as mentioned above,

other factors (e.g. visual cues) might have an important influence in mosquito orientation even at a long-range, thus hereafter short-range approach will be considered from the moment that behavioural changes (e.g. increased propensity of alighting) start to occur while mosquitoes are within visual range of a host (Gibson and Torr, 1999). Short-range orientation and landing cues are crucial for the control of mosquitoes' behaviour near odour sources. Surprisingly however, this is the part in host-seeking behaviour that is least understood (Gibson and Torr, 1999; Zhou *et al.*, 2018). As dominant vector control methods rely on the mosquito landing on treated surfaces or entering into the catching perimeter of a trap, the effectiveness of such control methods can be highly influenced by short-range behaviour. A study comparing four carbon dioxide-baited traps demonstrated that odours only produce part of the behavioural sequence that lead mosquitoes to a host, and odour alone does not always induce a close approach (Cooperband and Cardé, 2006). Thus, the efficiency of traps strongly relies on the presence of cues that guide the final approach. Therefore, it cannot be stressed enough the importance of understanding how these non-odour cues affect this part of host-seeking behaviour.

Of crucial relevance when considering short-range approach, is the preference of the species to feed either indoors or outdoors. If the species prefers to feed indoors (i.e. displays an endophagic behaviour), short-range stimuli will be detected only once the mosquito is inside the dwelling (Gibson and Torr, 1999). Two main traits control the entrance into a dwelling: the propensity to fly upward when encountering a vertical barrier and the tendency to enter into openings (Snow, 1987). It can be deduced then that some traits of short-range orientation are species-specific, and different responses may arise even in closely related species (Gibson and Torr, 1999). When close to the host, the odour plume formed both in still air and in a windy environment presents a gradual increase of odour concentration with proximity to the odour source (Cardé and Gibson, 2010). Thus, at a close range, mosquitoes could use longitudinal klinokinesis, i.e. orienting themselves using changes in odour concentration, as an additional strategy of orientation. Whether such a strategy is viable in an indoor

environment, where odours may accumulate with little obvious gradient, has not been explicitly investigated.

Apart from following the odour plume, thermal and humidity gradients, visual, and tactile chemical cues are also important keys that direct the final approach and may induce the alighting of the mosquito on the host (Costantini *et al.*, 1998). Thus, a brief overview of each one of the mentioned factors is provided below.

Visual stimulus

Aversion and avoidance of a solid object take place at a short distance from them (Bidlingmayer and Hem, 1979; Hawkes and Gibson, 2016). The expansion-avoidance paradigm indicates that when a flying insect comes in the vicinity of an object, the proportion occupied by this in the insect's field of view increases, as it expands in the frontal view; thus, the insect perceives the object as a barrier and initiates avoidance behaviour. This theory has been widely studied in flies (Tammero and Dickinson, 2002; Srinivasan and Zhang, 2004; Budick *et al.*, 2007). In mosquitoes, it has been proved that avoidance behaviour takes the mosquito to fly either to the left or to the right (Bidlingmayer, 1994; Cardé and Gibson, 2010) or upwards (Hawkes and Gibson, 2016) to avoid the object. However, in order to alight, the visual avoidance mechanism has to be suppressed. This change in behaviour seems to be triggered by additional stimuli detected at a close range. Thus, avoidance and attraction to the same object depend on the addition of a range of host cues, such as particular host odour, heat and humidity gradients (Cardé and Gibson, 2010). This has been demonstrated in field sampling, where odourless but visible directional flight traps appeared to be avoided by *An. melas*, while the same traps, when baited with a live calf, were attractive (Snow, 1976). Van Breugel *et al.* (2015) corroborated odour-gated visual attraction in *Ae. aegypti* females, which were significantly more attracted to a visually conspicuous feature when exposed to a carbon dioxide plume compared to females exposed to clean air. Furthermore, Hawkes and Gibson (2016) also reported similar results as *An. coluzzii* females flew directly towards a visually conspicuous object only when exposed to a host odour plume. It is interesting to note that in both of these studies, female

mosquitoes approached the visual feature hovering near the surface within centimetres without landing on it and subsequently flying away. This behaviour suggests that other important cues which may induce alighting were missing from the test object.

In the field, Haufe (1964) indicated that traps that use principles of visual attraction (e.g. traps with a higher contrast compared to the environment) are more successful in catching mosquitoes than other traps. This was corroborated by a second field study by Hawkes *et al.* (2017a) which reported that traps baited with human odour and high contrast visual stimuli caught more *Anopheles* mosquitoes than transparent traps.

Thermal stimulus

Thermoreceptors were first discovered in sensilla at the tip of the antenna of *Ae. aegypti* female mosquitoes (Davis and Sokolove, 1975). Here, two types of receptors were identified, one being warm-sensitive and the other being cold-sensitive. These thermoreceptors were found to have a phasic response to a temperature change as small as 0.2 ºC (Davis and Sokolove, 1975). Later, thermoreceptors were also reported in males of the same species (McIver and Siemicki, 1979). On the antennae of *An. gambiae*, Wang *et al.* (2009) found heat-activated channels that specifically respond to temperature gradients and are therefore responsible for the molecular transduction of temperature detection. A more recent study identified a cooling receptor (Ir21a) as the key mediator of heat-seeking in host-seeking *An. gambiae* females (Greppi *et al.*, 2020). Additionally, the proboscis of an Asian malaria mosquito, *Anopheles stephensi* (Liston, 1901), was found to also participate in thermo-sensation during the host-seeking process (Maekawa *et al.*, 2011), thus indicating that the thermal gradients are detected not only by the antennae.

Heat is always transferred from a higher-temperature medium to a lower-temperature medium and the transfer continues until the two bodies reach the same temperature. There are three different ways to transfer heat: conduction, convection, and radiation. Conduction occurs when the excited atoms of a heated body transfer the kinetic and potential energy to adjacent

atoms of another body. This can take place in solids, liquids or gases and it does not elicit a macroscopic movement of the matter (Mayhew and Rodgers, 1992; Yunus and Afshin, 2011; Ghassemi and Shahidian, 2017). Convection transfers heat through the movement of the fluids that surround the higher-temperature matter, thus it requires the presence of fluids. Radiation is the transfer of energy through photons in electromagnetic waves, e.g. infrared (IR) light. This can occur without physical contact between the two bodies and does not require the presence of an intervening medium, i.e. it can occur in a vacuum environment (Mayhew and Rodgers, 1992; Yunus and Afshin, 2011; Ghassemi and Shahidian, 2017). As the energy is transmitted by electromagnetic waves, it is not affected by turbulence or the position of the receiver and gives precise information on the location of the source (Zermoglio *et al.*, 2017). Nevertheless, not many animals seem to use IR radiation for host/prey location (Zermoglio *et al.*, 2017). A first study indicated that mosquitoes were not influenced by radiant heat but rather responded to currents of heated air (Howlett, 1910). Since then, a series of studies that used *Aedes* mosquitoes corroborated this hypothesis using behavioural approaches (Peterson and Brown, 1951; Zermoglio *et al.*, 2017) and sensory physiology approaches (Gingl *et al.*, 2005). These studies showed that mosquitoes did not orient towards heated bodies when radiant heat was the only available cue.

In convection heat transfer, the energy is passed to intermediate fluids (e.g. the air) creating what are defined as "convection currents", which can be explained as follows: the body warms the air in contact with it and as a result the density of the warmed air decreases, which causes the warm air mass to rise. The space left by the rising air is filled by surrounding colder air (Fig. 10). This creates a current of circulating air which continues to move until a uniform temperature is reached in the body-air system (Mayhew and Rodgers, 1992; Yunus and Afshin, 2011). Convection currents, if considered as cues for blood-sucking insects, pose a few disadvantages: first, these are only useful as a cue if the insect approaches the host from above, and second, wind can cause turbulence which disrupts the currents, and thus interrupts its signal (Guerenstein and Lazzari, 2009).

Air at ambient temperature

Air cools and sinks

Air warms and rises

Human hand ~34ºC
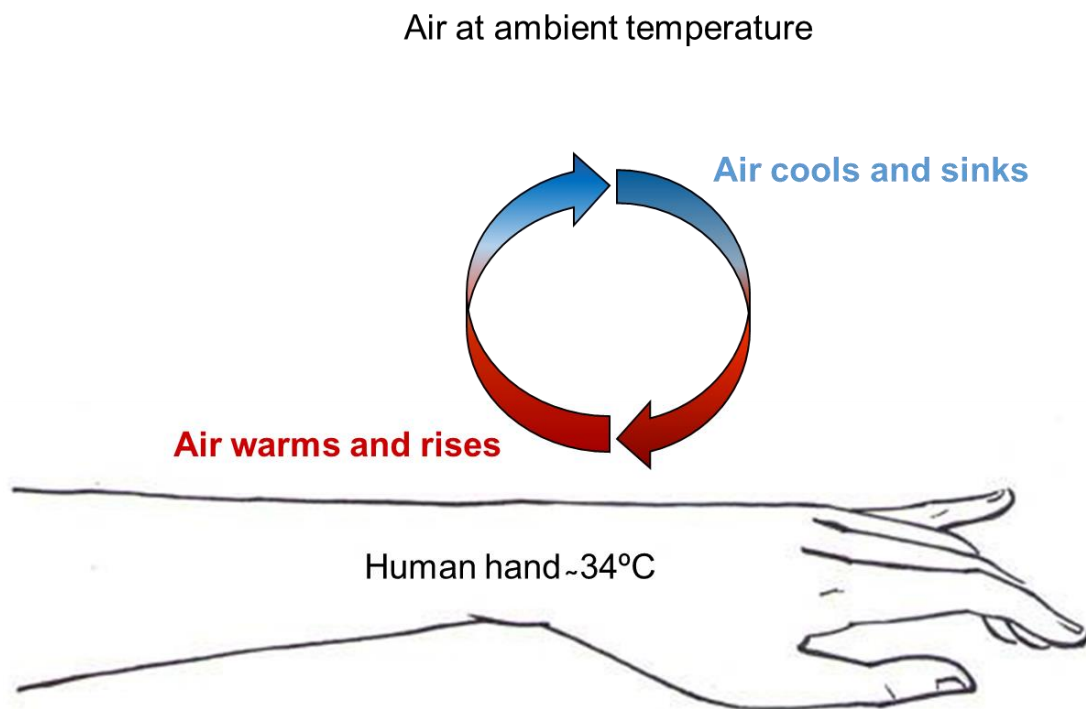
Figure 10: Convection currents rising from a human upper extremity. *Air in contact with a human arm warms up and moves upward while cold air sinks and fills the space surrounding the arm (drawn by author).*

Considering the sensitivity of the antennae thermal receptors, and provided that under stimulation all thermoreceptor sensilla will respond, it was estimated that mosquitoes would be highly sensitive to very small and rapid changes in temperature, equal to temperature changes found in thermal convection currents (Davis and Bowen, 1994). Convection currents rising from a human arm were demonstrated to rise and carry a local thermal difference of 1 º or 2 ºC at a distance greater than 40 cm (Wright, 1968). Warm convection currents rising from a two-kilogram rabbit resulted in a local thermal difference of 0.05 ºC over two metres away (Bowen, 1991). This temperature difference can elicit a response in mosquito thermoreceptors, thus mosquitoes could detect a thermal target from a metre or more of distance (Bowen, 1991; Davis and Bowen, 1994). Khan *et al.* (1966) reported a temperature gradient of 0.25 ºC up to a height of about 45 cm from a container filled with water at 34 ± 0.5 ºC. They also proved that the heat source was still effectively attractive to *Ae. aegypti* mosquitoes up to a height of 111 cm and lured one third and one fifth of the mosquitoes at a

height of 162 cm when presented in conjunction with carbon dioxide, and moist plus carbon dioxide, respectively.

Activated mosquitoes are positively attracted by warm bodies (Clements, 1999). When entering into contact with a warm, moist convection current, activated *Ae. aegypti* mosquitoes flew directly toward the source of the convection current and alighted on it (Kellogg and Wright, 1962a, 1962b). However, mosquitoes that were presented with a dry warm or a cold and damp object were not attracted to it (Wright and Kellogg, 1962). Similar results were obtained by Khan and Maibach (1966), who reported that heat alone can activate and attract mosquitoes but it is the addition of water vapour that enhances the landing response. In their study, *Ae. aegypti* females were presented with either one or a combination of the following stimuli: carbon dioxide, water vapour, and a warm body at 34 ± 0.5 °C. The females were introduced at the top of a 44-inch-tall tower and the stimuli were presented at the bottom of it. The number of females found on the bottom of the tower was counted every minute per ten minutes. Their results showed that heat alone was not crucial in eliciting landing, but the addition of moisture increased both landing and probing. Thus, based on these behavioural studies, it is reasonable to infer that heat is not the only attractive cue that mosquitoes use to finally land on a surface. In fact, convection currents arising from a host (Fig. 11) also carry other constituents such as vapour, carbon dioxide and organic volatiles which enhance the alighting response (Clements, 1999; Zermoglio *et al.*, 2017).
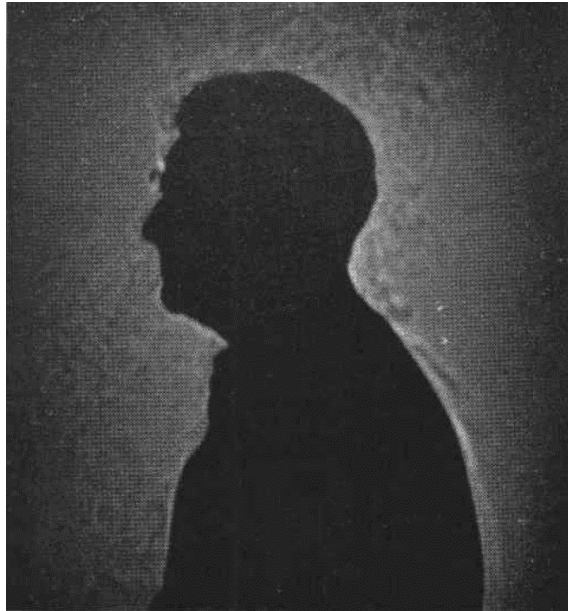
Figure 11: Schlieren photography of convection currents rising from the head and upper body of a fully-clothed man. *The rising streams of air are represented by the white and grey lines around the silhouette (after Cumming and Wright, 1967).*

Neutralising convection currents by raising the ambient temperature to the same temperature of a human hand (34 ºC) has been shown to completely suppress mosquito responses to a hand placed at the bottom of a cage (Khan *et al.*, 1968). Specifically, *Ae. aegypti* female mosquitoes did not land on the net above the hand or attempt probing it. Furthermore, when the convection currents were muffled, the number of mosquitoes taking-off also decreased. Thus, the authors concluded that muffling the convection currents suppressed the transport of odour, and therefore influenced the activation rate. This emphasised the importance of convection currents as vehicles of odour-transport. To verify the role of body odour in probing behaviour, Burgess (1959) conducted a laboratory experiment on *Ae. aegypti* females in which they eliminated the effect of body emanations by enclosing the human hand in an airtight plastic container. The hand was placed underneath a cage containing the mosquitoes and the number of females displaying a probing behaviour was counted. When exposed to the wrapped hand, mosquitoes showed no response, i.e. no probing behaviour, thus the plastic completely suppressed the mosquitoes' response to the heat. However, when carbon dioxide was introduced in the cage, the mosquitoes

became responsive and started to probe, suggesting that carbon dioxide triggers responsiveness to heat in the last phases of host-seeking behaviour.

The attraction of *Ae. aegypti* females to convection currents proved to be also enhanced by the introduction of human sweat odour and lactic acid (Eiras and Jepson, 1994). Similarly, when carbon dioxide was added into cages containing *Ae. aegypti* mosquitoes, it was noted that the gas provided synergistic improvement in mosquitoes' response to warm and moist convection currents, i.e. more mosquitoes were recorded at the bottom of the experimental tower where the stimuli were offered, compared with the number of mosquitoes found at the bottom of the tower when only one of the following stimulus was offered: carbon dioxide, heat, and moisture (Khan and Maibach, 1966; Khan *et al.*, 1966 as cited in Gillies, 1980). McMeniman *et al.* (2014) found that carbon dioxide gated *Ae. aegypti* response to a heat source, as in the absence of carbon dioxide, mosquitoes blood-fed from a heated membrane only when human odour was introduced in the cage. Two *Culex* species were also reported to have a heat-seeking response that was dependent on the presence of carbon dioxide (Reinhold *et al.*, 2022). In contrast, another study reported that carbon dioxide did not directly gate the attraction to warm objects, as *Ae. aegypti* mosquitoes showed a preference for warm objects even in clean air, compared with objects at ambient temperature (Van Breugel *et al.*, 2015). For the purpose of this thesis, a "gated response" refers to the neurophysiological mode by which the detection of one stimulus lowers the threshold of reception to other stimuli, thus enhancing their detection. Although the conclusions made by McMeniman *et al.* (2014) and Van Breugel *et al.* (2015) presented discrepancies, both studies suggested that in a multimodal context, heat is integrated with other host-associated cues. The interactions of different host cues enhance the probability of the mosquito coming near to the host, as demonstrated in *Ae. aegypti*, when highly efficient host location was triggered when two or more stimuli were presented simultaneously or in quick succession (Van Breugel *et al.*, 2015). This was corroborated in a later study, where once again *Ae. aegypti* mosquitoes were more attracted to a warm surface when this was presented with a small visual cue and carbon dioxide (Liu and Vosshall, 2019). It can be expected that a living

host releasing a complete set of cues will provide a more robust guiding signal than an artificial object. Thus, host-seeking mosquitoes will be more likely to successfully orient towards, locate, and alight on a living host than artificial objects that only provide a restricted range of cues. This has been demonstrated in two studies that were undertaken to differentiate the quantitative attractant effect of different cues, namely heat, humidity, carbon dioxide, and a human hand. It was found that *Ae. aegypti* mosquitoes responded faster and in a greater number when they were exposed to a palm of a human hand compared to any combination of other attractants (Khan and Maibach, 1966; Khan *et al.*, 1966). These results were corroborated in a later study on the same species conducted by Eiras and Jepson (1994).

The majority of the reported studies were undertaken using *Ae. aegypti* as the experimental subject. Given that only few studies considered anopheline species, it is worth briefly summarising their results. The first reported study that explored *Anopheles* response to convection current was undertaken by Wright and Kellogg (1964). In their experiment, they exposed females of *Anopheles quadrimaculatus* (Say, 1824) to warm bulb-like objects that emanated a column of convection currents. They found that the mosquitoes were non-responsive, i.e. did not alight, on the bulb emitting a narrow column of convection current but they alighted on the same bulb when the warm and moist currents were scattered in a wider area (Fig. 12). Considering that the main difference between the two treatments was only the extent of the convection currents produced, they deduced that there is an important size effect on host choice when two hosts are presented.
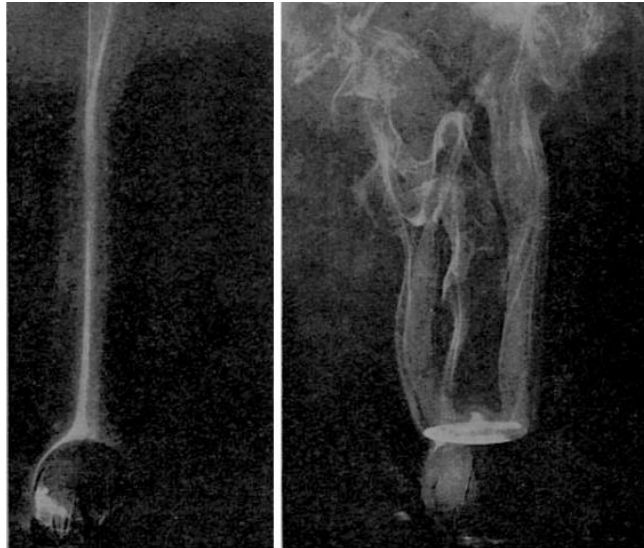
Figure 12: Visible convection currents rising from warm bulb-like targets. Anopheles quadrimaculatus *required a wider column of rising air (right) to display a landing behaviour, while* Ae. aegypti *was able to display the same behaviour on a narrower column (left) (after Wright and Kellogg, 1964).*

Dekker *et al.* (1998) reported that convection currents guided the descending and biting behaviour of three species of the *An. gambiae* complex (*An. gambiae s.s.*, *An. arabiensis*, and *An. quadriannulatus*). Following laboratory assays which used *An. gambiae* indicated that the level of landing response was temperature-dependent, with significantly more mosquitoes landing on an object that had a temperature within a range of 34 ± 2 °C (i.e. human skin temperature) compared to objects at 27 ° and 41 °C (Healy *et al.*, 2002). Spitzen *et al.* (2013) found that *An. gambiae* mosquitoes exposed to both human foot odour and heat treatment (34 °C) had longer, faster, and more tortuous flights, which ended with a higher proportion of mosquitoes landing on the odour and heat source compared to when odour or heat treatments were used alone. This suggests that the combination of these cues would elicit behaviours that result in an increased chance of alighting on a host. They also found that heat reduced flight speed near the source (at less than 15 cm from it), producing a more tortuous flight, which provides more opportunities for the mosquito to scan the environment and accurately assess the location and the quality of the stimuli before proceeding to land.

A behavioural assay conducted with *An. gambiae* mosquitoes in a dual-port tent olfactometer reported that heat and moisture enhanced in a synergistic manner the attractiveness of a non-occupied tent that was baited with human odour (both synthetic and natural blend) (Olanga *et al.*, 2010). Likewise, the addition of a warm surface to a visually conspicuous odour-baited trap increased the number of *Anopheles* mosquitoes caught compared with the number of mosquitoes caught in an odour-baited and visually conspicuous trap set at ambient temperature (Hawkes *et al.*, 2017a).

Humidity stimulus

Where ambient temperatures are elevated, for example in tropical areas, human skin releases sweat as a mechanism of thermoregulation. The aqueous component of the perspiration product evaporates, thus producing water vapour. Therefore, convection currents arising from potential hosts are generally associated with humidity gradients. The ability of mosquitoes to sense moisture gradients is crucial in order to differentiate warm objects (e.g. rocks heated by the sun) from animals (Van Breugel *et al.*, 2015). Consequently, linking the alighting behaviour with a responsiveness to the humidity cue increases the likelihood of the mosquito to land on a suitable host.

Mosquito hygroreceptors were first identified on *Ae. aegypti* antennae (Bar-Zeev, 1960). A later study determined that these antennal sensilla could detect an increase of two per cent in the relative humidity (Kellogg, 1970). Relative humidity is defined as the ratio between the amount of water vapour actually present in an air volume and the greatest amount possible needed to saturate the air at the same temperature. The ratio is then converted into a percentage. Hygro-sensilla in mosquitoes were described to generally contain three neuroreceptors: a cold receptor, a dry receptor, and a moist receptor (Altner and Loftus, 1985). More recent studies have reported the presence of hygro-sensitive sensilla in *An. gambiae* antennae (van den Broek and den Otter, 2000; Meijerink *et al.*, 2001). These hygro-sensilla were found to respond to both the presence of high and low percentages of water vapour, which indicates their role in humidity detection (van den Broek and den Otter, 2000).

A behavioural study demonstrated that *An. gambiae* possesses a high sensitivity to humidity gradients and that relative humidity plays an important role in host-seeking behaviour (Takken *et al.*, 1997b). After the observation that warm moist air attracts mosquitoes better than warm dry air, Davis and Bowen (1994) proposed that humidity enhances thermal stimulation. They suggested that this could be explained by the fact that some antennal receptors are moisture dependent, that is to say, that they lose sensitivity when humidity falls below a threshold value. For this reason, moist air seems to carry additional information that enhances temperature sensitivity. Similarly, in their behavioural experiments using *Ae. aegypti* females released in a wind tunnel, Van Breugel *et al.* (2015) found that mosquitoes had a significantly stronger response at a higher distance (up to 6 cm higher) to a warm object when this was presented along with a moist tissue paper. Without the addition of the moist tissue paper, mosquitoes were responding to the warm object only when they flew very close to the object (2 cm above it). Many studies that considered the effect of thermal stimuli on mosquito behaviour also reported how orientation downwards during the final approach, and in particular, alighting behaviour, was mediated and augmented by the presence of water vapour in convection currents (Wright and Kellogg, 1962; Khan and Maibach, 1966; Eiras and Jepson, 1994).

Chemo-tactile stimulus

It has been suggested that the final stage of host-seeking requires chemo-tactile stimulation. Mosquitoes receive this through contact with the surface of the host's body via the distal tip of the legs, i.e. the tarsi. Wild mosquitoes activated by carbon dioxide were reported to touch the surface of an artificial target by extending a leg before landing (Browne and Bennett, 1981). The authors attributed this behaviour as a sensory function; however, no further studies confirmed this hypothesis and therefore further research is needed to clarify this. Moisture receptors that mediate reactions to dry or wet stimuli were also found on the tarsi of *Ae. aegypti* (Bar-Zeev, 1960). Furthermore, as mentioned in section 2.2, the gustatory sensilla presented in the tarsi were shown to be involved in oviposition behaviour, nectar-feeding, and mating (Sparks *et al.*, 2013; Montell and Zwiebel, 2016). Thus, it can be expected that a similar

mechanism could mediate landing on the host. However, further investigation is required to support this idea.

2.5. *Anopheles* mosquitoes as vectors

To better understand how to disrupt malaria transmission, it is important to understand the role that mosquitoes play in its transmission. Thus, a brief summary of the parasite life cycle and the characteristics that make mosquitoes effective vectors is provided below.

2.5.1. The life cycle of Plasmodium

There are about 80 *Plasmodium* species that infect vertebrates but only five species are able to cause clinical malaria in humans (Becker *et al.*, 2010; CDC, 2021). The life cycle of *Plasmodium* species that cause malaria in humans is complex and is divided into two phases of replication: the sexual replication takes place in the invertebrate host (mosquitoes of the genus *Anopheles*) while the asexual replication occurs in the vertebrate host (Fig.13).
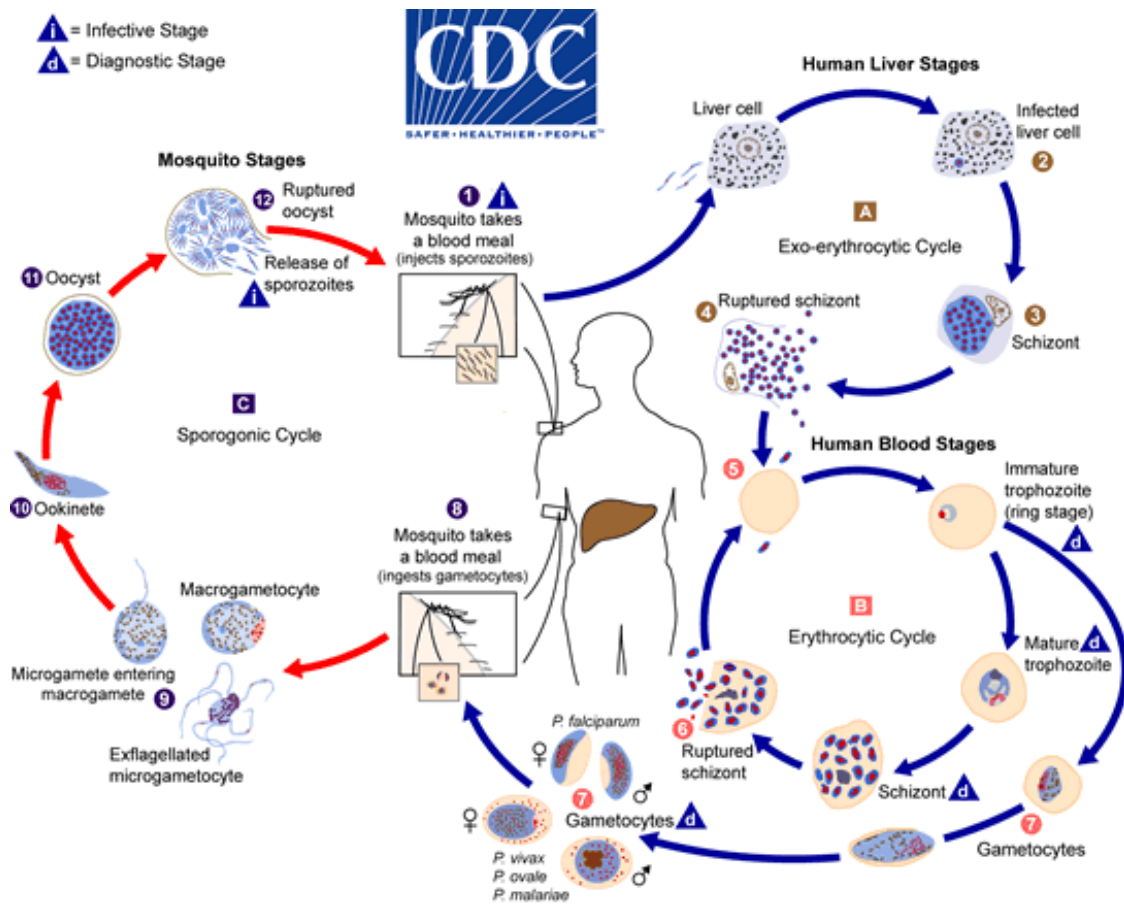
Figure 13: Human malaria parasite life cycle. *The life cycle of* Plasmodium *species consists of two stages; sexual replication occurs in mosquitoes and asexual replication in humans* (after CDC, 2021)*.*

A mosquito becomes infected after it ingests blood containing gametocytes, the sexual form of the parasite. Once in the mosquito's midgut, the gametocytes fuse forming a zygote, which develops into a motile form called the ookinete that then migrates across the peritrophic membrane of the mosquito's midgut and forms an oocyst (Service, 2008). Here, sexual reproduction, called sporogony, takes place, and after several meiotic and mitotic divisions, many haploid sporozoites are formed. The sporozoites migrate to the mosquito's salivary gland and their inoculation to a new human host takes place when the mosquito has its next blood meal (Service, 2008). Sporozoite-containing saliva is injected into the host's tissue via the hypopharynx, alongside other proteins that anaesthetize the host and prevent coagulation of the blood (Gullan and Cranston, 2014; CDC, 2021).

Inside the human body, the sporozoites infect the liver cells. Here they undergo a series of changes and divisions (asexual reproduction, called exo-erythrocytic schizogony), forming thousands of merozoites which are then released into the bloodstream where they find and invade the erythrocytes (Becker *et al.*, 2010). Inside the erythrocytes a second asexual reproduction (called erythrocytic schizogony) takes place and new merozoites are formed and released with the rupture of the erythrocytes. The rupture of the infected erythrocytes occurs synchronously, releasing tens of thousands of new merozoites each time, which will infect new erythrocytes, repeating the asexual reproduction several times. It is at this stage, with the synchronous rupture of the erythrocytes, that malaria symptoms, such as fever, are displayed (PATH, 2015). After many erythrocyte cycles, some parasites differentiate into gametes. These can be acquired by another mosquito during its blood meal, completing the cycle of transmission (Becker *et al.*, 2010; Gullan and Cranston, 2014; CDC, 2021).

Understanding the life cycle of the parasite provides valuable insights into the transmission chain. A few important aspects of the parasite's transmission should be noted:

- The mosquito, in order to pass the parasite to the human host, has to live long enough to allow the gametes of the parasite to fully complete the sexual reproduction. This takes an average time of 10 to 21 days at 25 ºC (CDC, 2021).

- Below 15 ºC and above 33 ºC, the part of the cycle in the mosquito cannot be achieved and therefore malaria cannot be transmitted. Consequently, malaria is confined to specific areas of the world where temperatures that allow this cycle to take place are maintained (CDC, 2021).

- Given that the part of the cycle in humans takes approximately six to 15 days, it is highly unlikely that the mosquitoes that pick up recently formed gametes are from the same generation of mosquitoes that inoculated the parasite in the first place. In fact, adding the duration of both parts of the cycle (in the mosquito and in the human host) the mosquito would have to

be from two weeks to over a month old, and in the wild *Anopheles* vectors very rarely survive for that long (Esposito and Habluetzel, 1997). Thus, for a constant transmission of the parasite, the mosquito population needs to have a quick generation turn-over to guarantee a constant presence.

- Humans and mosquito populations have to be in relatively close contact in order to perpetuate parasite transmission (CDC, 2021).

Thus, in malariological entomology, a few parameters are considered important for the prediction of malaria transmission intensity, including the number of females that approach a human host for biting, the origin of the blood meal in the mosquito midgut to evaluate host preference, and the age of the mosquito population (Esposito and Habluetzel, 1997). A metric that combines some of the above-mentioned parameters is the entomological inoculation rate (EIR), which indicates the number of bites by infectious mosquitoes per person in a given time (Kelly-Hope and McKenzie, 2009). This is calculated using vector density, human blood (extracted from mosquitoes gut) index, and the sporozoite rate in mosquitoes' salivary glands (Esposito and Habluetzel, 1997). This summarises the intensity of malaria transmission in a certain area (Esposito and Habluetzel, 1997; Kelly-Hope and McKenzie, 2009). The EIR also shows that it is not necessary to aim for a complete eradication of mosquitoes in order to reduce malaria transmission, as it is sufficient to lower single parameters (e.g. biting rate) to lower the EIR to a level where transmission is no longer sustained (Scholte *et al.*, 2006; Gullan and Cranston, 2014; Hiscox *et al.*, 2016).

2.5.2. Characteristics required for being effective vectors

Not all mosquitoes are effective vectors of malaria. The vectorial capacity of each species is defined as the daily number of future infectious bites that would arise from a single infective patient, if all the females biting that patient become infected (Massad and Coutinho, 2012; Brady *et al.*, 2016). This depends on the combination of different parameters and is associated with the following characteristics:

- Competence: the mosquito must be able to acquire the parasite and allow its sexual reproduction. Sufficient numbers of sporozoites must be present in the salivary glands to ensure inoculation of the host (Gullan and Cranston, 2014; The malaria atlas project, 2018; CDC, 2021).

- Host preference: the more a mosquito population is in contact with humans, the more it is likely for the species to be a suitable vector. This factor is defined by the anthropophily of the mosquito: anthropophilic and endophagic species have more opportunities to be in contact with humans, as they are more attracted to humans than other vertebrates and tend to acquire their blood meals inside of human-inhabited dwellings. Thus these species would be more suitable vectors than zoophilic and exophagic species (Gullan and Cranston, 2014; The malaria atlas project, 2018; CDC, 2021).

- Abundance: the mosquito population has to be large and with a rapid generation turn-over to maintain the constant presence of a high number of potential vectors (Gullan and Cranston, 2014; The malaria atlas project, 2018).

- Longevity: the mosquito must survive long enough to allow reproduction and development of the parasite (Esposito and Habluetzel, 1997; Gullan and Cranston, 2014; The malaria atlas project, 2018). In general, it is considered that a mosquito has to live a minimum of two to three weeks to transmit malaria (Crutcher and Hoffman, 1996).

- Feeding rate: this indicates how often the females approach and feed on vertebrate blood and is dictated by climate and female adult body size (Gullan and Cranston, 2014). Smaller females that have experienced overcrowding, high temperatures, and poor nutrition conditions during larval development lack metabolic energy reserves, which are essential not only for egg development but also for basic metabolic processes (Scott and Takken, 2012). Thus, smaller females tend to have a higher feeding rate. Due to the nonlinear positive relationship between host biting and

transmission, a small increase in the number of blood meals taken per day quadratically increases the opportunities for parasite transmission (Scott and Takken, 2012).

Changes to these parameters can affect the vectorial capacity of a mosquito population and therefore can be targeted for vector control interventions.

2.6. Vector control methods

With the discovery in 1897 of mosquitoes' role in malaria transmission, the idea of designing control methods against mosquitoes became the focus of much research (Becker *et al.*, 2010). Since then, substantial effort has been put into reducing mosquito populations to eliminate malaria. However, the adaptability of both the vector and the parasite, in addition to political, economic, and geographical reasons have negatively impacted the vector control techniques that have been suggested by scientists (Becker *et al.*, 2010).

It is important to consider the psychological and sociological impact of a proposed control tool on a personal and community level, in order to ensure that such tool is used in an appropriate manner and as often as recommended to achieve the expected result (Hiscox *et al.*, 2016; Ingabire *et al.*, 2017). Furthermore, vector control programs that include community participation have proved to be more enduring, cost-effective and sustainable (Bryan *et al.*, 1994). Community engagement and strong health systems are at the base of the pyramid to achieve high coverage of preventive and curative interventions (Rabinovich *et al.*, 2017).

Decades of research have provided an array of different types of vector control approaches. Many aspects (e.g. effectiveness, cost, feasibility of application, and sustainability) have to be considered when developing a control method and an exhaustive review of this field cannot be provided here. Therefore, the following section is intended as an overview of the different techniques that have been used and the potential new methods that could be developed.

2.6.1. Chemical control

The first global strategy for malaria control took place in the 1950s and 1960s, after the discovery of the pesticide dichlorodiphenyltrichloroethane (DDT). The program consisted of wide-scale coverage of DDT via spray application in malaria-endemic countries (Sokhna *et al.*, 2013). However, this approach did not succeed in all countries included in the program. Moreover, due to the devastating side effects of DDT on non-target organisms (humans included) and on the environment, its use has been greatly reduced in all countries (Becker *et al.*, 2010; Kamareddine, 2012).

Current malaria prevention relies heavily on vector control techniques (i.e. preventing the population at risk from being bitten by mosquitoes) (WHO, 2017; Carrasco *et al.*, 2019; Kendie, 2020), and most scientists involved in malaria control programs agree that vector control plays a fundamental part in malaria elimination (Sokhna *et al.*, 2013), being responsible for 81% of the reduction of infection prevalence between 2000 and 2015 (Bhatt *et al.*, 2015). Over the past three decades, there has been a significant decline in malaria incidence which was largely attributed to the widespread use of ITNs and IRS, along with drug therapy (Ranson *et al.*, 2011; Sokhna *et al.*, 2013; Kweka *et al.*, 2017; Riveron *et al.*, 2018). Insecticide-treated nets are bed nets that received a treatment with insecticides, thus they provide chemical protection along with the physical protection that prevents vector-host contact. To avoid the loss of insecticidal effects due to degradation or washing, new nets were developed by impregnating the pyrethroid insecticides in the polyethylene fabric that form the net. These nets are called Long Lasting Insecticide-treated Nets (LLINs) as they are certified to retain their insecticidal effectiveness for at least three years (Gullan and Cranston, 2014). Further, these nets may also contain synergist compounds that increase the effectiveness of the insecticide (Allossogbe *et al.*, 2017). Indoor residual spraying refers to the application of residual insecticides on the internal walls and surfaces of dwellings to kill mosquitoes that come to rest on them (WHO, 2015).

Even if the level of coverage of ITNs and IRS has increased, in 2020 only 65% of sub-Saharan households had at least one ITN, and this percentage decreases to 34% if only the households that owned at least one ITN for every two people are considered (WHO, 2021). It has been estimated that between the years 2000 and 2015 the incidence of clinical malaria was reduced by 40% (WHO, 2014) and ITNs and IRS are thought to have contributed respectively 68% and 13% to this decline (Bhatt *et al.*, 2015). Although great effort is being put into malaria eradication, in 2016 the decline rate of malaria incidence and mortality has slowed and since 2014, in some African regions the decline rate appeared to be reversed (WHO, 2017). This is thought to be caused by the rapid spread of insecticide resistance (Ranson *et al.*, 2011; Homan *et al.*, 2016; Riveron *et al.*, 2018; WHO, 2021) and behavioural resistance (Ranson *et al.*, 2011; Sokhna *et al.*, 2013; Gullan and Cranston, 2014; Ranson and Lissenden, 2016).

Insecticide resistance can be defined as the ability of an insect to survive the toxic effects of an insecticide after being in contact with it (Riveron *et al.*, 2018). There are several endogenous and exogenous factors (e.g. genetic, metabolic, in-life adaptability, environmental) that contribute to the emergence and spread of a resistance trait. How these factors interact and contribute to the overall resistance trait is still not well understood and several studies are currently being undertaken to fill this gap (Ranson *et al.*, 2011; Hancock *et al.*, 2018). There are two major mechanisms that enable insects to withstand insecticides: the modification of the insecticide target, thus reducing its binding with insect receptors, and the increase of metabolic rates of detoxification enzymes that degrade the insecticide (Ranson *et al.*, 2011; Sokhna *et al.*, 2013; Riveron *et al.*, 2018). The continuous use and, most importantly, the misuse of insecticides has resulted in a selection pressure that favours resistant phenotypes, thus causing a wide and rapid spread of the resistance trait (Riveron *et al.*, 2018). Resistance to pyrethroids, the only insecticide authorised by WHO to be used in ITNs, has been widely reported in 81% of malaria-endemic countries (WHO, 2017). In 2018, 60 out of the 73 countries with reported malaria transmission recorded mosquitoes resistant to at least one type of insecticide and 50 countries reported mosquitoes with resistance to two or more classes of insecticides (Riveron *et al.*,

2018). Worryingly, the level of insecticide resistance among mosquito populations is increasing continuously (WHO, 2020).

The indoor application of insecticides is thought to select for mosquitoes that present more exophilic and exophagic traits, that tend to bite earlier in the evenings and after sunrise (i.e. when people are not under bed nets), and that display a more zoophagic behaviour (Reddy *et al.*, 2011; Padonou *et al.*, 2012; Yohannes and Boelee, 2012; Sokhna *et al.*, 2013; Thomsen *et al.*, 2017; Abong'o *et al.*, 2018). This behavioural adjustment falls into the category of behavioural resistance, which is defined as the changes in vector behaviour that enables the avoidance of or the reduction of insecticide exposure (Sokhna *et al.*, 2013; Riveron *et al.*, 2018; Carrasco *et al.*, 2019). As a result of this behavioural change, the frequency of non-protected biting by malaria-transmitting mosquitoes has increased and this phenomenon seriously jeopardises the current vector control strategies (Ranson *et al.*, 2011; Thomsen *et al.*, 2017; Riveron *et al.*, 2018; Carrasco *et al.*, 2019). Thus, there is an urge to improve old vector control techniques and an impelling need to develop new and more effective tools for vector control, which should also include non-insecticide methods to delay and reduce the spread of insecticide resistance (Farenhorst *et al.*, 2009; Ranson *et al.*, 2011; Sokhna *et al.*, 2013; Homan *et al.*, 2016; Ranson and Lissenden, 2016; Thomas, 2018). New strategies should also aim to reduce outdoor malaria transmission, which is worryingly high and is an area currently underrepresented by control techniques (Hawkes and Gibson, 2016; Degefa *et al.*, 2017; Hawkes *et al.*, 2017a; Thomsen *et al.*, 2017; Abong'o *et al.*, 2018; Chaumeau *et al.*, 2020; Sougoufara *et al.*, 2020). Along with vector control techniques, research should also focus on the development of field monitoring and surveillance tools (Ferguson *et al.*, 2010; James *et al.*, 2014; Cooke *et al.*, 2015; Ranson and Lissenden, 2016; WHO, 2021).

2.6.2. Outdoor traps and attractive toxic sugar bait

In the past decade, several new outdoor traps were developed to overcome the problem caused by the insecticide resistance and the behavioural shift; these

traps are also important in providing a monitoring tool for determining outdoor vector abundance, composition, and biting time (Cooperband and Cardé, 2006; Jawara *et al.*, 2009; Homan *et al.*, 2016; Charlwood *et al.*, 2017; Hawkes *et al.*, 2017a, 2017b). Many of the effective sampling and control strategies rely on the catching of mosquitoes during their host-seeking behaviour (Hawkes *et al.*, 2017a), exploiting therefore natural cues used in blood-seeking behaviour as attractants. The majority of the outdoor traps consist of an attractant component, which releases stimuli (e.g. light, carbon dioxide, host odour) that are attractive to the mosquitoes even over long distances, and a catching or a killing component, which traps or kills the mosquitoes. The attractive stimuli lure the insects in proximity of the device and the type of attractants changes depending on the type of trap or bait (e.g. sugar baits present different attractants compared to light traps). Nevertheless, irrespectively of the luring method, all traps and baits require the mosquitoes to get close enough so that they can be caught. For example, two common types of traps are sticky traps and suction traps. In traps that use sticky surfaces, the mosquitoes get caught when they come into contact with the surface, while in suction traps, the fan draws air from the outside of the trap towards the inside, creating an air current that is strong enough to ensure that mosquitoes entering the current are dragged inside the trap. Attractive toxic sugar baits attract mosquitoes of both sexes and drive them to land and feed on a solution that contains an oral toxin (Traore *et al.*, 2020; Stromsky, V. E., Hajkazemian *et al.*, 2021). Accordingly, both traps and attractive toxic sugar baits need to display short distance and landing cues in order to ensure a close approach of the mosquitoes (Hawkes and Gibson, 2016; Abong'o *et al.*, 2018).

2.6.3. Alternative control methods

It has become evident that to achieve a sustainable malaria control program it is necessary to develop alternative methods. Integrated Vector Management (IVM) considers the combination of several approaches in order to control different stages of the mosquito life cycle (Thomas and Read, 2007; Hancock, 2009; Kamareddine, 2012; Koenraadt and Takken, 2018; Thomas, 2018). One of these approaches is referred to as biological control (or biocontrol), which

encompasses the use of other organisms (e.g. parasites, pathogens, predators, or competitors), or products of other organisms, to control a target population (Eilenberg *et al.*, 2001). Biocontrol methods have a great potential to become crucial assets in the malaria eradication program and several studies have been carried out in order to optimise their application (Kamareddine, 2012; Abagli *et al.*, 2019; Kendie, 2020). Even though the potential of biological control has been extensively proven, there has been a gap between scientific reports and the actual application of such techniques in the field (Thomas and Read, 2007; Knols *et al.*, 2010; Thomas, 2018).

Biocontrol agents can interfere with malaria transmission by either eliminating the vector (or changing the behaviour that takes it to transmit the parasite) or obstructing the parasite development inside the vector (Kamareddine, 2012). There are several different biocontrol agents that could be exploited for malaria interventions. A brief list that summarises them is provided below:

- Entomopathogenic fungi (EPF): they represent the most promising and explored biocontrol method for malaria control. These agents do not require ingestion and a brief external contact with the insect is sufficient to cause infection, a characteristic shared with chemical insecticides and that makes their application practical and easy (Scholte *et al.*, 2005; Thomas and Read, 2007; Kamareddine, 2012; Bilgo *et al.*, 2018b). These bioinsecticides are also effective on mosquitoes that are resistant to chemical insecticides, making them a suitable candidate for insecticide-resistant management approaches (Farenhorst *et al.*, 2009; Knols *et al.*, 2010; Kamareddine, 2012; Thomas, 2018; Lovett *et al.*, 2019). Furthermore, mosquitoes infected with EPF presented a lower expression of resistance to chemical insecticides (Farenhorst *et al.*, 2009), which could lead to a synergistic mode of action between EPF and insecticides (Hancock, 2009; Mnyone *et al.*, 2012; Bilgo *et al.*, 2018a). Moreover, these agents cause an effect also in parasite viability as it has been demonstrated that fewer infected surviving mosquitoes presented sporozoites in their mouthparts compared with the control (malaria-only

infected mosquitoes) (Thomas and Read, 2007). In addition, EPF infections cause changes in the behaviour of the mosquito, leading to a significant reduction of blood-feeding propensity and lifetime fecundity (Scholte *et al.*, 2006). Thus, the overall reduction of malaria transmission is considerable, with both field and modelling-based studies estimating a drop of over 75-80% in the EIR (Scholte *et al.*, 2005; Mnyone *et al.*, 2012). Lastly, because of their mode of action, EPF are thought to impose just a minimal selection pressure on the mosquitoes and therefore no substantial resistance against EPF is expected to arise (Thomas and Read, 2007).

- Bacterial agents: most studies focus on the larvicidal activity of bacterial strains (Becker *et al.*, 2010; Kamareddine, 2012; Ingabire *et al.*, 2017), although adult mortality and adult behavioural changes (e.g. reduction of blood-feeding propensity or fecundity) have also been reported (Gnambani *et al.*, 2020). The use of microbial agents that naturally express or are genetically engineered to express toxins that could affect either the mosquito or the parasite has also been explored (Knols *et al.*, 2010; Kamareddine, 2012). A good example of this tool is given by *Bacillus thuringiensis* (Berliner, 1915) serovariety *israelensis*, which has become a well-established larvicide, that has been widely used for over 30 years to control mosquito populations in the United States and other countries (Centers for Disease Control and Prevention (CDC), 2007; Lacey, 2007).

- Predatory fish: could be used for larval control. This technique can be used for mosquito species that show a preference for ovipositing in well-defined water bodies and where adults exhibit a strong exophilic behaviour and therefore avoid indoor control strategies (Kamareddine, 2012; Kendie, 2020).

- Viruses, microsporidian parasites, and nematodes: these could act by either expressing antagonistic molecules for the parasite and/or the vector, or directly impacting the survival and the fecundity of the vector (Kamareddine, 2012; Kendie, 2020).

It is also worth mentioning that several research groups have been studying the possibility of using gene drive systems to genetically modify the mosquitoes, aiming to render them less capable of transmitting the parasite or to reduce their population (e.g. through sterilisation) (James *et al.*, 2020). This approach has enormous potential as the transgenic construct could persist in the mosquito population, therefore providing a high-impact, low-cost, durable and self-sustaining tool (James *et al.*, 2018). However, this technology faces community opposition on ethical grounds.

2.7.   The importance of behavioural studies

Studies that rely exclusively on a narrow set of physiological responses are not always indicative of the overall reaction of an organism, as they do not take into consideration the multiple factors that interact when an organism responds (or does not respond) to a stimulus. On the other hand, behavioural studies are a powerful tool to investigate how the insect sensory system works, what stimuli are detected, and how insects react to such stimuli (Gibson, 1995). It is important to consider that the final response measured by, for example, trap catches, is in fact a combination of several responses to different cues that drive the different stages of the host-seeking behaviour (e.g. activation, attraction, close-approach, and possibly, landing), and the organism's response is always the result of a multimodal integration of multiple sensory information (Spitzen and Takken, 2018). Thus, the exact role of single cues is difficult to assess, and therefore, it is essential to carry out a wide range of different assays to separate and quantify the various types of responses so as to have a comprehensive understanding of the insect's behaviour (Torr, 1994).

Torr and Vale (2015) emphasised how knowledge of vector behaviour is fundamental to develop new control tools, upgrade old methods, and improve the overall control and surveillance programs. This idea is also supported by other authors (Hawkes and Gibson, 2016; Cribellier *et al.*, 2018, 2020; Tananchai *et al.*, 2019). A good example of how understanding single specific responses that form part of an overall behavioural response can lead to identifying potential vector control methods or improvements in existing

programs is given by the history of tsetse fly control (Vale, 1993; Torr, 1994). Specifically, acquiring a deeper understanding of the fundamental biology of the insect provided the knowledge to radically improve the techniques used to control this vector. Similarly, a detailed understanding of mosquito behaviour would be essential to successfully implement control tools (Spitzen and Takken, 2018; Tananchai *et al.*, 2019). Furthermore, studying the natural behaviour of mosquitoes permits identifying behavioural patterns that allow the vector to avoid control tools, and this aspect is crucial when estimating the effectiveness of the interventions (Cribellier *et al.*, 2018, 2020; Spitzen and Takken, 2018; Tananchai *et al.*, 2019).

Additionally, applying the knowledge of vector behaviour has the potential to provide more efficient and cost-effective interventions (Torr and Vale, 2015). For example, before discovering the many aspects that drove tsetse flies towards their hosts, the control of this vector was implemented by spraying insecticides from aircraft onto large areas. As can be imagined, this strategy was very expensive and it required the use of large quantities of insecticides. However, after unveiling the response of tsetse to their hosts, the strategy switched to spraying insecticides only on baited targets, which decreased the cost of the operation, the side effects to the environment, and the amount of work that had to be put in to control an area (Torr, 1994).

The idea of applying basic behavioural knowledge to develop a trap was at the base of the creation of the Host Decoy Trap (HDT), an outdoor trap that captures mosquitoes which are led to approach and land on a sticky surface by a combination of different stimuli (odour, high visual contrast, and heat) (Hawkes *et al.*, 2017a). Field studies using this trap have demonstrated its efficacy and therefore the validity of this approach (Hawkes *et al.*, 2017a; Abong'o *et al.*, 2018). Nonetheless, vector behaviour and specifically anophelines behaviour is poorly exploited in mosquito surveillance and control programs (Hawkes *et al.*, 2017a). Although landing cues represent an important part of the final stage of host-seeking behaviour, little research has been done with regards to quantifying

the effects of these cues in inducing landing in *An. gambiae s.l.* or analysing its behaviour in response to these cues.

Considering that understanding behavioural responses is crucial for the development of effective control and surveillance strategies, this project will mainly employ behavioural techniques in its research methodology.

2.8. Summary

Mosquito species belonging to the *An. gambiae s.l.* complex are major African vectors of the *Plasmodium* parasite, which causes human malaria. Currently, malaria affects one-fifth of the world population. Current control methods rely heavily on the use of insecticides. However, insecticide resistance traits are starting to arise and spread in mosquito populations, undermining the effort put into malaria control. It is therefore urgent to develop new strategies that target mosquitoes to reduce malaria incidence. It has been demonstrated that for a control strategy to be efficient, it is essential to develop it taking into account the ecology, biology, and behaviour of the vector. The majority of the studies conducted on mosquito host-seeking have focused on the initial phases of this behaviour, while short-distance and landing behaviour have been neglected. This project arises from the need to better understand the last phases of host-seeking behaviour and the cues that elicit it.

3. GENERAL METHODS

3.1. Mosquito colony

A colony of *An. coluzzii* mosquitoes was established at the Natural Resources Institute (NRI), University of Greenwich, Kent, UK, in 2017. The original mosquitoes derived from eggs provided by the Institut de Recherche en Sciences de la Santé, Burkina Faso, from a colony established the same year from wild gravid females collected in Vallée du Kou (Bama, southwestern Burkina Faso, 11°23'14"N, 4°24'42"W) while at rest in inhabited human dwellings. Filial (F_1) females were placed individually in cages and provided with oviposition dishes. After oviposition, they were identified to species level by routine polymerase chain reaction (PCR) (Fanello *et al.*, 2002). Eggs were transported to NRI's laboratories for subsequent rearing. The colony was maintained in a climate-controlled insectary at 26 ± 2 °C and 60 ± 10% RH. The photoperiod was set with a 12:12 h LD cycle. All water used for rearing purposes was an isotonic water solution of 0.1% aquarium salts (Tropic Marin®, Germany), made by mixing 10 L of deionised water with 10 g of aquarium salt.

For experiments carried out in Chapters 4 and 5, adult females of five to ten days old were offered a human blood meal (i.e. an arm was inserted in the cage) for 15 min at the start of the scotophase for two non-consecutive days with one day of rest in between. Twelve hours after the second blood meal, egg dishes were placed in adult cages to allow oviposition. Egg dishes consisted of moist filter paper (9 cm of diameter) placed on isotonic water-soaked cotton wool, which was laid on a plastic dish (10 cm of diameter). After 48 h, eggs were transferred to larval trays (15.5 cm x 15 cm x 27.5 cm) that contained 1 L of isotonic water solution. Non-hatched eggs were removed the following day and placed into a separate larval tray with the same water volume and were kept for two additional days, thus allowing for further hatching opportunities. After that time, non-hatched eggs were removed from the tray. Larvae were monitored daily and fed powdered organic baby rice (4-6+ months, Aptamil©, Netherlands) and fish flakes (TetraMin©, Tetra Werke, Germany) *ad libitum*. Four days after hatching,

larvae were separated into groups of approx. 100 individuals. Each group was put into a clean larval tray with 1 L of isotonic water solution. Pupae were separated daily using disposable plastic pipettes and placed in plastic cups (10 cm of diameter) containing approx. 100 mL of isotonic water. These dishes were transferred into adult cages, consisting of a metal wireframe (30 cm x 30 cm x 30 cm) covered with medical tube gauze. Each cage was placed inside a transparent plastic bag to maintain moisture inside the cage and provide additional containment in the event of an escape. For normal colony maintenance, each cage contained approximately 200 adult mosquitoes, however, when additional insects were required for experimental work, the density of mosquitoes in cages increased to approximately 400 adults per cage. A feeder containing a solution of deionised water with 10% sucrose was placed in each cage from which adults fed *ad libitum*. Feeders were replaced every five to seven days unless mould was noted on the surface of the feeder, in which case the feeder was immediately replaced. General rearing practice (e.g. cleanliness, general maintenance, colony management) was based on indications for *Anopheles* laboratory rearing in Benedict (2015).

For experiments carried out in Chapter 6, a new colony of *An. coluzzii* females fed on an artificial membrane-feeding system (Hemotek, UK) using defibrinated horse blood (TCS Biosciences Ltd, UK) was established. This colony derived from the *An. coluzzii* colony used in Chapters 4 and 5 and was reared in the same manner as described above, with the only variation being that mature females were offered the blood meal only via an artificial feeder, which each time was prepared with a new batch of defibrinated horse blood and was sealed with swine intestine membrane (i.e. sausage casing). The feeder unit was inserted in the cage and was kept in position for approx. 15 mins, whilst a person breathed on the cage. This adjustment was done as experiments in Chapter 6 involved the use of blood at different temperatures and therefore required the use of an artificial feeder that could be set to a wide range of temperatures. For rearing purposes, the feeder temperature was set to 35 ± 2 ºC. The colony was reared using the artificial feeding system for over 60 generations, which allowed the manipulation of feeding preferences as suggested by Lyimo and Ferguson

(2009). A satisfactory level of acceptance to feed on the artificial feeder was reached (i.e. no difference was noted in the number of eggs collected in the colony reared on human blood and in the colony reared with the artificial feeder).

Adult mosquitoes reared and kept for experiments were maintained in lightboxes set to 12:12 h LD cycles, with transitions set to occur at different times during the working day, so that assays could be performed throughout the day while ensuring each cohort of mosquitoes used in the experiment was always in the first three hours of their scotophase. Mosquitoes were transferred to the lightboxes when collected as pupae, so that prior to being used for the experiments they were exposed to the new light-cycle for at least three days, which proved to be sufficient in *An. gambiae* to completely reset the circadian rhythm (Jones *et al.*, 1967). Humidity and temperature conditions in the lightboxes were regularly checked with a USB Data Logger (EL-USB-2-LCD, Lascar Electronics, UK) and found to be similar in all lightboxes and matched the conditions of the main mosquito-rearing laboratory. Lighting in the boxes was provided by strips of LEDs (4100058-WW, LE Lighting ever, UK). The illumination level was measured with a light meter (LUX Meter LX101, Lutron Electronic, USA) and set to be equal to light levels in the laboratory room. Thus, conditions were consistent in all rearing environments.

3.2. Wind tunnel

One way to assess the behaviour of an organism is to study the organism directly in its natural environment. However, it is very challenging to observe and analyse mosquito behaviour directly in the field (Cardé and Gibson, 2010). Thus, it is important to develop laboratory assays that reproduce some aspects of the environment in which mosquitoes live to obtain observations that reflect more faithfully their natural behaviour.

Small arenas pose external constraints on mosquito behaviour and therefore might influence their overall response to presented stimuli. Thus, the experiments described in Chapters 4 and 5 were carried out in a large wind tunnel (Fig. 14) to allow mosquitoes to execute flight manoeuvres (Kennedy,

1977). The flight arena (1.2 m wide x 1.2 m high x 2 m long) was kept at 25 ± 2 °C and 65 ± 5% RH. The air was drawn in by an impelling fan (Fischbach GmbH, Germany) from outside the building, to avoid using room air where human emanations can accumulate (Clements, 1999). Before entering the experimental arena, the air was purified by passage through activated charcoal filters to remove the majority of organic compounds (Fig.15). The background level of carbon dioxide was periodically assessed with a carbon dioxide meter (EGM-4 Environmental Gas Monitor for carbon dioxide, PP System, USA) in the most downwind part of the wind tunnel prior to the commencement of the assays. The concentration was found to be between 403 and 553 ppm, with a mean of 457.58 ± 10.13 ppm (n=19).

After passing through the filters, the air was warmed and humidified using a 2 kW fan heater (Glen, UK) and atomising humidifier (Hydrofogger, USA) (Fig. 15). Both the heater and the humidifier were controlled by a thermo-hygrostat (ReptiZoo, model THC09, China) which was set at the desired temperature and humidity. The air was then forced to pass through a screen of brushed cotton (Fig. 14), which created a laminar airflow into the flight arena (Hawkes, 2013). The wind speed was measured at the upwind end of the wind tunnel with an anemometer (PSI Prosser Scientific Instruments, model AVM501TC, UK) and found to be less than 0.2 m/s, a velocity congruent with similar experimental setups (Beeuwkes *et al.*, 2008; Spitzen *et al.*, 2013; Hawkes and Gibson, 2016).

During experiments, mosquitoes were released at the downwind end of the wind tunnel from a release cage (15 cm x 15 cm x 15 cm), which was possible to open and close from a distance of about 1 m from the wind tunnel (Fig.15). This allowed for the cage to be operated without influencing mosquitoes' behaviour, i.e. without breathing close to them or jolting the cage. The release cage was at the centre of the X-axis (Fig. 14), 60 cm from the lateral walls of the flight arena and at approx. 35 cm from the wind tunnel floor (Fig. 15).
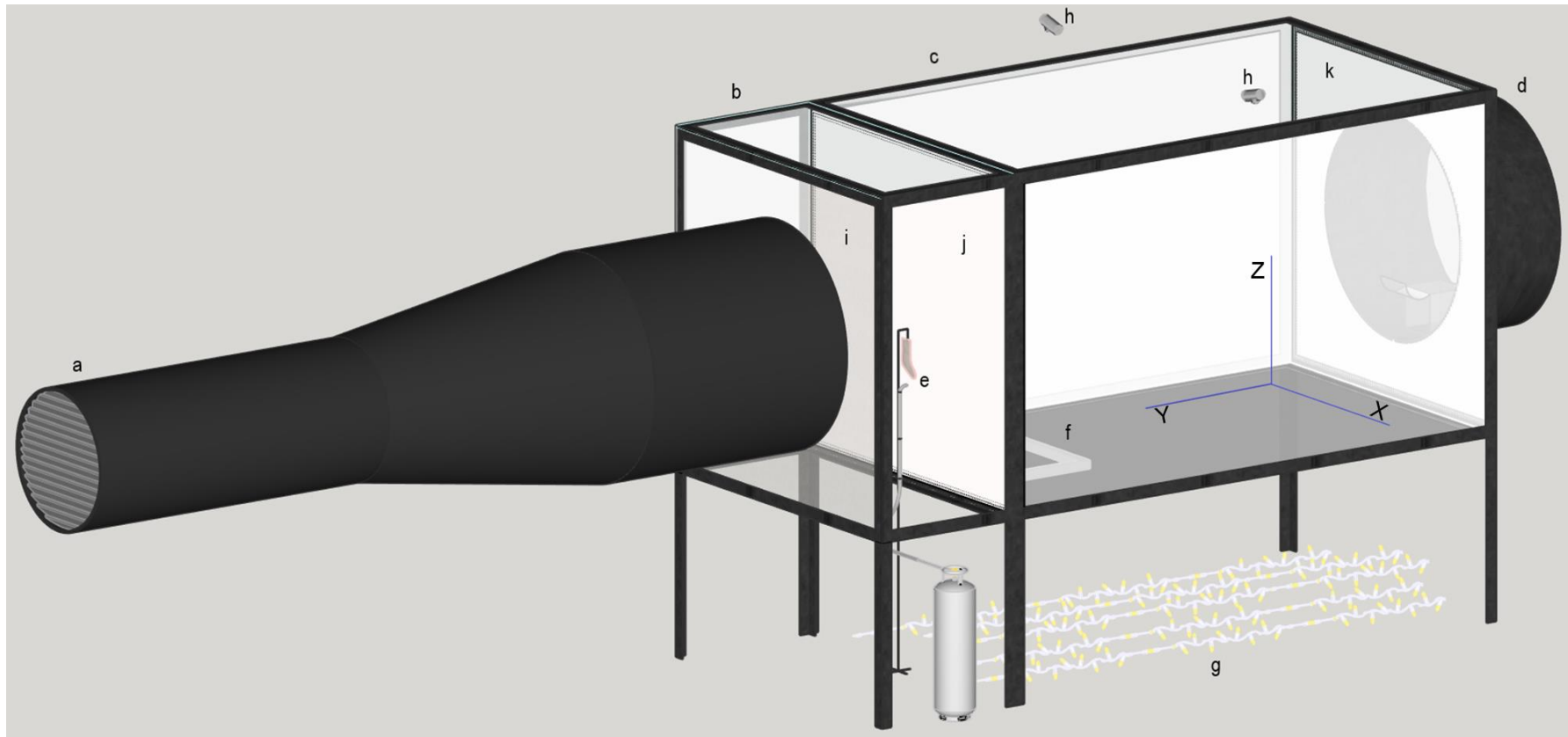
Figure 14: Schematic view of the wind tunnel. *The diagram shows the three axes (X, Y, and Z) and the main components: (a) air duct system which draws the air from outside the building, (b) odour release chamber, (c) large flight arena (1.2 m wide x 1.2 m high x 2 m long) which permits the mosquitoes to fly and respond to host stimuli, (d) mosquito release section, (e) worn socks and carbon dioxide release tube, (f) landing target, (g) array of LED in the visible spectrum, (h) video cameras, (i) brushed cotton screen, (j) upwind white net screen, (k) downwind white net screen (drawn by author using SketchUp 3D modelling software and Paint 3D).*
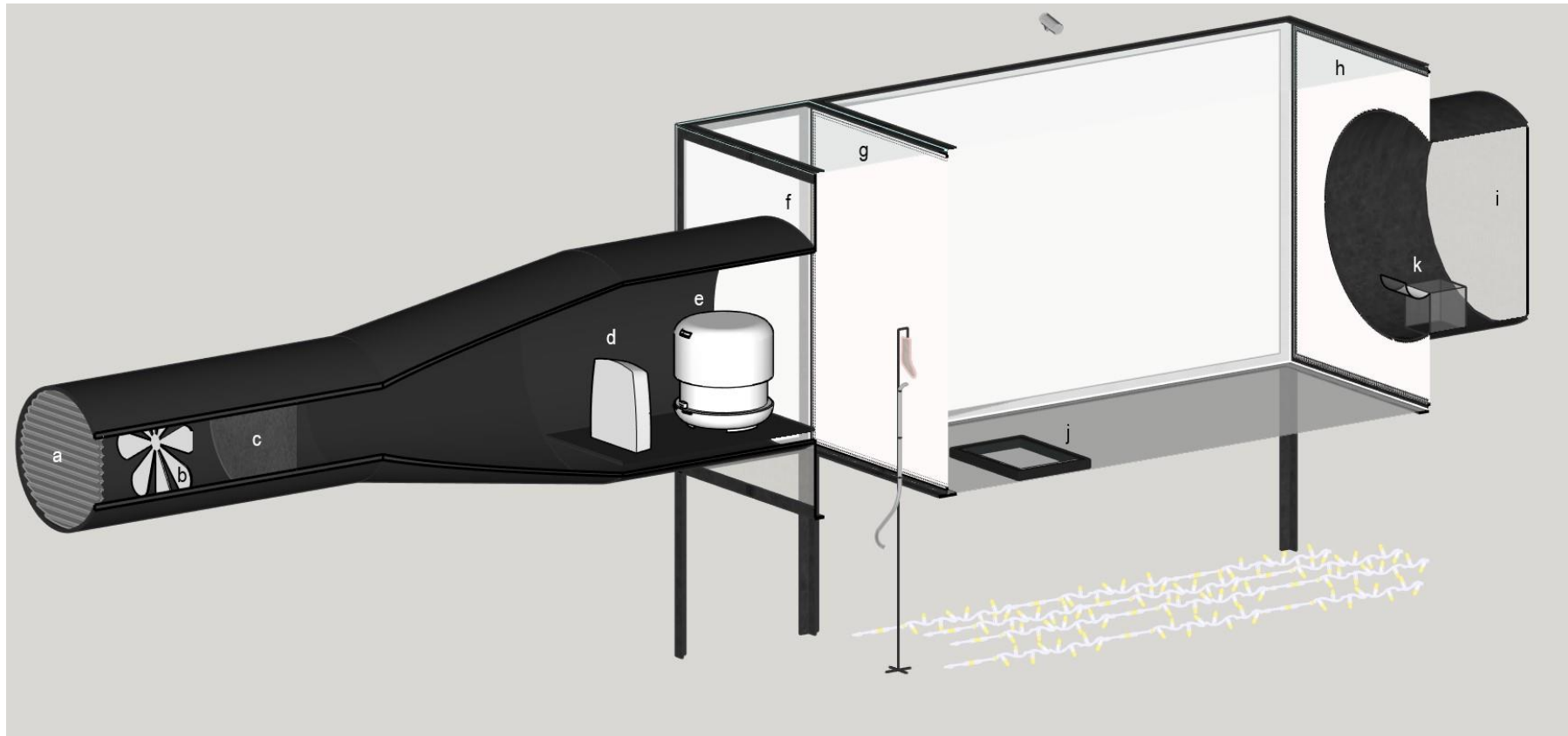
Figure 15: Schematic view of transverse section of the wind tunnel. *The internal components of the wind tunnel: (a) shutter, (b) impelling fan, (c) charcoal filters, (d) fan heater, (e) atomising humidifier, (f) brushed cotton screen, (g) upwind white net screen, (h) downwind white net screen, (i) terminal downwind netting, (j) landing surface, (k) release cage (drawn by author using SketchUp 3D modelling software and Paint 3D).*

The assays were carried out in the period when *An. coluzzii* are most active (i.e. during the first three hours of the scotophase) (Hawkes *et al.*, 2012). During the experiments, the lights of the laboratory room were turned off and the only source of illumination consisted of a series of warm white LEDs (Kontsmide, Sweden) which were homogeneously placed on the laboratory floor, below the floor of the flight arena. The lights employed were the same used by Hawkes (2013), which provided a homogenous light level of 0.001 W/m^2 of visible light in the range between 420 and 680 nm, which is similar to full moonlight illumination. The wind tunnel walls and floor consisted of panels of opal Perspex (The Plastic Shop, UK), while the ceiling was made of a transparent Perspex panel (The Plastic Shop, UK).

To permit mosquitoes to orient themselves and navigate using the optomotor mechanism, nine small visually conspicuous squares (10 cm per side) and two large squares (20 cm per side) were placed randomly on the floor of the wind tunnel (Fig. 16). The squares consisted of IR transmitting black-coloured plastic (Instrument Plastic Limited, UK) which appears black to both human and mosquito eyes, i.e. it absorbs all the waves in the visible spectrum, but permits IR light to pass through. This special material was used as the 3D tracking system employed in Chapter 4 required the background (i.e. the walls and floor of the arena) to be of a similar shade of colour. This was necessary to allow sufficient contrast between the dark silhouette of the mosquitoes and the background so that mosquitoes could be distinguished by the IR sensitive cameras. More details on the setup of the 3D tracking system are reported in section 5.2.6.
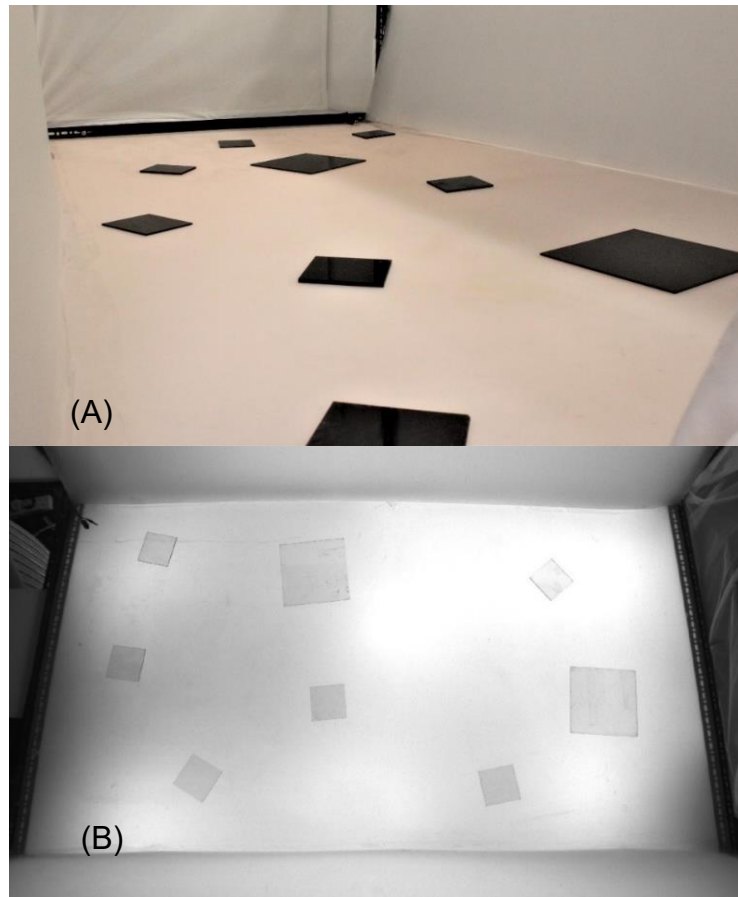
Figure 16: Black IR transmitting plastic squares were positioned randomly on the panel floor of the wind tunnel. This system allowed to create a visually conspicuous pattern over the white panel floor. The photo above (A) was taken with a camera in normal daylight equivalent conditions, while the photo below (B) was captured with IR sensitive cameras with IR illumination coming from below the wind tunnel floor. In this case, the markers appear only of a light grey colour as they allow the IR light to pass through.

3.3. Randomisation of the treatments

Throughout the experimental chapters, the treatments examined for each experiment were tested in a quasi-randomised manner, where the order of the treatments was chosen with the randomised function in Excel (version 1910, Microsoft Office, USA) that accounted for the following restrictions: firstly, the same treatment could not be repeated more than twice on an experimental day; secondly, the exact order of treatments performed on one day could not be repeated for at least four experimental days, meaning that the order of the treatments was always different for each experimental week.

This was done to ensure that each treatment was carried out with equal representation of mosquitoes deriving from the different light cycles, and also to ensure that treatments were not repeated at the same time in different working days, thus excluding any possible temporal effect or any effect caused by differences deriving from the specific conditions of each cage.

3.4. Data analysis and statistics

Statistical analyses were performed using RStudio (versions comprised from 3.6.0 to 4.1.2, R Development Core Team, 2013). Plots were produced using R (versions comprised from 3.6.0 to 4.1.2, R Development Core Team, 2013) and Excel (version 1910, Microsoft Office, USA). The R packages used were "corrplot" for plotting residuals (Wei *et al.*, 2021), "gplots" for plotting charts (Warnes *et al.*, 2016), "lme4" for linear mixed effects models (Bates *et al.*, 2015), "MASS" for generalised linear models (GLM) (Venables and Ripley, 2002), "multcomp" for Tukey's multiple comparisons (Hothorn *et al.*, 2008), and "vegan" for principal component analysis and redundancy analysis (Oksanen *et al.*, 2019).

Throughout the thesis, prior to the use of an ANOVA test, data sets were assessed for normality and homogeneity of variance using respectively a Shapiro-Wilk test on residuals and Barlett's test. Where these tests resulted in a P-value < 0.05, a graphical assessment was carried out to confirm compliance with ANOVA's assumptions. Likewise, prior to using a GLM, different distributions were tested and, in each case, the distribution that fit best (i.e. with residual deviance levels deemed satisfactory) was chosen. Multiple comparisons between treatments were done using a Tukey's *post hoc* tests, which allowed corrections of P-values against inflation of Type I errors. The Tukey's *post hoc* tests were run on the model (i.e. the GLM or the linear model) where the analysis indicated a significant difference between treatments. Thus, the produced t-values reported for each Tukey's *post hoc* test are not the result of t-tests conducted on the raw data.

4. QUANTIFICATION OF THE LANDING BEHAVIOUR OF *ANOPHELES COLUZZII* MOSQUITOES IN RESPOSE TO HOST-ASSOCIATED STIMULI [1]

4.1. Introduction

Long-range detection and orientation of female mosquitoes towards hosts has been widely investigated, especially with regards to responses to different odorants (Zwiebel and Takken, 2004). However, little research has been conducted to thoroughly identify the role played by different host cues in eliciting orientation and landing in the last phase of host-seeking behaviour (Gibson and Torr, 1999; Zhou *et al.*, 2018). Cribellier *et al.* (2018) suggested that the absence of short-range cues could drive mosquitoes that are flying towards an object to actively fly upwards as a form of avoidance behaviour. The addition of short-range and landing cues promotes a close approach of mosquitoes to traps or treated surfaces, thus increasing the effectiveness of these control methods. Consequently, there is an impelling urge to gain a better understanding of the events that take a mosquito to land. Published studies present only the response to one or two stimuli at a time. Furthermore, the vast majority of the studies that examined the response to physical close-range cues (e.g. heat, humidity) were carried out with the diurnal *Aedes* mosquitoes, which display some differences in their behaviour compared to *Anopheles* mosquitoes (Baik *et al.*, 2020). Little is known on the effect of close-range cues on malaria vectors. Thus, it is imperative to explore this field in order to have a better understanding on malaria vector behaviour for the construction of more targeted control interventions.

In proximity to the host, apart from the host odour, female mosquitoes use other stimuli such as visual, tactile, temperature, and humidity gradients to orient towards and finally land on a surface (Costantini *et al.*, 1998). In laboratory studies, visually conspicuous surfaces have been demonstrated to attract host-seeking females of *An. coluzzii* (Hawkes and Gibson, 2016) and *Ae. aegypti* (Van Breugel *et al.*, 2015). However, in both these cases, the mosquitoes were recorded to hover above the surface without alighting on it. This suggests that the experimental objects lacked other important stimuli that may be required for triggering landing behaviour. Field trials that studied the attractiveness of visually conspicuous surfaces indicated that traps with high contrast compared to the environment are more successful in catching female mosquitoes (Haufe, 1964; Hawkes *et al.*, 2017a).

It is widely accepted that thermal and humidity gradients are important cues used by mosquitoes during the close-range and landing phase (Khan and Maibach, 1966; Khan *et al.*, 1968). After the discovery of thermoreceptors in mosquito's antennae (Davis and Sokolove, 1975; Wang *et al.*, 2009) and proboscis (Maekawa *et al.*, 2011), and hygroreceptors in mosquito's antennae (Bar-Zeev, 1960; Kellogg, 1970; Meijerink *et al.*, 2001), laboratory studies were carried out to determine the reactions of host-seeking mosquitoes in response to thermal and humidity cues presented alone (Kellogg and Wright, 1962a, 1962b; Wright and Kellogg, 1962, 1964; Khan and Maibach, 1966) and in combination with host odour cues (Khan and Maibach, 1966; Khan *et al.*, 1968; Eiras and Jepson, 1994; Takken *et al.*, 1997b). However, only recent studies conducted using tracking systems offered a broader understanding of mosquito behaviour when stimulated with thermal cues (Healy *et al.*, 2002; Spitzen *et al.*, 2013; McMeniman *et al.*, 2014; Van Breugel *et al.*, 2015). Nonetheless, little is known about the specific range of temperatures that elicit landing behaviour in *Anopheles* mosquitoes. A study conducted on *Ae. aegypti* reported that the number of mosquitoes alighting on a heated Peltier plate increased monotonically with the increase of the Peltier plate temperature, which ranged from 28.5 °C to 40 °C, while an avoidance behaviour was registered when the plate was heated at temperatures > 45 °C (Corfas and Vosshall, 2015). Similar

results were obtained with two species of *Culex* mosquitoes that also responded with a monotonical increase in the landing rate on plates heated from 30 °C to 45 °C, with the highest landing rates obtained at 40 °C and 45 °C (Reinhold *et al.*, 2022). Yet, information on the thermal range that elicits landing behaviour in anopheline species is limited. Healy *et al.* (2002) described how *An. gambiae* females displayed a landing behaviour that was dependent on the target temperature, showing a preference for targets set at human temperatures ± 2 °C. However, no further studies have confirmed these results or have explored these response in other *Anopheles* species.

When studying the behavioural response of an insect it is crucial to keep in mind that the measured response is, with the exception of basic reflex responses, the result of a complex integration of several stimuli (Wessnitzer and Webb, 2006). The integration process allows the insect to generate flexible and context-dependant behaviours. In particular, some behaviours such as pattern recognition and avoidance, and spatial navigation require the integration of information from many sensory modalities (Wessnitzer and Webb, 2006). For example, it has been proposed that visual and odour information is integrated in odour-seeking *Drosophila* flies (Gilbert and Kuenen, 2008). This was then supported by the results of laboratory studies, in which fruit flies were only able to remain headed into the odour plume and therefore to find the odour source if stimulated with appropriate visual cues (Duistermars and Frye, 2008, 2010). Similar results were reported in a previous study based on moths (Vickers and Baker, 1994). For mosquitoes, a few studies have examined multimodal integration of information in host-seeking females (McMeniman *et al.*, 2014; Corfas and Vosshall, 2015; Van Breugel *et al.*, 2015; Liu and Vosshall, 2019; Vinauger *et al.*, 2019). However, most of these studies investigated the attraction and not the landing response, thus little is known on the integration mechanism that leads to alighting. Furthermore, the role of singular stimuli in driving landing behaviour has not yet been quantified. The difficulty in assessing the exact role of single stimuli lies in the fact that in a multimodal integration mode, a response might be generated only when two or more stimuli are presented, or the response itself might change its characteristics, e.g. velocity of response, precision of

response, etc., when a combination of stimuli are presented. Thus, in this study, a multimodal step approach was adopted, where stimuli were presented first alone, then as a combination. This allowed to have an overview of the effect of single stimuli and allowed to determine how the effect changed as a result of multimodal integration. The study presented here explored the effect of host odour, thermal gradient, and visual contrast, i.e. three host-associated stimuli, in eliciting landing behaviour in *An. coluzzii* host-seeking females. As indicated by Vale (1993), to assess ways of improving a trap is important to divide the overall stimulus of the trap into its constituents and examine them separately. Thus, an effort was put into quantifying the effect of the stimuli when presented alone and when presented as combinations. The following hypotheses were set out:

- H_0: The proportion of activated mosquitoes is not different in assays with and without the addition of host odour.
  H_1: The proportion of activated mosquitoes is higher in treatments where host odour is offered compared to treatments where host odour is absent.

- H_0: The number of mosquitoes landing on the downwind half of the target is the same as the number landing on the upwind half of the target.
  H_1: The number of mosquitoes landing on the downwind half of the target is greater than the number landing on the upwind half of the target.

- H_0: There is no difference in the number of mosquitoes that land in assays where all three host-associated stimuli are presented simultaneously compared to assays where the stimuli are presented alone or in pairs.
  H_1: The number of mosquitoes that land is greater in assays where all three host-associated stimuli are presented simultaneously compared to assays where the stimuli are presented alone or in pairs.

- H_0: The overall effect of two or more host-associated stimuli simultaneously presented is given by the sum of the individual effect of each stimulus.
  H_1: The overall effect of two or more host-associated stimuli

simultaneously presented is greater than the sum of the individual effect of each stimulus.

The explanation behind the second hypothesis lies in the fact that, if the stimuli that drive the landing phase elicit an immediate response, i.e. the mosquito lands immediately on the first adequate surface, then when the target was presented with visual and thermal cues, the first adequate landing surface that the mosquitoes encountered is the downwind half of the target. Thus, if the landing response is immediate, the number of mosquitoes recovered on the downwind half of the target should be greater. To the author's best knowledge, this is the first study that evaluates and quantifies the effect of different surface temperatures, visual, and odour cues in triggering landing behaviour in *An. coluzzii* host-seeking females.

4.2.   Materials and Methods

4.2.1. Mosquitoes

Mosquitoes were reared as indicated in section 3.1. Sugar feeders were removed from adult cages four to five hours prior to the commencement of assays, with the exception of nine cases where the sugar feeders were removed one to three hours prior to the experiment.

Each experimental group consisted of about 25 female mosquitoes, all randomly selected from one cage and therefore, all being of the same age. The mosquitoes used were between four and twelve days old. This was based on Sheppard *et al.* (2017) work, as they used four to ten days old *An. gambiae* females, which they assumed were inseminated and ready to host-seek. The age of the mosquitoes used in each replicate was recorded. The mosquitoes were collected between one and two hours before assays started using a mouth-aspirator and were then kept in small WHO test tubes (4.5 cm of diameter and 12 cm of height) in darkness to allow eye adaptation to darkness, i.e. increase in pupil width and increase of light sensitivity (Sato, 1957; Moon *et al.,* 2014). The assays were

carried out in the period when *An. coluzzii* was found to be most active (i.e. during the first three hours of the scotophase) (Hawkes *et al.*, 2012).

4.2.2. Landing target

To test landing response on a surface that could incorporate different combinations of thermal and visual stimuli, a landing target was designed based on a transparent heated glass unit (E-GLAS sample, Saint Gobain, UK). The unit (3 cm x 40 cm x 30 cm) consisted of a sheet of transparent glass which converts electrical energy to heat and can reach a maximum temperature of 50 °C.The landing target was positioned horizontally on the floor of the flight arena, at the centre of the X-axis (40 cm from the lateral walls) and 30 cm from the upwind end of the flight arena (Y-axis).

To capture mosquitoes landing on the target, the surface was covered by a layer of transparent adhesive film (FICSFIL, Barrettine, UK) (Fig. 17), which consists of a transparent plastic sheet coated with a transparent layer of strong glue. The layer of adhesive film was replaced every eight to ten assays to avoid the accumulation of mosquito's body parts and possible retention of chemical signals emanated by caught mosquitoes.

Thermal properties

For experimental purposes, the surface temperature of the landing target was controlled using a probe and thermostat (ReptiZoo, model THC09, China) which maintained the desired temperature (± 1 °C). To maximise the contact between the glass surface and the probe, and therefore to achieve a highly accurate temperature reading, a layer of heat sink paste (RS Components, UK) was interposed between the two surfaces (Fig. 17). Thus, a range of experimental temperatures could be achieved. The temperature of the landing target was recorded with an IR laser gun thermometer (Beha-Amprobe IR-710, Germany) both prior to the commencement and at the end of each replicate. The surface was thoroughly scanned in a clockwise circular motion, starting always from the same point, and the minimum and maximum temperatures of the surface were

recorded. This confirmed that the entire surface of the landing target maintained a temperature of within ± 2 °C from the desired experimental temperature.

Visual properties

Visual properties of the target could also be controlled; the glass could be either left transparent to provide a very low contrast stimulus or made highly visually conspicuous with the addition of black IR transmitting plastic tiles secured underneath the glass (Fig. 17). It has to be taken into account that objects with different visual characteristics (e.g. transparent or black) might also have different thermal properties, i.e. they might absorb and transmit heat differently. To limit this, in this study, the black panels that provided the visual cue were positioned underneath the target where they were not heated, as the target only emitted heat from its upper surface. Thus, convection currents produced by the upper heated surface of the target were unlikely to be different, as the part that produced and maintained heat was unaltered in both target types.

Figure 17: Experimental landing surface. On the left, the surface is transparent while on the right, the same surface is made visually conspicuous with the addition of black tiles. The edges of the surface were covered in white tape to camouflage them (i.e. white tape over white background). From both settings, the probe of the thermostat can be seen laying on top of the surface. A layer of heat sink paste was interposed between the probe of the thermostat and the glass surface to achieve a highly accurate reading of the temperature. The cables of both the thermostat and the transparent heater were also covered in white tape to disguise them over the white background. At the top of both images, the arrows indicate the reflection of the transparent adhesive film that could be seen under normal laboratory lighting; these reflections were not present under experimental lighting conditions.

4.2.3. Odour treatment

The odour treatment consisted of a combination of human foot odour and carbon dioxide. Human foot odour was sourced from 100% polyamide nylon socks (15

deniers sheer knee-high socks, Wilko, UK) worn by the author for 24 h. To limit changes in body odour, the socks were worn by the same person (Pates *et al.*, 2001) and with the same pair of shoes throughout the experiment. Prior to wearing the socks, the author washed her feet with water and fragrance-free soap (Baby Wash Unfragranced, Childs Farm, UK). Throughout the duration of the experiment, the author abstained from eating food with spices and alcohol, and using perfumes, strong perfumed-soaps and clothes detergents as these substances can affect human body odour (Shirai *et al.*, 2002; Lefèvre *et al.*, 2010; Verhulst *et al.*, 2011b). Even though it has been demonstrated that the effect on mosquitoes of human body odour collected on socks remains active for several weeks after collection (Zwiebel and Takken, 2004), in preliminary experiments it was noticed that after seven days of use, the mosquitoes did not respond to the socks. Thus, it was decided to offer a new pair of socks in each experimental week. When not in use, the socks were kept in a sealed zip-lock bag at -20 °C to minimise variation of the odour components (Beeuwkes *et al.*, 2008; Jawara *et al.*, 2009). The socks were presented at the upwind end of the wind tunnel in the odour delivery chamber, ~5 cm behind the netting, in the centre of the X-axis (at 60 cm from the lateral walls) and at 40 cm above the arena floor (Fig. 18).
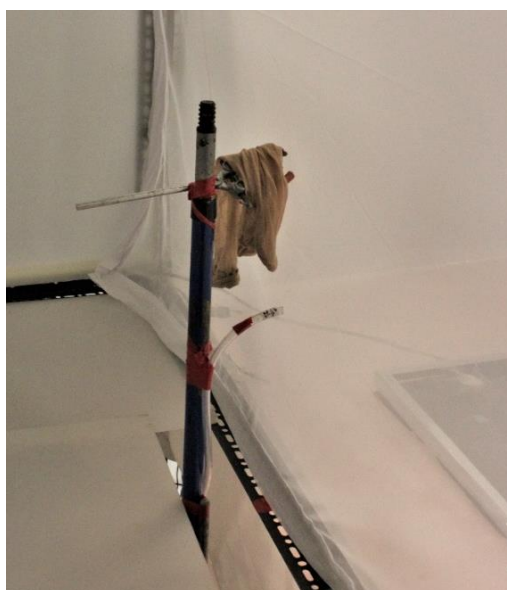
Figure 18: Odour delivery system at the upwind side of the flight arena. *A pair of worn socks was hung at 40 cm from the floor of the wind tunnel, while the tube that delivered the carbon*

dioxide was positioned at 25 cm above the wind tunnel floor. Both stimuli were positioned at a distance of about 5 cm from the net.

In addition to human body odour, carbon dioxide was also presented in the odour treatments. This was presented as a constant flow of approx. 4.5% concentration at a flow rate of 5 L/min, as this was shown to be approximately the concentration in human breath (Pates *et al.*, 2001) and that elicited behavioural responses in the form of upwind surges in host-seeking mosquitoes (Dekker and Carde, 2011). To achieve this concentration, the 100% carbon dioxide source was mixed with the room air and pumped through a Dreschel bottle containing deionised water to humidify and mix the gases. The tube that released carbon dioxide was positioned in the centre of the X-axis (at 60 cm of distance from the lateral walls) and at 25 cm of height above the wind tunnel floor (Fig. 18).

4.2.4. Experimental procedure

The mosquitoes were transferred into the release cage by simply opening one end of the WHO test tube and allowing the mosquitoes to exit the tube. Each group consisted of approx. 25 mosquitoes and was given five to ten minutes to habituate to the wind tunnel environment, after which the release cage was gently opened from a distance to avoid disturbing the mosquitoes, i.e. avoid causing an escape response, and to avoid introducing human odours into the arena. In assays requiring it, the odour treatment started immediately after the release cage was opened. After 30 min, the assay was terminated, the release cage was closed, and the number of mosquitoes recovered in different parts of the wind tunnel were counted as follows: number of mosquitoes stuck to the landing target (N_t), number of mosquitoes caught in the wind tunnel beside the release cage and the landing target (N_w), and number of mosquitoes in the release cage (N_c). The target surface was divided into four equal quadrants (Fig. 19) and the number of mosquitoes caught on each quadrant was recorded for each replicate. This was done to assess whether the presence of the thermostat's probe, which was always placed on the left side of the target on quadrant 3, influenced the landing response, as well as to determine whether

mosquitoes landed more on the downwind side compared to the upwind side (second hypothesis).
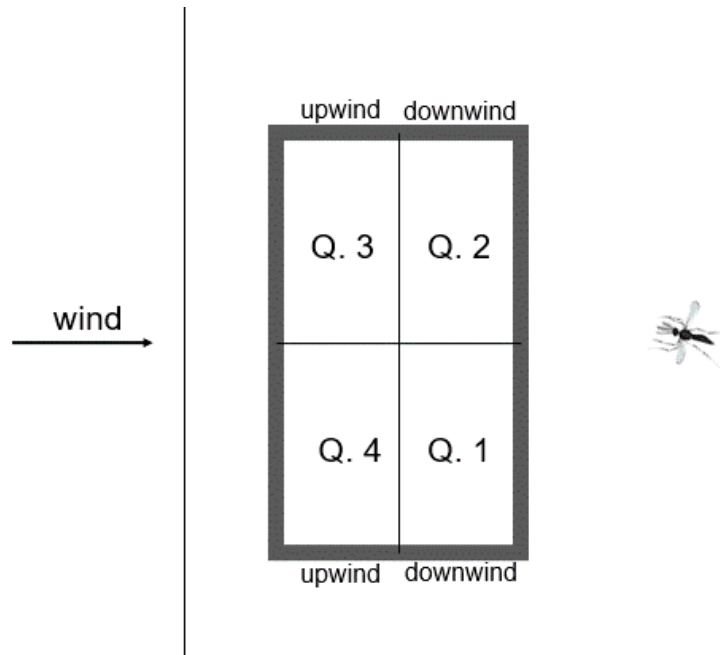
Figure 19: Landing target surface divided into four equal quadrants. Quadrants 1 and 2 are downwind compared to quadrants 3 and 4, which are upwind. The arrow indicates the direction of the wind and the point where the carbon dioxide was released. Thus, the notation "upwind" and "downwind" refers to the position of a section of the target with respect to the release cage. Note: figure not to scale (drawn by author).

At the end of each replicate, the atmospheric pressure (i.e. proxy of weather) recorded by a barometer on the University campus was noted.

Each experimental group was subjected to a single treatment. In total, twelve different treatments were tested (Table 1), each of which consisted of a combination of the following cues:

- Visual cue: either a transparent target surface or a visually conspicuous target surface (i.e. solid black).

- Thermal cue: the target surface was set at either 25 °C, 35 °C, or 45 °C. The middle temperature in this range was chosen to reflect the temperature of a human host, as this is comparable with human skin temperature (Healy and Copland, 1995; Menger *et al.*, 2014). The lowest

temperature corresponded to ambient temperature, and therefore acted as the negative control, while the highest temperature represented a high extreme temperature.

- Host odour cue: a combination of human foot volatiles and constant carbon dioxide at 4.5% concentration was either present or absent.

A minimum of ten replicates were carried out for each treatment. The treatments were tested in a quasi-randomised order (see section 3.3), between and within days, to exclude the effect of testing sequence.

Table 1: Summary of the different treatments tested.

Treatment	Visual cue	Thermal cue	Odour cue
1 (negative control)	Transparent	25 °C	Absent
2	Transparent	35 °C	Absent
3	Transparent	45 °C	Absent
4	Black	25 °C	Absent
5	Black	35 °C	Absent
6	Black	45 °C	Absent
7	Transparent	25 °C	Present
8	Transparent	35 °C	Present
9	Transparent	45 °C	Present
10	Black	25 °C	Present
11 (positive control)	Black	35 °C	Present
12	Black	45 °C	Present

Prior to the commencement of sets of replicates with no odour cue, the wind tunnel surfaces were washed with deionised water, then wiped with pure ethanol, and left to air dry. All fabric components of the wind tunnel (i.e. nettings and brushed cotton screen) were washed at high temperature with a fragrance-free detergent (Surcare, UK). Furthermore, clean surgical gloves were worn at all times when touching the equipment, to minimise contamination of human skin odour (Okumu *et al.*, 2010a; Spitzen *et al.*, 2013; Reinhold *et al.*, 2022).

4.2.5. Analysis and statistics

The number of mosquitoes considered activated into host-seeking behaviour (N_a) was designated as the number of mosquitoes found in the flight arena (N_w) plus the number found on the landing target (N_t), i.e. $N_a = N_t + N_w$.

Activation

A GLM with quasi-binomial errors and a logit link was used to analyse the proportion of activated mosquitoes over the total number of mosquitoes released. Visibility of the trap, surface temperature, and presence or absence of host odour were introduced into the model as factors. Mosquito age, length of starvation, length of darkness habituation period, and atmospheric pressure were analysed as covariates.

Landing

The landing analysis was carried out on the number of mosquitoes recovered on the target. A GLM with negative binomial errors and a log link was used to analyse the data. Residual deviance levels were satisfactory (residual deviance: 160, 111 degrees of freedom).

A three-way analysis of deviance was used to assess differences in the number of mosquitoes found on the target in different treatments. Multiple comparisons of means using Tukey's *post hoc* test were carried out to compare results from treatments that had different combinations of factors. Visibility of the trap, surface temperature, and presence or absence of host odour were introduced into the

model as factors. Mosquito age, length of starvation, length of darkness habituation period, and atmospheric pressure were analysed as covariates.

Landing position

A GLM with negative binomial errors and log link was used for the data collected. The difference in the number of mosquitoes landing on different quadrants was assessed using a one-way analysis of deviance. The analysis was carried out individually for each separate treatment in order to reduce the effect of different stimuli on the landing response. Where the comparisons with the analysis of deviance resulted in a significant difference, the differences between the number of mosquitoes that had landed on different quadrants were then further assessed with a chi-square test. This allowed to pair-compare the number of mosquitoes caught on different sections of the landing target.

Effect of the interaction of host-associated stimuli

To determine whether the effect of two or more stimuli was synergistic or additive, the observed results were compared with a hypothetical data set that reflected an additive effect. To do this, the recorded numbers of landings on treatments where two or more stimuli were presented together were compared with the predicted numbers of mosquitoes that were expected to land if each cue acted in an additive manner. The expected additive landing numbers were derived from assays where each singular component was tested, with these results added to give a predicted additive effect. For example, to create a simulated data point for the additive effect of all three cues presented together, the number of mosquitoes recovered from an assay where only the visual cue was offered was added to the number of landings from an assay where only odour was provided, and was then added to the number of landings from an assay where only the thermal cue was presented. To select which data points to use to create the simulated data set, the assays were grouped together according to closest chronological proximity. This limited the effects of potential covariates that fell outside of experimental control. In this way, a simulated data set of ten points was constructed for each of the four potential treatment combinations (i.e. a simulated data set for all three cues, for the visual plus odour

cues, for the visual plus thermal cues, and for the thermal plus odour cues). All data (recorded from experimental observation and predicted from the simulated data sets) were tested in a GLM with negative binomial errors and a log link. An analysis of deviance was used to assess if the recorded mean number of landed mosquitoes in treatments where cues were presented together was greater than the mean number of mosquitoes expected to land if the total effect was given by the sum of each component.

4.3. Results

4.3.1. Overview

A total of 139 assays were conducted using 3454 *An. coluzzii* females. After an initial screening, 124 replicates, for a total number of 3074 mosquitoes, were deemed satisfactory to be used in the statistical analysis. The remaining 15 replicates were discarded as some technical issues occurred during the experiment and the conditions were not maintained constant throughout the assay.

The results of the assays used in the analysis are summarised in Table 2. Throughout all the replicates in which the temperature of the landing target was set at 25 °C, the mean ± SEM temperature recorded on the target surface was 25.17 ± 0.09 °C. Similarly, for all replicates where the landing target was set at 35 °C, and for replicates where the target was set at 45 °C, the mean ± SEM temperature recorded on the surface was 33.99 ± 0.25 °C and 43.66 ± 0.33 °C, respectively.

Table 2: Summary of the data used in the statistical analysis. *Mean percentages (in brackets) reflect the mean proportion of mosquitoes found in the different parts of the wind tunnel at the end of each replicate. These were first calculated for each replicate, taking into consideration the exact number of mosquitoes placed in the wind tunnel at the beginning of the replicate, and then the percentages were averaged for each treatment.*

Treatment	N. replicates	Tot. mosquitoes released	Mean ± SEM number of mosquitoes released per replicate	Mean ± SEM number of mosquitoes activated	Mean ± SEM number of mosquitoes on landing target	Mean ± SEM number of mosquitoes in the release cage
No odour + 25 °C + transparent	10	246	24.6 ± 0.4	20.9 ± 1.09 (84.89%)	0.2 ± 0.13 (0.80%)	3.7 ± 1.00 (15.11%)
No odour + 35 °C + transparent	10	247	24.7 ± 0.60	18.0 ± 1.35 (72.44%)	0.4 ± 0.16 (1.57%)	6.7 ± 1.10 (27.56%)
No odour + 45 °C + transparent	10	250	25.0 ± 0.00	20.6 ± 0.83 (82.40%)	1.1 ± 0.59 (4.4.%)	4.4 ± 0.83 (17.60%)
No odour + 25 °C + black	10	251	25.1 ± 0.10	19.2 ± 1.11 (76.57%)	2.0 ± 0.82 (7.98%)	5.9 ± 1.16 (23.43%)
No odour + 35 °C + black	10	250	25.0 ± 0.00	18.8 ± 1.21 (75.20%)	1.7 ± 0.62 (6.8%)	6.2 ± 1.21 (24.80%)
No odour + 45 °C + black	10	243	24.3 ± 0.71	16.8 ± 1.63 (69.29%)	1.2 ± 0.42 (4.94%)	7.5 ± 1.63 (30.71%)
Odour + 25 °C + transparent	12	301	25.1 ± 0.23	17.6 ± 0.92 (70.19%)	4.8 ± 0.88 (19.14%)	7.5 ± 0.96 (29.81%)

Treatment	N. replicates	Tot. mosquitoes released	Mean ± SEM number of mosquitoes released per replicate	Mean ± SEM number of mosquitoes activated	Mean ± SEM number of mosquitoes on landing target	Mean ± SEM number of mosquitoes in the release cage
Odour + 35 °C + transparent	10	252	25.2 ± 0.13	21.7 ± 0.52 (86.20%)	13.3 ± 1.41 (52.77%)	3.5 ± 0.62 (13.80%)
Odour + 45 °C + transparent	10	252	25.2 ± 0.13	19.3 ± 1.07 (76.57%)	11.8 ± 1.24 (46.80%)	5.9 ± 1.04 (23.43%)
Odour + 25 °C + black	10	250	25.0 ± 0.00	19.8 ± 0.77 (79.20%)	10.4 ± 1.77 (41.60%)	5.2 ± 0.77 (20.80%)
Odour + 35 °C + black	10	240	24.0 ± 0.68	19.3 ± 1.08 (80.53%)	15.9 ± 1.05 (66.47%)	4.7 ± 1.00 (19.47%)
Odour + 45 °C + black	12	292	24.3 ± 0.38	19.3 ± 0.86 (79.61%)	15.2 ± 1.00 (62.67%)	5.0 ± 0.9 (20.39%)
Tot.	124	3074				

4.3.2. Activation

An overview of the number of mosquitoes activated in each treatment, and an overview of the distribution of the data is given in Fig. 20. The visual properties of the target did not significantly affect the activation behaviour of *An. coluzzii* females that were released in the assay (GLM, chi-square=0.32, d.f.=1, P=0.84). Similarly, the temperature of the target and the presence or absence of host odour did not significantly alter the activation behaviour of the mosquitoes released (GLM, chi-square=0.34, d.f.=2, P=0.57; chi-square=0.51, d.f.=1, P=0.48, respectively).
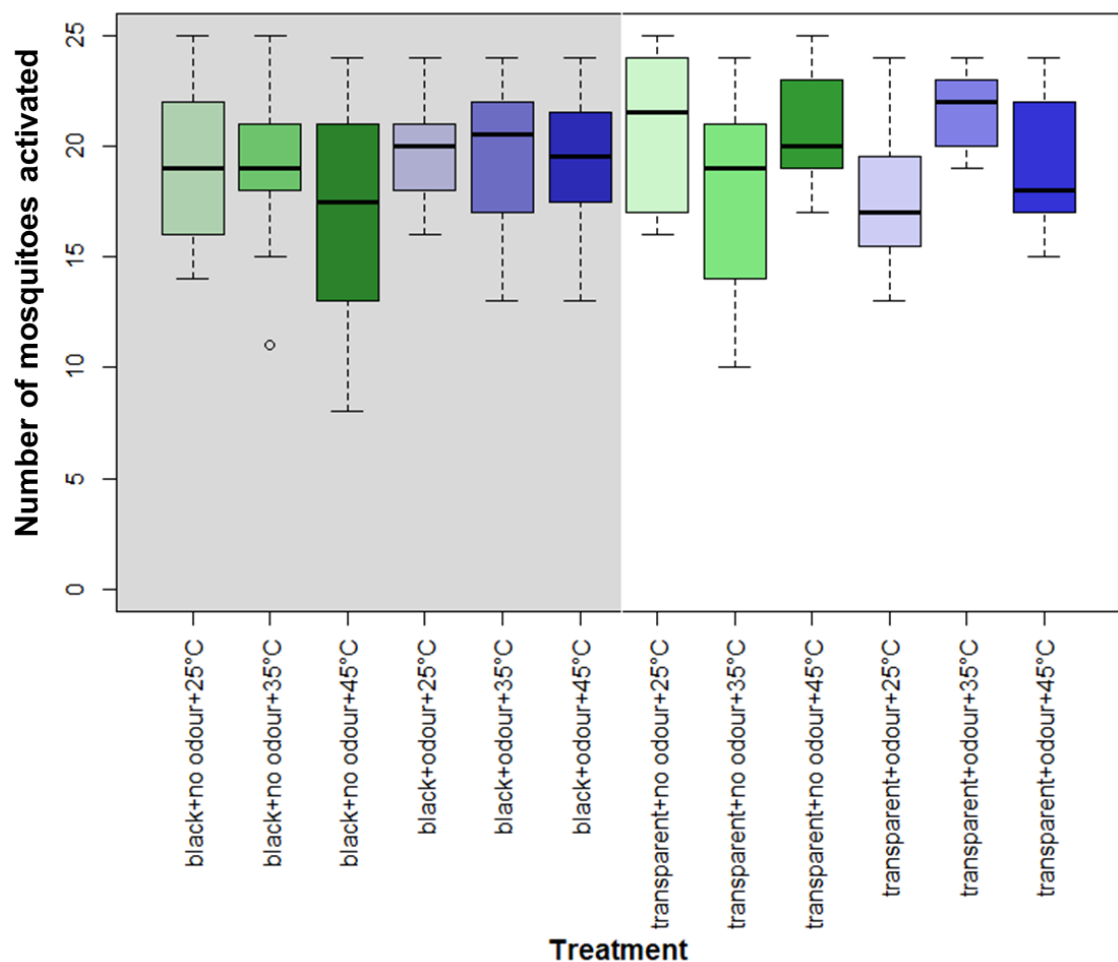
Figure 20: Number of mosquitoes activated at the end of the experiment for each treatment. *The black bars indicate the medians, the upper and lower limits of the boxes indicate the interquartile range. The whiskers indicate the maximum and minimum data points excluding outliers which are represented as small circles. The Y-axis shows the twelve different treatments,*

Age and starvation time, when introduced in the model as covariates, had no significant effect on the activation of the mosquitoes (GLM, chi-square=1.01, d.f.=1, P=0.314; chi-square=2.39, d.f.=1, P=0.12, respectively). Similarly, the atmospheric pressure (i.e. proxy of weather) did not affect the activation rate (GLM, chi-square=2.94, d.f.=1, P=0.09). However, the time spent in darkness prior to the experiment, i.e. either one or two hours, affected the number of mosquitoes that were activated during the assay, as a longer exposition to darkness prior to the experiment led to the recovery of more activated mosquitoes (for exposure of one hour: mean ± SEM=18.58 ± 0.40, for exposure of two hours: mean ± SEM=20.24 ± 0.49; GLM, chi-square=7.3, d.f.=1, P=0.006). This suggests that mosquitoes are more prone to be activated on the third hour of the scotophase, after being kept for two hours in darkness.

4.3.3. Landing overview

An overview of the number of mosquitoes that landed on the target in each treatment, including the distribution of the data, is given in Fig. 21.
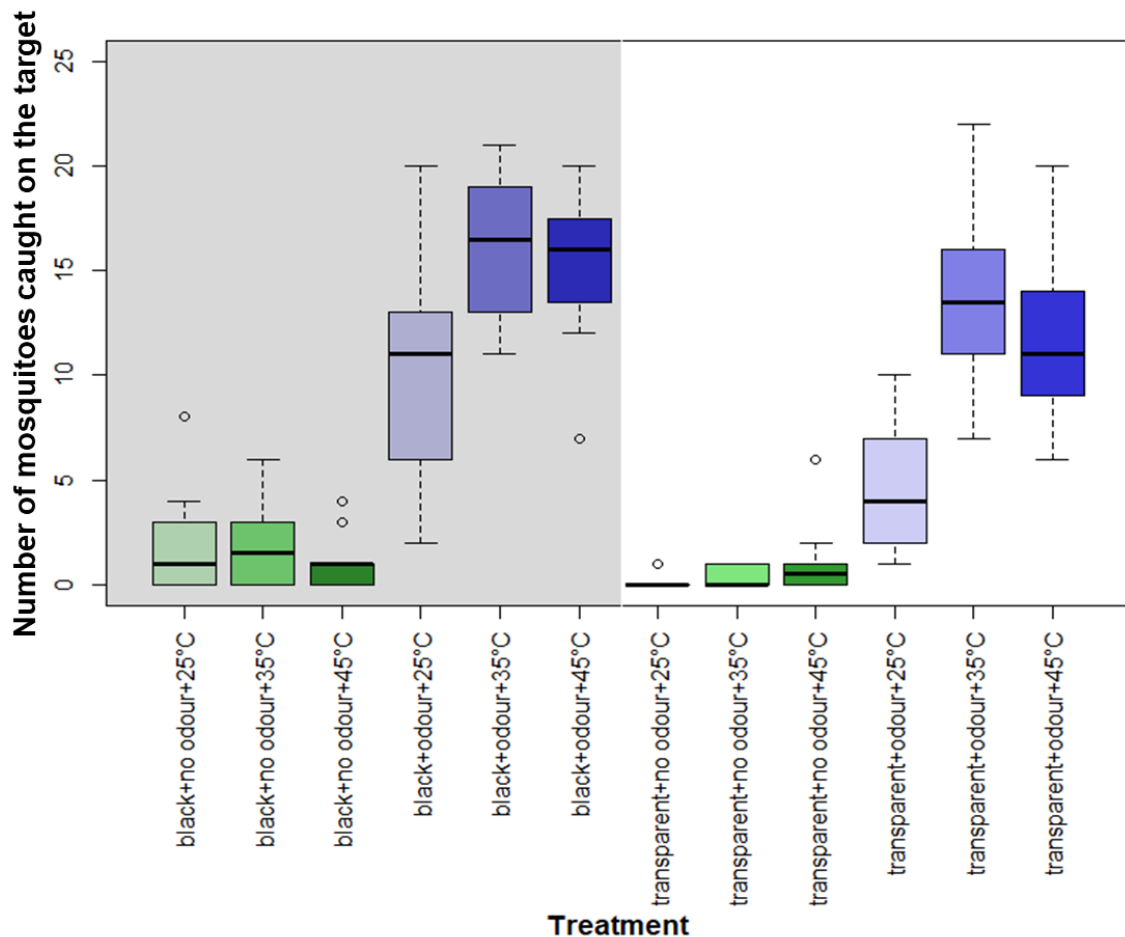
Figure 21: Number of mosquitoes found on the target surface at the end of the experiment for each treatment. *The black bars indicate the medians, the upper and lower limits of the boxes indicate the interquartile range. The whiskers indicate the maximum and minimum data points excluding outliers which are represented as small circles. The Y-axis shows the twelve different treatments, where "black" and "transparent" indicate the different visual cues offered, "no odour" and "odour" indicate the absence or presence of host odour, and the temperature indicates the thermal cue used. Blue bars indicate that host odour was present during assays, green bars indicate it was absent. Paler colours denote lower temperatures and darker colours indicate higher temperatures. The background colour indicates the presence (grey) or absence (white) of the visual cue.*

The three tested variables, when considered as independent factors, had a significant effect on the landing behaviour of *An. coluzzii* females (GLM, for temperature: chi-square=41.89, d.f.=2, P<0.001; for visibility: chi-square=29.11, d.f.=1, P<0.00; for host odour: chi-square=548, d.f.=1, P<0.001).

Adult age and the atmospheric pressure (i.e. proxy of weather), when introduced into the model as covariates had no significant influence on the number of

mosquitoes that landed on the surface in the different treatments (for adult age: GLM, chi-square=3.27, d.f.=1, P=0.07; for atmospheric pressure: GLM, chi-square=162.53, d.f.=1, P=0.21). However, starvation time and time spent in the darkness prior to the experiment did have a significant effect on the landing behaviour of *An. coluzzii* females (correspondingly GLM, chi-square=3.99, d.f.=1, P<0.05; chi-square=6.89, d.f.=1, P<0.001).

In the three treatments where host odour was presented in combination with a transparent surface, the number of mosquitoes recovered on different quadrants was significantly different (Table 3). Figure 22 shows the total number of mosquitoes caught on each quadrant for treatments where a significant difference was detected in the number of mosquitoes landing on different parts of the target. The majority of the mosquitoes were recovered on quadrant 2 (downwind position). However, the number that landed on the other downwind quadrant (quadrant 1) was not significantly different from that of the two quadrants in the upwind position (comparison with quadrant 3: chi-square=3.22, P=0.20; comparison with quadrant 4: chi-square=1.59, P=0.45). Furthermore, the combined number of mosquitoes that landed on the two downwind quadrants was not different to the total number of mosquitoes caught on the upwind quadrants (chi-square=0.86, P=0.64). For the rest of the treatments, mosquitoes were equally recovered in all quadrants of the target surface (Table 3). Note that one treatment, i.e. transparent target set at 45 °C in the absence of odour, approached significance, but similar number of mosquitoes were recovered on the upwind and downwind side of the target. Altogether, these results indicate that mosquitoes did not land significantly more on the first two quadrants encountered (i.e. the two downwind quadrants, quadrants 1 and 2) and it also suggests that the thermostat probe (positioned on quadrant 3) did not influence the landing behaviour. Overall, these results confirm the symmetrical conditions of the landing target.

Table 3: Comparisons of the number of mosquitoes recovered in different quadrants of the landing target. *The analysis (GLM) was done separately for each treatment to neutralise the variability of the landing numbers given by the effect of host cues. The asterisk indicates a significant difference at a level of least at P<0.05.*

Treatment	d.f.	Chi-square	P-value
No odour + 25 °C + transparent	3	2.77	0.43
No odour + 35 °C + transparent	3	4.50	0.21
No odour + 45 °C + transparent	3	7.70	0.052
No odour + 25 °C + black	3	2.05	0.56
No odour + 35 °C + black	3	6.31	0.10
No odour + 45 °C + black	3	1.36	0.72
Odour + 25 °C + transparent	3	12.14	0.007*
Odour + 35 °C + transparent	3	24.30	<0.001*
Odour + 45 °C + transparent	3	25.42	<0.001*
Odour + 25 °C + black	3	0.55	0.91
Odour + 35 °C + black	3	4.10	0.25
Odour + 45 °C + black	3	3.30	0.35
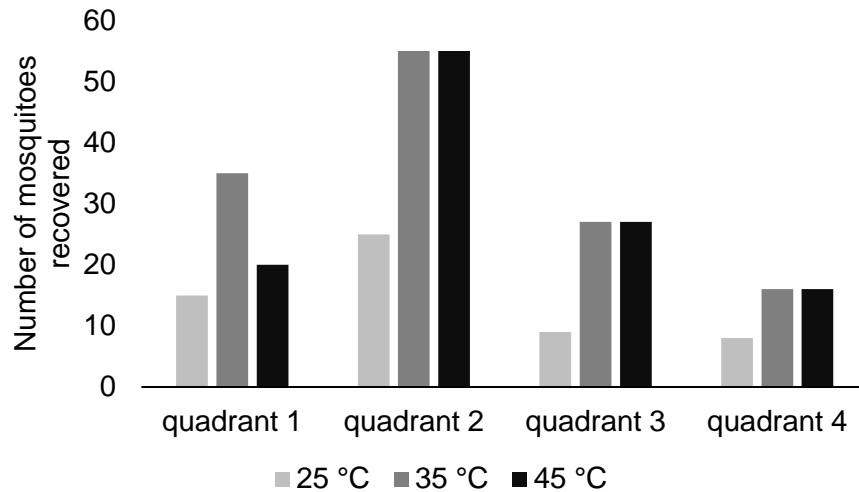
Figure 22: Total number of mosquitoes recovered in each one of the quadrants for treatments where host odour was dispensed and the landing target was presented as transparent, i.e. the treatments where mosquitoes landed in significantly different numbers on the different quadrants.

To establish the role of each host-associated cue, presented separately or in combinations, a series of comparisons between treatments were carried out. The results are visually summarised in Fig. 23, while the numeric outcome of the analysis are reported in Table 4. The positive control was taken as the treatment where all three host-associated stimuli were presented, i.e. the target was visually conspicuous, with a surface temperature of 35 °C, and in presence of host odour. The negative control was taken as the treatment where none of the host-associated stimuli were offered, having the target surface transparent and at room temperature, and in absence of host odour.
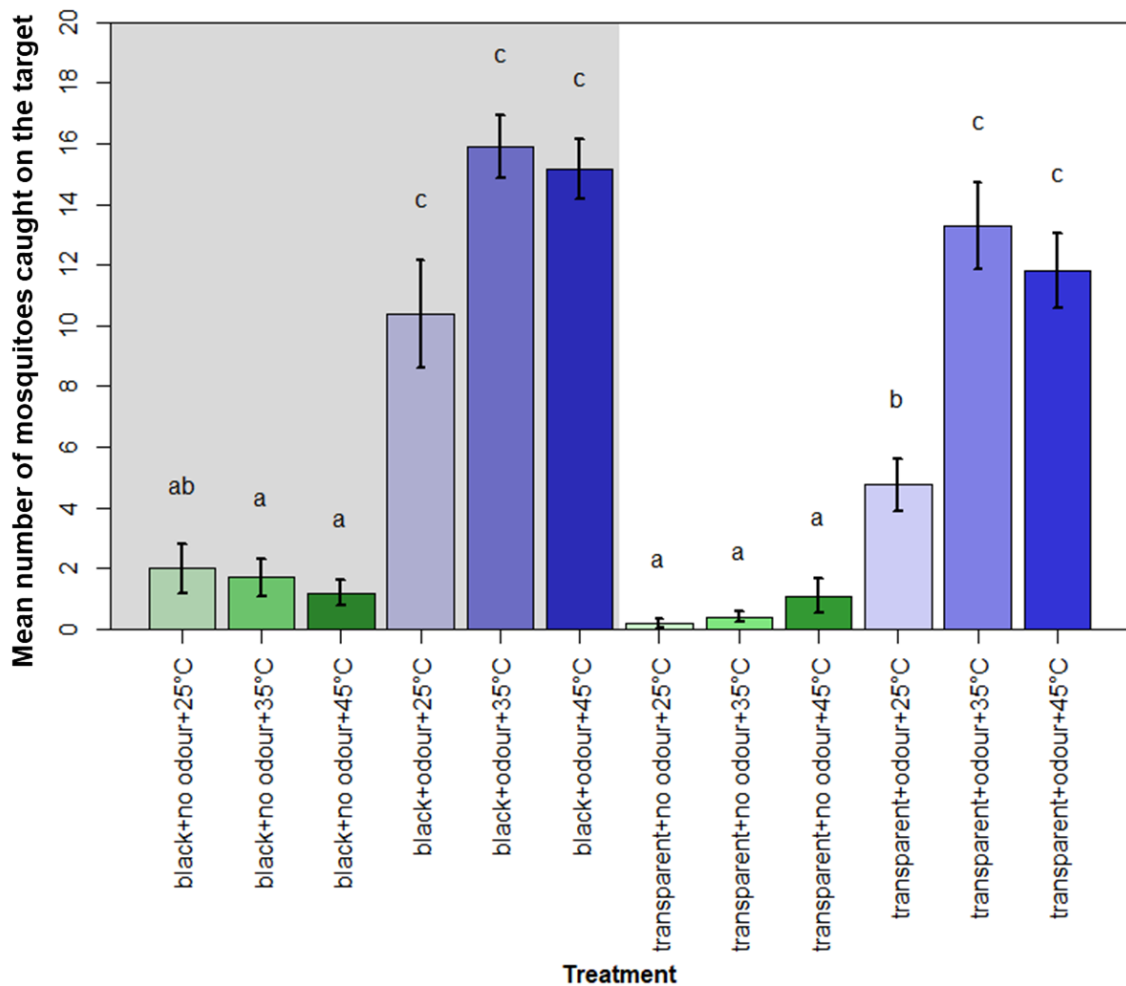
Figure 23: Mean number of mosquitoes found on the landing surface in each treatment. *The Y-axis shows the twelve different treatments, where "black" and "transparent" indicate the different visual cues offered, "no odour" and "odour" indicate the absence or presence of host odour, and the temperature indicates the thermal cue used. Blue bars indicate that host odour was present during assays, green bars indicate it was absent. The error bars indicate the SEM. Paler colours denote lower temperatures and darker colours indicate higher temperatures. The background colour indicates the presence (grey) or absence (white) of the visual cue. Different letters denote significant differences between treatments (Tukey's test, significance threshold of at least P<0.05).*

Table 4: Results from Tukey's test between treatments. *The two columns showing the mean ± SEM refer to the mean number of mosquitoes recovered on the trap for each corresponding treatment. In bold is highlighted the changing variable in each comparison. The asterisk denotes significant difference between the treatments compared at a level of at least P<0.05.*

Treatment 1	Mean ± SEM	Treatment 2	Mean ± SEM	t-value	P-value	Factor compared
Odour + 25 °C + transparent	4.8 ± 0.88	**No odour** + 25 °C + transparent	0.2 ± 0.13	4.37	0.001*	Odour cue
No odour + 25 °C + **black**	2.0 ± 0.82	No odour + 25 °C + **transparent**	0.2 ± 0.13	-3.08	0.07	Visual cue
No odour + **25 °C** + transparent	0.2 ± 0.13	No odour + **35 °C** + transparent	0.4 ± 0.16	0.80	1	Thermal cue
No odour + **25 °C** + transparent	0.2 ± 0.13	No odour + **45 °C** + transparent	1.1 ± 0.59	2.20	0.49	Thermal cue
No odour + **35 °C** + transparent	0.4 ± 0.16	No odour + **45 °C** + transparent	1.1 ± 0.59	1.71	0.83	Thermal cue
Odour + 35 °C + transparent	13.3 ± 1.41	**No odour** + 35 °C + transparent	0.4 ± 0.16	6.79	0.001*	Odour cue in presence of thermal cue

Treatment 1	Mean ± SEM	Treatment 2	Mean ± SEM	t-value	P-value	Factor compared
The Odour + 45 °C + transparent	11.8 ± 1.24	**No odour** + 45 °C + transparent	1.1 ± 0.59	7.21	0.001*	Odour cue in presence of thermal cue
Odour + 25 °C + black	10.4 ± 1.77	**No odour** + 25 °C + black	2.0 ± 0.82	6.30	0.001*	Odour cue in presence of visual cue
Odour + 35 °C + black	15.9 ± 1.05	**No odour** + 35 °C + black	1.7 ± 0.62	8.21	0.001*	Odour cue in presence of visual and thermal cues
Odour + 45 °C + black	15.2 ± 1.00	**No odour** + 45 °C + black	1.2 ± 0.42	8.14	0.001*	Odour cue in presence of visual and thermal cues
Odour + 25 °C + **black**	10.4 ± 1.77	Odour + 25 °C + **transparent**	4.8 ± 0.88	-4.17	0.001*	Visual cue in presence of odour cue
No odour + 35 °C + **black**	1.7 ± 0.62	No odour + 35 °C + **transparent**	0.4 ± 0.16	-2.57	0.25	Visual cue in presence of thermal cue

Treatment 1	Mean ± SEM	Treatment 2	Mean ± SEM	t-value	P-value	Factor compared
No odour + 45 °C + **black**	1.2 ± 0.42	No odour + 45 °C + **transparent**	1.1 ± 0.59	-0.20	1	Visual cue in presence of thermal cue
Odour + 35 °C + **black**	15.9 ± 1.05	Odour + 35 °C + **transparent**	13.3 ± 1.41	-1.18	0.99	Visual cue in presence of thermal and odour cues
Odour + 45 °C + **black**	15.2 ± 1.00	Odour + 45 °C + **transparent**	11.8 ± 1.24	-1.69	0.84	Visual cue in presence of thermal and odour cues
No odour + **25 °C** + black	2.0 ± 0.82	No odour + **35 °C** + black	1.7 ± 0.62	-0.47	1	Thermal cue in presence of visual cue
No odour + **25 °C** + black	2.0 ± 0.82	No odour + **45 °C** + black	1.2 ± 0.42	-1.35	0.96	Thermal cue in presence of visual cue
No odour + **35 °C** + black	1.7 ± 0.62	No odour + **45 °C** + black	1.2 ± 0.42	-0.90	1	Thermal cue in presence of visual cue

Treatment 1	Mean ± SEM	Treatment 2	Mean ± SEM	t-value	P-value	Factor compared
Odour + **25 °C** + transparent	4.8 ± 0.88	Odour + **35 °C** + transparent	13.3 ± 1.41	5.64	0.001*	Thermal cue in presence of odour cue
Odour + **25 °C** + transparent	4.8 ± 0.88	Odour + **45 °C** + transparent	11.8 ± 1.24	4.92	0.001*	Thermal cue in presence of odour cue
Odour + **35 °C** + transparent	13.3 ± 1.41	Odour + **45 °C** + transparent	11.8 ± 1.24	-0.76	1	Thermal cue in presence of odour cue
Odour + **25 °C** + black	10.4 ± 1.77	Odour + **35 °C** + black	15.9 ± 1.05	2.69	0.19	Thermal cue in presence of visual and odour cues
Odour + **25 °C** + black	10.4 ± 1.77	Odour + **45 °C** + black	15.2 ± 1.00	2.47	0.30	Thermal cue in presence of visual and odour cues
Odour + **35 °C** + black	15.9 ± 1.05	Odour + **45 °C** + black	15.2 ± 1.00	-0.33	1	Thermal cue in presence of visual and odour cues

4.3.4. Effect of host odour

Host odour, when considered as the only cue offered, had a strong effect on the landing behaviour. Significantly more mosquitoes landed on the target in presence of host odour compared with the number of mosquitoes that landed on the same surface but in absence of host odour.

4.3.5. Effect of target visual properties

The visibility of the target, when presented alone, did not significantly affect the number of mosquitoes landing on the target. Although a small number of individuals did land on the high contrast black target, this was not significantly different to the mean number of mosquitoes that landed on a transparent target. This indicates that with no other stimuli, the visual cue alone does not induce landing.

4.3.6. Effect of target temperature

The temperature of the target, when considered as the only cue offered, did not influence the landing behaviour of the female mosquitoes. In particular, no significant effect was noticed when comparing the number of mosquitoes that had landed on a transparent target at 25 °C and the number of mosquitoes that had landed on the same target at 35 °C and at 45 °C. In the same manner, no significant difference was noticed when comparing the number of mosquitoes that had landed on the target at 35 °C and at 45 °C. This indicates that with no other stimuli, the thermal cue is not sufficient to induce landing.

4.3.7. Effect of stimuli-interactions

Effect of the odour cue when presented with the thermal cue
Host odour and the thermal cue, when presented together, did have a significant effect on the landing behaviour of *An. coluzzii* host-seeking females. Significantly more mosquitoes landed on a surface at 35 °C in the presence of host odour compared with the number of mosquitoes that landed on a surface at 35 °C in

the absence of host odour. Similarly, significantly more mosquitoes landed on a surface at 45 °C in the presence of host odour compared with the number of mosquitoes that landed on a surface at 45 °C in the absence of host odour.

Effect of the odour cue when presented with the visual cue

When host odour and the visual cue were presented simultaneously, significantly more mosquitoes landed on the surface compared to the treatment where the surface was still visually conspicuous but host odour was absent.

Effect of the odour cue when presented with the thermal and visual cues

Host odour, when presented together with thermal and visual cues did have a significant effect on the landing behaviour of *An. coluzzii* host-seeking females. Significantly more mosquitoes landed on a black surface at 35 °C in the presence of host odour compared with the number of mosquitoes that landed on a black surface at 35 °C in the absence of host odour. Similarly, significantly more mosquitoes landed on a black surface at 45 °C in the presence of host odour compared with the number of mosquitoes that landed on a black surface at 45 °C in the absence of host odour. These results suggest that the presence of the odour cue is the prerequisite for a substantial landing response.

Effect of the visual cue when presented with the odour cue

Significantly more mosquitoes landed on black target surfaces in the presence of host odour compared to the number of mosquitoes that landed on transparent targets in presence of host odour.

Effect of the visual cue when presented with the thermal cue

No significant effect was recorded on the landing behaviour when, in the absence of host odour, *An. coluzzii* host-seeking females were presented with a black surface at 35 °C compared with landings on a transparent surface at 35 °C. Similarly, no significant effect was recorded on the number of mosquitoes that in the absence of host odour landed on a black surface at 45 °C compared with landings on a transparent surface at 45 °C. This indicates that, in absence of host odour, the visibility of a heated target surface does not influence the landing behaviour of the mosquitoes.

Effect of the visual cue when presented with the thermal and odour cues

No significant difference was found in the number of landings between treatments where the visually conspicuous target surface was set at 35 °C and host odour was offered when compared with the treatment whereas conditions were identical apart from having a transparent surface. Equally, landings were not significantly different when comparing the treatment where the surface was black and at 45 °C in the presence of host odour with the treatment where the surface was transparent and at 45 °C in the presence of host odour. These results indicate that the visibility of the target did not significantly increase the landing behaviour if the other two factors, i.e. host odour and thermal cue, were present. Thus, visibility does not play a major role in eliciting landing behaviour if other host-associated stimuli are present, i.e. if considering only the landing response, mosquitoes can bypass the absence of the visual cue if sufficient non-visual host-associated stimuli are present.

Effect of the thermal cue when presented with the visual cue

In treatments where host odour was absent and the target surface was visually conspicuous, the temperature of the surface did not influence the landing behaviour of the female mosquitoes. Specifically, no significant effect was noticed when comparing the number of caught mosquitoes on a black surface at 25 °C with the number of caught mosquitoes on the same surface at 35 °C and 45 °C. In the same manner, no significant difference was noticed in the number of landings between the treatment at 35 °C and at 45 °C. This indicates that in the absence of host odour, the effect of the thermal and visual cues is minimal.

Effect of the thermal cue when presented with the odour cue

The thermal cue, when provided simultaneously with host odour, had a significant effect on the landing response. A significantly higher number of mosquitoes were caught in treatments where the transparent target was set at 35 °C and 45 °C and was presented with host odour when compared to the treatment where, in presence of host odour, the transparent target was set at 25 °C. However, no significant difference was observed between treatments where, in presence of host odour and with a transparent target, the temperature

was set at 35 °C and 45 °C. This indicates that mosquitoes did not show a preference for landing on surfaces with a temperature near to human body temperature, and that as long as the surface was warmer than the environment, it elicited a similar landing response.

Effect of the thermal cue when presented with the visual and odour cues

No significant difference in the number of landings was observed in treatments where host odour was offered and the black target surface was at either 35 °C or 45 °C compared with the respective treatment where the temperature was 25 °C. Similarly, no difference was detected in the number of landings between treatments where the target was set to either 35 °C or 45 °C. These results indicate that the thermal cue does not significantly increase landing if other host-associated stimuli, i.e. host odour and visual cue, are present. Thus, if considering the final outcome (i.e. landing response), mosquitoes can bypass the absence of the thermal cue if they receive other sufficient information to locate the host. Furthermore, no preference was detected for landing on targets set at human body temperature.

4.3.8. Quantification of the role of each stimulus on triggering landing behaviour and synergistic effect of combined stimuli

Figure 24 illustrates the effect of each stimulus, presented both alone or in combination with other host-associated stimuli. As there was no significant difference between the number of mosquitoes recovered on target surfaces set at 35 °C and 45 °C, the latter temperature was removed from this analysis.

The thermal stimulus, i.e. surface at 35 °C, when presented alone elicited 1.57% of mosquitoes to land. Visibility alone, i.e. making the target visually conspicuous, elicited 7.98% of mosquitoes to land. Host odour, when presented as the only cue, elicited 19.14% of the mosquitoes to land on the target surface.
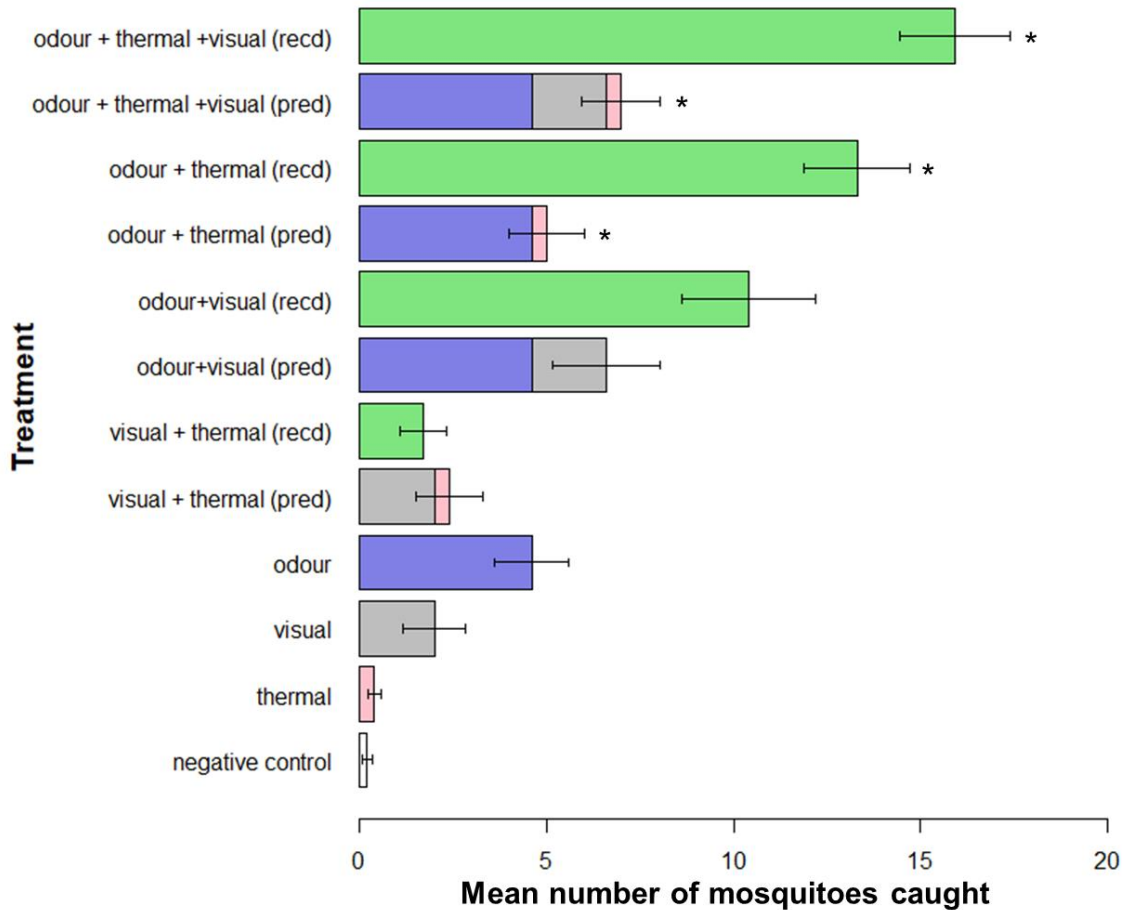
Figure 24: Predicted and recorded effects of host-associated stimuli on the mean landing response of host-seeking** An. coluzzii, **when stimuli were presented alone and in combination. *The negative control is set based on the number of mosquitoes landing on a target when no thermal, visual, or odour stimuli are presented. The error bars indicate the SEM. Abbreviations: recd=recorded mean from experiments; pred=additive predicted mean derived from simulated data set produced using recorded baseline values. The asterisk denotes significant difference between the recorded effect and the predicted effect for each respective treatment (GLM, P<0.001).*

When the visual and thermal cues were combined, 6.8% of the mosquitoes landed on the target surface. Host odour and visual cue, when presented simultaneously, elicited 41.60% of the mosquitoes to land, while 52.77% of the mosquitoes landed when they were presented with host odour and the thermal cue. Finally, when the three cues were presented simultaneously, 66.47% of the mosquitoes landed on the surface. This was the treatment that received the highest proportion of mosquitoes landing on the target surface.

To understand the role of the different host-associated stimuli when presented in combination with other stimuli, the number of mosquitoes that landed when exposed to different treatments was compared with the number of mosquitoes expected to land if the effect of each stimulus in that combination would act in an additive manner. From this analysis, it resulted that the effect of certain combinations of cues exceeded considerably the predicted effect, thus indicating that host-associated stimuli act in a synergistic manner in eliciting landing behaviour in host-seeking *An. coluzzii* females. The recorded number of mosquitoes that landed when the three host-associated cues were presented simultaneously (mean ± SEM=15.9 ± 1.05) was significantly higher when compared to the number of mosquitoes that were expected to land if the effect was given by the addition of the singular effects of each stimulus (mean ± SEM=7.0 ± 1.48; GLM, chi-square=24.15, d.f.=1, P<0.001). Similarly, the odour cue acted synergistically with the thermal cue in eliciting landing behaviour, as the recorded number (mean ± SEM=13.3 ± 1.41) significantly exceed the expected number (mean ± SEM=5.0 ± 1.01; GLM, chi-square=26.19, d.f.=1, P<0.001). However, no synergism was found between the odour and the visual cue (mean ± SEM recorded landings=10.4 ± 1.77, expected=6.6 ± 1.43; GLM, chi-square=2.84, d.f.=1, P=0.09) or the thermal and the visual cue (mean ± SEM recorded landings=1.7 ± 0.62, expected=2.4 ± 0.90; GLM, chi-square=0.42, d.f.=1, P=0.52).

4.4. Discussion

4.4.1. Activation

Several studies conducted on different species of mosquitoes reported that the activation was significantly higher when mosquitoes were exposed to either single compounds or combinations of compounds of host odour compared with activation levels of mosquitoes that were exposed to clean air only (Omer, 1979; Eiras and Jepson, 1991; Dekker and Carde, 2011; Hawkes *et al.*, 2012; Hawkes and Gibson, 2016). For example, in their study with *Ae. aegypti,* Eiras and Jepson (1991) reported that one minute after the commencement of the olfactory

stimulation, about two mosquitoes (approx. 6.67% of the total mosquitoes exposed to the treatment) were recorded to take off in clean air, while about twelve mosquitoes (approx. 40% of the mosquitoes) were activated in the treatment where carbon dioxide was presented. In another study with the same species, Dekker *et al.* (2005) reported that after two minutes of stimulation with 100% human skin odour, i.e. full skin-odour laden air, more than 80% of the mosquitoes were scored as activated. They also reported similar percentages of mosquitoes activated after two minutes of exposure to different concentrations (from 0.05% to 0.3%) of carbon dioxide. Interestingly, 40% of the mosquitoes were also regarded as activated after two minutes of being exposed to clean air. In a more recent study conducted on *An. coluzzii* females, Hawkes and Gibson (2016) found that 53% of the mosquitoes were activated when exposed to clean air. This activation level significantly increased to 70% when human odour was introduced in the wind tunnel. In the study reported here, host odour, when dispensed, was given as a combination of carbon dioxide at 4.5% concentration and human foot odour collected on a pair of worn nylon socks. Both these stimuli are proven to act as activators and attractants (Dekker *et al.*, 2005; Dekker and Carde, 2011), thus it was expected to obtain a higher proportion of mosquitoes activated in treatments where host odour was presented. However, the results reported here show that the activation was not different in treatments where mosquitoes were either exposed or not to host odour.

This anomaly could be partially explained by the fact that the experiments were carried out during the phase in which the colony was reported to be most active (Hawkes *et al.*, 2012). In Hawkes *et al.* (2012) it was described that during the scotophase, *An. coluzzii* females showed a periodicity in spontaneous activation even when not presented with host cues. Thus, it could be that in the reported study, *An. coluzzii* females were spontaneously activated by their internal circadian rhythm even in the absence of host odour. Furthermore, it is interesting to note that Dekker *et al.* (2005) and Hawkes and Gibson (2016) also recorded an activation level of 40% and 53% correspondingly, in mosquitoes that were exposed to a moving stream of only clean air. Other wind tunnel studies conducted on *An. gambiae s.s.* reported that the mosquitoes generally flew

upwind even when not exposed to host odours (Takken *et al.*, 1997a; Costantini *et al.*, 2001). This could be explained by the predisposition of mosquitoes to take-off and fly upwind when exposed to air currents (Cardé and Gibson, 2010). Results from the current study support this view.

4.4.2. Landing

Both the carbon dioxide release method and the wind tunnel conditions used in this study were comparable with the conditions used by Hawkes (2013). Hawkes (2013) reported that 50 cm downwind from the carbon dioxide release point, the gas was confined in a 25 cm circle plume. Translating this information into this study, it follows that the 25 cm circle plume was found approx. in the middle of the landing target on the X-axis, 10 cm from the downwind edge of target in the Y-axis, and with the bottom side of the plume edge touching the landing target (Fig. 14 for axes position, Fig. 25 for diagram of carbon dioxide plume over landing target).
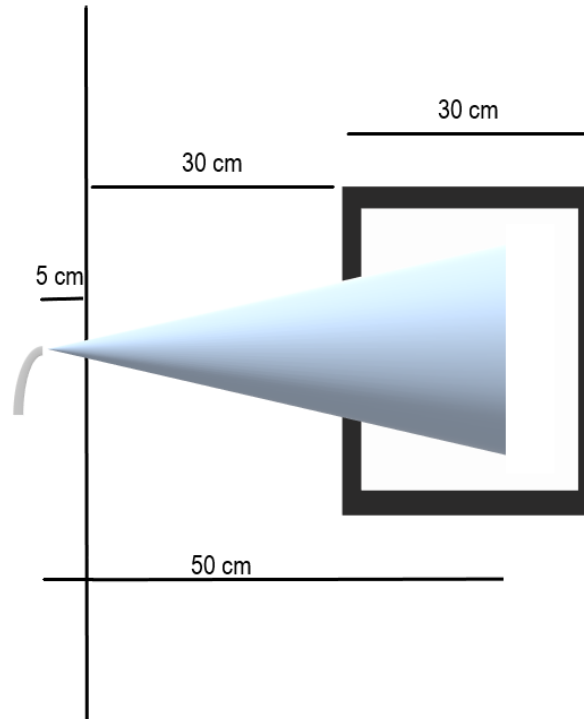
Figure 25: Diagram of carbon dioxide plume over landing target. *The carbon dioxide was released 25 cm above the wind tunnel floor and 5 cm from the upwind net. At 50 cm downwind*

from the release point, the diameter of the plume should be approx. 25 cm, meaning that the bottom border of the plume touched the floor of the wind tunnel and therefore the surface of the landing target (drawn by author using Paint 3D).

If considered that after encountering an odour plume mosquitoes normally surge upwind until they exit the plume (Beeuwkes *et al.*, 2008), it can be supposed that in this study, host-seeking mosquitoes flying upwind in the carbon dioxide plume will first encounter the downwind side of the landing target. Contrary to the hypothesis laid out at the beginning of this study, there was no difference between the number of mosquitoes landing on the downwind side of the target and the number of mosquitoes landing on the upwind side. This indicates that, even when thermal and visual cues were offered, mosquitoes did not land immediately on the surface where the stimuli were assumed to be first detected, suggesting that the response to short-range cues is not instantaneous. Thus, it appears that landing behaviour is not a mere reflex but is rather a complex behaviour that requires the integration of information at higher neuronal levels (Wessnitzer and Webb, 2006). It is to be considered that the experimental set-up of the present study recorded only the endpoint of the landing behaviour. Thus, it is not possible to infer the short-range behaviour from this dataset. Further investigation using an approach that allows to accurately track mosquitoes' movements is needed to better understand and fully characterise the landing behaviour of host-seeking *An. coluzzii* females.

Effect of different cues on landing behaviour

The results reported here indicate that visual, thermal, and odour cues are all important factors that affect landing in *An. coluzzii* host-seeking mosquitoes. When the landing target was presented as visually conspicuous and at a host temperature, but without the host odour, 6.8% of the female mosquitoes landed, indicating that these two cues mediate landing behaviour but only in a minimal manner. The greatest effect was generated by host odour. The addition of host odour to visually conspicuous and at host temperature targets increased the landing rate from 6.8% to 66.47%, thus causing a 10-fold increase in the response. Previous research showed that *An. gambiae* females landed significantly more, with a 25-fold increase, on a surface heated to 34 °C when

carbon dioxide pulses were introduced in the arena (Kröber *et al.*, 2010). The warm surface, when presented as the only cue, did not lead the female mosquitoes to land. Similar conclusions were drawn in an experiment that used *Ae. aegypti* with regards to carbon dioxide and visual stimuli (Van Breugel *et al.*, 2015). In their experiment, *Ae. aegypti* females spent a considerable amount of time exploring the visual feature only when exposed to a carbon dioxide plume. Similar results on host odour and the visual cue were also obtained with *An. coluzzii* (Hawkes and Gibson, 2016). Healy and Copland (1995) reported that if exposed to clean air, only 4% of the released *An. gambiae* mosquitoes arrived and landed on a transparent cylinder heated at 34 °C. This percentage significantly increased to 20.5% if presented with carbon dioxide with a concentration of 0.01% above background. In their experimental set-up, the heated cylinder was positioned below the carbon dioxide release point. It was observed that mosquitoes took a long time to arrive and land on the cylinder, as they firstly flew directly towards the carbon dioxide release point, and the cylinder was approached only secondarily. Thus, they suggested that other stimuli involved in close-range approach were missing. It is interesting to note that the heated object lacked visual cues, which are known to signal important medium to short-range information (Cardé and Gibson, 2010). It could be that the addition of visual cues would have improved the competence of mosquitoes to find the thermal cue.

The studies described above support the view that landing behaviour is gated by the presence of carbon dioxide (Webster *et al.*, 2015). The behavioural study of Van Breugel *et al.* (2015) reported for the first time that *Ae. aegypti* showed an odour-gated visual attraction. This was further substantiated by a sensory physiology study, which proved the role of carbon dioxide as a modulator of the mosquito's response to visual stimuli (Vinauger *et al.*, 2019). In their study, they reported that the carbon dioxide modulated the response of the lobula neuropil, a region dense of synaptic connections in mosquito's optic lobe, to discrete visual stimuli. Interestingly, the modulation of these sensory stimuli was reported to be asymmetric, with carbon dioxide regulating responses to visual stimuli but not vice versa (Vinauger *et al.*, 2019). Although the interaction between odour

and visual stimuli seems better understood, it remains unknown whether a similar mechanism regulates the interaction between odour and thermal cues.

It has been suggested that carbon dioxide may also gate the landing response elicited by thermal cues (McMeniman *et al.*, 2014; Webster *et al.*, 2015). However, Van Breugel *et al.* (2015) proposed a contrasting view, in which different cues might be able to interact increasing a behavioural response while remaining independent from each other. Under this view, *An. coluzzii* mosquitoes might still approach and land on a warm object, even in absence of host odour. In the study reported here, female mosquitoes were significantly more attracted and prone to land on a visually conspicuous and warm object when exposed to host odour. However, even in absence of the odour cue mosquitoes were recovered from the landing target, indicating that despite the absence of an olfactory cue, a small proportion of the tested mosquitoes responded to only thermal and visual cues. Thus, the results reported here support Van Breugel *et al.* (2015) view. It is interesting to note that Van Breugel *et al.* (2015) extrapolated their conclusion using results obtained from assays carried out on *Ae. aegypti*, a diurnal mosquito species, while the results presented here were obtained using a nocturnal species, *An. coluzzii*. Thus, it appears that this view might apply to several mosquito species, with very different ecology and behavioural traits.

The visibility of the target played an important role in attracting and eliciting landing behaviour. Interestingly, a small proportion of mosquitoes (7.98%) were recovered on the target surface when only the visual cue was given. This could be caused by an intrinsic attraction of mosquitoes towards visually conspicuous features, which has been reported in previous studies (Bidlingmayer and Hem, 1979, 1980; Liu and Vosshall, 2019). This could result from the preference of mosquitoes to rest in dark areas, as in the wild these could signal the presence of a protected area in which to shelter, such as a crevice or other small hole. However, further studies are required to explore this potential explanation.

Visual cues are thought to evoke a range of different behavioural responses in mosquitoes. The optomotor mechanism enables mosquitoes to spatially orient

themselves relative to visual cues in the environment (Kennedy, 1940; Gibson, 1995). However, visual cues may also be associated with potential hosts, and so can be attractive depending on the insect's physiological condition, the presence of other host-associated stimuli, and the precise nature of the visual cue. In the present study, the target was considerably larger when compared to the black tiles that were provided on the wind tunnel floor to facilitate optomotor anemotaxis. Additionally, the target could also present other host cues. Thus, although the black tiles could elicit some attraction, it can be expected that most of the host-seeking response displayed by mosquitoes was elicited by the target. When flying close to an object, visual cues may elicit an avoidance mechanism, i.e. flying either to the sides or upwards to avoid the object that is perceived as a barrier (Cardé and Gibson, 2010; Hawkes and Gibson, 2016; Cribellier *et al.*, 2018, 2020). On the other hand, when additional host cues are presented, the visual avoidance mechanism is suppressed and visual cues act as attractants (Van Breugel *et al.*, 2015; Hawkes and Gibson, 2016). Without short-range cues however, mosquitoes only hover above the visually conspicuous surface without landing on it (Van Breugel *et al.*, 2015; Hawkes and Gibson, 2016).

In a female mosquito located within centimetres of a visually conspicuous body, the frontal and lower field of view would be completely occupied by the view of the body. Thus, this stimulus would likely not provide any significant information that suggests that the object is in fact a host, and so would be unlikely to trigger landing behaviour. Furthermore, nocturnal mosquitoes that feed inside human dwellings are expected to operate under very limiting light conditions, thus it is no surprise that the landing response is not governed by visual cues alone. Altogether, these elements indicate that in the context of host-seeking, visual cues serve as attractants and their role is important in the medium to close-range phase of location, while their effect in triggering landing behaviour is likely to be negligible, due to diminished resolution at very close range. A study conducted on *Ae. aegypti* also suggested that visibility is a characteristic that increments the facility by which the target is found by odour-induced searching mosquitoes, however, the "permission to land" is elicited by other stimuli, such as warm, wet convection currents (Kellogg and Wright, 1962a). Interestingly, a recent study

reported how mosquitoes might use self-induced airflow patterns to avoid collision with surfaces (Nakata *et al.*, 2020), suggesting that other cues (such as airflow) might provide directional information during the landing phase. Further studies to explore this are recommended.

Thermal cues have been proven to be efficient landing cues, eliciting a temperature-dependent landing response in *An. gambiae* mosquitoes (Healy *et al.*, 2002), *Ae. aegypti* mosquitoes (Corfas and Vosshall, 2015), and *Culex* mosquitoes (Reinhold *et al.*, 2022). In the present study, thermal stimulus when added to other host-associated cues was an important factor that guided landing behaviour. However, when presented alone, only 1.57% of the mosquitoes landed on the target. This could be due to the fact that, in absence of other cues, mosquitoes were less likely to encounter the thermal stimulus as the thermal cue is thought to be detected within a close-range from the host (Spitzen *et al.*, 2013; McMeniman *et al.*, 2014; Cardé, 2015; Van Breugel *et al.*, 2015; Greppi *et al.*, 2020). This supports the idea that thermal cues mostly play a role in directing the final phases of mosquitoes' approach to a host. It is interesting to note that, although fewer mosquitoes were recovered from the target surface when it was set at 45 °C, no significant difference was found with the number of mosquitoes recovered on the surface when this was set at 35 °C or 45 °C. This suggests that mosquitoes did not differentiate between targets set at host temperature and targets set 10 °C higher than host temperature. These results disagree with previous findings obtained by Healy *et al.* (2002), in which *An. gambiae* mosquitoes showed a significant preference for landing on a target set at host temperature (34 ± 2 °C) when compared with targets at lower (27 °C) and higher (41 °C) temperatures. Two other studies that were conducted in still air using *Ae. aegypti* recorded a heat avoidance behaviour for high temperatures, while both studies recorded a preference (intended as either directional choice or time spent closer to the thermal cue) for temperatures closer to human body temperature (Corfas and Vosshall, 2015; Zermoglio *et al.*, 2017). As the study presented here was conducted in a wind tunnel with flowing air, it is possible that the moving air might have contributed to altering the thermal cue given by the heated target, not by altering the surface's temperature as this was maintained

stable throughout the assay, but by cooling the temperature of the air surrounding the target. This might in part explain the differences between our results and previous studies.

On the other hand, the results presented here are in accordance with those of a recent study conducted on *Culex* mosquitoes, where it was indicated that the proportion of heat-seeking females landing on a warm object was highest when the temperature of the object was set at 40 °C and 45 °C (Reinhold *et al.*, 2022). Other blood-sucking insects (e.g. triatomines) were described to exhibit host-seeking behaviours in response to objects if these presented a temperature from approx. 2 °C above ambient temperature and up to 47 °C (Guerenstein and Lazzari, 2009). Thus, landing on targets that are warmer than the physiological temperature of a host agrees with reports presented for other blood-sucking insects and it might be a trait that strictly depends on the thermopreferences of different species (Reinhold *et al.*, 2022). It would be interesting to further investigate what are the upper and lower limit temperatures that elicit a landing behaviour in *An. coluzzii* host-seeking females.

Synergistic effect and multimodal sensory interaction

The finding reported here demonstrate for the first time a synergistic effect between odour, visual, and thermal cues in eliciting landing behaviour in host-seeking *An. coluzzii* females. When all the host-associated cues were provided, the total number of mosquitoes landing on the surface was more than twice the number that would have been theoretically expected to land, based on the simple addition of the landing effect attributable to each cue individually. Similarly, a synergistic effect was also recorded when the odour cue was combined with the thermal cue. However, the odour and the visual cue acted in a non-synergistic manner.

Although Liu and Vosshall (2019) reported an enhanced attractiveness to warm stimuli when presented with a visual cue, we reported no synergistic effect between the visual and thermal cues when presented alone. This might be a behavioural discrepancy due to species-specific responses, as their model

organism was *Ae. aegypti*, however, further investigation on this topic is suggested.

The are many advantages of using several different cues that signal the same information to produce a behavioural response. For instance, the integration of redundant information is important as it enhances the reliability of singular stimuli and increases the chances of an accurate host location (Fischer *et al.*, 2001; Gilbert and Kuenen, 2008; McMeniman *et al.*, 2014). Furthermore, the sensitivity to a certain stimulus may be further increased by integrating information deriving from other cues, thus, enhancing the response to the first cue (Lehane, 2005; Guerenstein and Lazzari, 2009). In addition, relying on several cues may allow the insect to have a parsimonious use of sensory organs (Guerenstein and Lazzari, 2009), thus acquiring the most accurate information in the least costly way (Fawcett and Johnstone, 2003). In environmental conditions where one cue may not be detectable, the presence of another cue carried on a parallel sensory system might still provide sufficient information to allow successful resource location.

In a natural context, vertebrate hosts emit a wide variety of different sensory cues (Guerenstein and Lazzari, 2009). Not only are mosquitoes able to detect and respond to these cues to locate and land on a host, but they are also able to integrate different sensory information, to provide a more accurate and efficient host localisation. Central integration of multimodal sensory information is a fundamental requirement for explaining how insects are able to produce complex and relevant behaviours in response to a wide variety of environmental situations (Wessnitzer and Webb, 2006). For example, a recent study on *Drosophila* larvae showed how these animals integrated the information of different thermo-sensory cells to produce context-dependent navigation, which allowed them to respond to different stimuli in a highly flexible manner (Hernandez-Nunez *et al.*, 2021). Furthermore, it has been demonstrated that many host-seeking species rely on the integration of information which derives from many different stimuli to locate their host. For instance, it was found that some parasitic wasps integrate the information deriving from vibrational and visual

cues in order to locate their host (Fischer *et al.*, 2001). In another study carried out on host-seeking bark beetles it was demonstrated that multimodal sensory information deriving from visual and olfactory cues were integrated at a close-range distance ($\leq$ 2 m), while the insect was flying towards its hosts, regulating the insect's landing behaviour (Campbell and Borden, 2006). From these results, the authors proposed that during host selection the insect shows a "flexible continuum of sensory integration", which allows it to accurately discriminate possible hosts. In their review on triatomines foraging behaviour, Guerenstein and Lazzari (2009) described how these blood-sucking insects make use of a convergence system, where information deriving from multimodal signals is integrated, and each context is defined by a particular combination of cues. For example, feeding behaviour in these insects is determined by the presence of both host odorants and heat. These are only a few examples of the many studies available in the literature that report how multimodal cues interact to modify the overall behavioural response of the insect. Thus, is not surprising that mosquitoes also integrate sensory cues to find human hosts.

4.5. Conclusions

In absence of host odour, only a small proportion of mosquitoes landed on the target surface, even if this was presented as visually conspicuous and at host temperature. This strongly indicates the crucial role of host odour in eliciting landing behaviour in host-seeking *An. coluzzii* female mosquitoes. The strong synergistic effect between odour, visual, and thermal cues indicate the robust interaction of these elements, which incremented the landing response. Understanding the role of different cues used in triggering attraction and landing may be essential for the development or improvement of tools for delivering insecticides on contact, as well as traps for surveillance and control (Kline and Lemire, 1995). By unravelling tsetse responses to different cues and their specific characteristics, researchers were able to identify key components that were then incorporated into an improved vector control approach (Torr and Vale, 2015). The results described here bring new information that could be used to modify surveillance and control tools against mosquitoes, particularly where

landing or contact is required. As demonstrated in this study, the addition of a visual, but more importantly, of a thermal cue in an odour releasing trap could greatly increase the number of mosquitoes caught, improving the efficiency of control methods (Homan *et al.*, 2016; Hawkes *et al.*, 2017a; Cribellier *et al.*, 2020).

Furthermore, the results reported here support the view which considers landing behaviour as the result of a series of stimuli integration, where the information deriving from different stimuli is integrated to permit a flexible, yet highly accurate context-relevant behaviour.

5. QUANTIFICATION OF PRE-LANDING AND LANDING BEHAVIOUR IN RESPONSE TO VARIATIONS IN PHYSICAL HOST-ASSOCIATED PROPERTIES

5.1. Introduction

As insecticide-resistant traits are spreading at an alarming rate in wild populations of *Anopheles* mosquitoes (Reddy *et al.*, 2011; Ranson and Lissenden, 2016; WHO, 2017, 2021; Riveron *et al.*, 2018), it has become evident that to achieve a sustainable malaria control program it is necessary to develop alternative control methods (Padonou *et al.*, 2012; Cooke *et al.*, 2015; Sougoufara *et al.*, 2020; Sanou *et al.*, 2021). To bypass the obstacle of insecticide resistance, the new methods should include non-insecticide approaches (Homan *et al.*, 2016; Ranson and Lissenden, 2016; Thomas, 2018) and should also aim to reduce outdoor malaria transmission (Degefa *et al.*, 2017; Hawkes *et al.*, 2017a; Thomsen *et al.*, 2017; Abong'o *et al.*, 2018), given that malaria control methods are currently underrepresented at this level (Sougoufara *et al.*, 2020).

As both the vector and the parasite have demonstrated adaptations to the control measures that have been put in place in the last few decades, it is unlikely that any novel control tool will prove to be a "silver bullet" (Rabinovich *et al.*, 2017; Nolan, 2021). In fact, it is thought that eradication will probably not be achieved with a single tool, but rather using an IVM approach (Stromsky, V. E., Hajkazemian *et al.*, 2021). This highlights the importance of finding new versatile methods that can be used in conjunction with current control methods. In that respect, in the past few years, a range of traps and targets have been developed as new methods to reduce the vector population, with the ultimate aim of reducing the EIR (Mmbando *et al.*, 2015; Homan *et al.*, 2016; Cribellier *et al.*, 2020; Gnambani *et al.*, 2020; Kessy *et al.*, 2020; Sanou *et al.*, 2021). Results from these studies are promising and suggest that mass-trapping mosquitoes could play an important role in reducing malaria transmission if included in vector control programmes (Homan *et al.*, 2016).

Attracting mosquitoes at short-range is important both for traps, which kill or capture mosquitoes and so must lure the insect to within range of their trapping mechanism, and lethal targets, which rely on mosquito contact with a surface to deliver insecticides or biocontrol agents. As discussed in Chapter 4, host odour, thermal gradient, and visual contrast play an important role in eliciting close-range attraction and landing response in host-seeking females, which are often the primary target of attractive traps. In particular, the combination of the thermal and odour cue drastically increases the landing response (Carnaghi *et al.*, 2021). This supports the finding of a field study that systematically tested the effect that visual, thermal, and olfactory cues had on the capture rate of a sticky trap (Hawkes *et al.*, 2017a). Here, the authors reported that adding a high contrast visual stimuli to an odour-baited trap augmented the catches, and the addition of a thermal stimulus to the visually conspicuous odour-baited trap further enhanced the performance. A more recent study that used a different mosquito trap (i.e. a counter-flow odour-bated trap) corroborates this concept, as it reported that by adding a thermal cue the trap capture rate improved drastically (Cribellier *et al.*, 2020).

Current trap-designs have a range of practical limitations (e.g. the dependence on a power source, a bulky design, high cost of production) that hamper their application at a large-scale (Hoshi *et al.*, 2019; Meza *et al.*, 2019; Sougoufara *et al.*, 2020) and in remote locations (Peck *et al.*, 2018). Thus, effort should be made to improve current trap designs to both facilitate their utilisation in the field and find more cost-effective solutions (Hawaria *et al.*, 2016). Optimising the attraction of traps and targets could increase the efficiency of such tools, which could lead to a reduction of the number of traps or targets needed in an area, thus reducing the cost associated with the operation.

Given that insect responses are a complex combination of several responses to different cues, to better understand the overall response it is important to divide it into its constituents and examine the individual response to different stimuli when presented separately. In doing so, it is possible to gain a better understanding of how mosquitoes respond to specific cues, and by integrating

this knowledge on trap design it is possible to improve potential catch rates (Vale, 1993; Kline and Lemire, 1995). This approach was used to develop and improve traps and targets used to control tsetse flies and proved to be particularly beneficial as it provided information relative to each characteristic so that each one was then refined at its optimal condition (Torr, 1994). Some of the most common characteristics considered when developing a trap are its size, colour, temperature, and orientation. An overview of the most relevant studies conducted for some of the mentioned characteristics is given below.

A key aspect to consider in a target is the full configuration of each stimulus that is presented. Not only the presence/absence of the cues is important, but also the magnitude, dimensions, intensity, and other variations in attributes of the cues presented could influence specific behavioural responses. For example, a study conducted on stingless bees indicated that during the landing phase, the flight parameters of these insects are regulated by different factors, including the size of the landing target (Tichit *et al.*, 2020). Contrary to most flying animals (including birds, insects, and bats) that decelerate before touchdown (Riskin *et al.*, 2009; van Breugel and Dickinson, 2012; Baird *et al.*, 2013; Provini *et al.*, 2014), stingless bees were reported to accelerate during the landing phase (Tichit *et al.*, 2020). This is thought to be an adaptation product resulting from the combination of a remarkably small landing target (i.e. the hive entrance is typically ~ 20 mm in diameter and ~ 20 mm in length) and a high traffic flow that accumulates at the nest entrance. Through mathematical modelling, the authors demonstrated that by adopting an "accelerated landing" strategy the traffic congestion was reduced, which resulted in an advantage for the colony. However, if the size of the landing platform was increased or the traffic flow was reduced, the model suggested that this strategy would no longer be beneficial. Thus, target size appears to have contributed forging specific landing behaviours in this species.

The idea that the target size influences landing behaviour was also reported in a previous study conducted on *Drosophila* flies, where it was indicated that the size of the visual target determined the moment in which the insect started

decelerating during landing (van Breugel and Dickinson, 2012). Thus, target size plays an important role in inducing specific behavioural responses during landing in different insect taxa, suggesting that its effect in mosquitoes is worth being examined.

For a target that presents multiple cues, it can be expected that changing the size of the area that delivers a particular cue might alter the insect's response to the target, thus interfering with the response that other cues might have elicited. The only study that has examined this aspect in mosquitoes was carried out by Wright and Kellogg (1964). They reported that although *Ae. aegypti* could be driven to land on a small target (a cylinder of 2.5 cm in diameter and 5 cm long), *An. quadrimaculatus* females would not alight on the same small target when this was emitting a narrow column of convection currents. However, they found that aside from landing on the experimenter's arm (thus proving to be responsive to hosts), *An. quadrimaculatus* alighted on the target if the convention currents rising from it were scattered over a wider area. From this, the authors deduced that the size of the area of certain stimuli, in this case convention currents, can affect attraction and landing responses, and they concluded that stimulus size might have a considerable effect on host-choice. However, this interesting conclusion has not been corroborated in further studies.

The size of targets or traps is often the subject of a trade-off, as on one hand, the target has to be sufficiently large to be detected by the insect and elicit attraction, and on the other hand, it strongly influences the transportability and the cost of the target. Thus, determining the optimal size that maximises the target cost-effectiveness is crucial, as demonstrated by a study conducted on targets used for the control of tsetse flies (Lindh *et al.*, 2009). Here, the authors showed that the size of the target could be reduced up to 16 times without drastically reducing the number of tsetse flies that were caught. It follows that the cost-effectiveness per square centimetre was considerably greater for smaller targets. This finding had important implications for control strategies and the authors called for more studies to optimise target design for these vectors. Recently, two studies investigated the effect that different trap sizes had on

mosquito catches. The first study evaluated the performance of large mosquito electrocuting traps capable of containing a person. These large traps were compared with small mosquito electrocuting traps, where only the lower portion of a person (i.e. legs and feet) was introduced in the trapping mechanisms (Meza *et al.*, 2019). It is to be noted however that for both target sizes a full person was used as bait, so it can be expected that the range of stimuli presented remained constant, and the only variable that changed was the size of the killing mechanism (i.e. the size of the electrocuting net). The second study assessed the performance of different sizes of the funnel (i.e. the catching mechanism) of the Lehmann Funnel Entry Trap (Sanou *et al.* 2021). Similarly to what described in the previous study, the range and intensity of the attractant cues were maintained constant, and the only variable that changed was the size of the trapping mechanism. Although both studies evaluated the performance of different trap sizes, no emphasis was put on examining whether changes in the size of the area that provided the stimuli influenced the behavioural response of mosquitoes. Thus, it is still unclear how the size of different cues (e.g. visual, thermal) might affect landing behaviour in mosquitoes and there are no reports that explore the effects of target sizes specifically on host-seeking *Anopheles* females. Investigating these factors could provide some important insights into mosquito behaviour, which might then translate into opportunities for improvements in the attractiveness and efficiency of vector control or surveillance tools.

Along with the size, other physical characteristics of the target and the environment in which the target is placed may also influence trap catches (Bidlingmayer and Evans, 1987). For example, the spatial orientation of targets (i.e. positioned vertically or horizontally) can affect the distance at which the trap is spotted by mosquitoes and might indicate whether an object is a barrier or not (Bidlingmayer, 1975). Extensive investigations on how a physical barrier is perceived by mosquitoes, what behavioural responses it elicits in the insects, and the role of visual stimuli in traps were carried out by Bidlingmayer and colleagues between the '70s and '90s (Bidlingmayer, 1975, 1994; Bidlingmayer and Hem, 1979, 1980; Bidlingmayer and Evans, 1987). In one of these studies,

the effect that vertical and horizontal panels had on trap catches was examined and it was found that different mosquito species had remarkably different responses to these barriers, as mosquitoes that inhabit woodland habitats approached visible objects closely (<30 cm), whilst mosquitoes that inhabit open field habitats kept a larger distance from the objects. This was probably because the vertical target elicited an avoidance behaviour at a greater distance in species that inhabit open fields (Bidlingmayer, 1975; Bidlingmayer and Hem, 1979). These early reports suggest that the spatial orientation of visual cues strongly influence mosquito response to traps.

A study aimed to investigate the biting site location in three *Anopheles* species (*An. gambiae s.s.*, *An. arabiensis*, and *An. quadriannulatus*) reported that, independently of the spatial orientation of the offered host (a person sitting on a stool and therefore in a vertical position, or a person lying on the ground in a horizontal position), mosquitoes always fed on body parts that were closer to the ground (Dekker *et al.*, 1998), suggesting that *Anopheles* mosquitoes land more on objects nearer the ground. Thus, as horizontal targets placed on the ground have larger areas in the space where mosquitoes land more, it can be supposed that these targets might elicit a higher landing response compared to vertical targets. In contrast, a study conducted on *An. gambiae* reported that these mosquitoes often first approached the head region of a human seated on a stool, and then moved to the feet at a later stage (de Jong and Knols, 1995). This indicated that mosquitoes approached the target even if this was in a vertical position, and moreover, they approached the target at an appreciable distance from the ground. The discrepancies found in the studies reported above reveal that much is still unclear on the possible effects that the target's spatial orientation might have on *Anopheles* attraction. It is important to note that the two last-mentioned studies used a whole human as the target, thus the surface might have offered a full array of cues that had slight variations depending on the body part (e.g. different perspiration rates on different parts of the body, different temperatures). As these factors could influence mosquito response, it would be recommended to carry out a structured study with targets that present

defined and controlled set of cues, if one aims to elucidate landing preferences with respect to the spatial orientation of the target.

In the past, the use of 3D tracking systems has substantially augmented the understanding of mosquitoes host-seeking behaviour (Beeuwkes *et al.*, 2008; Dekker and Carde, 2011; Spitzen *et al.*, 2013; Hawkes and Gibson, 2016; Cribellier *et al.*, 2018, 2020). For example, results from these studies have described *Anopheles* flight behaviour when inside and outside of odour plumes (Beeuwkes *et al.*, 2008), the behavioural responses to visual cues (both in presence and absence of an odour cue) (Hawkes and Gibson, 2016), and the flight response to thermal cues (Spitzen *et al.*, 2013). Recent studies demonstrated how by using a tracking system it was possible to break down mosquitoes flight behaviour around odour-baited traps alone (Cribellier *et al.*, 2018) and traps augmented with heat and humidity (Cribellier *et al.*, 2020). These studies highlighted the importance of understanding flight dynamics around trapping devices if one is to develop trap improvements. The authors demonstrated that the capture rate of a trap was significantly influenced by its orientation (i.e. standing traps captured fewer mosquitoes compared to hanging traps) (Cribellier *et al.*, 2018). This phenomenon was completely attributed to the stereotypical flight response that mosquitoes displayed when they approach the odour-baited traps (i.e. mosquitoes rapidly flew upwards when close to the trap). Furthermore, the tracking system allowed the researchers to quantify the time mosquitoes spent in the proximity of the trap, which helped demonstrate that the higher capture rates in traps augmented with heat and humidity were the result of the increased time spent flying around the trap (Cribellier *et al.*, 2020). These findings would not have been possible without a moment-to-moment tracking system that allowed the collection of accurate 3D coordinates at a high temporal resolution. Ultimately, detailed information on flight dynamics enables accurate characterisation of mosquito host-seeking and flight behaviour (Jones *et al.*, 2021). Although the potential of 3D video tracking technology has been clearly demonstrated, only few studies have considered this approach when exploring mosquitoes response to different targets (Cribellier *et al.*, 2020). Thus, many aspects of trap attraction are still unclear and open to be explored.

Building from these considerations and results obtained in the previous chapter, this following study sets out to explore mosquito landing response in relation to specific target properties. In particular, three target characteristics were examined, each in a separate subset of experiments: 1) the size (spatial dimensions) of a target, 2) the spatial orientation (i.e. vertical position and horizontal position) of the target, and 3) the proportion of surface area heated on a consistent visual target. The first two characteristics were studied using behavioural choice-assays to determine mosquito preference for the different variants of the characteristics, while for the third characteristic a similar approach as taken in Chapter 4 was adopted, with a no-choice assay to determine the threshold of the size under which the thermal cue was no longer affecting the landing response. The following hypotheses were set out:

- H_0: The number of mosquitoes landing on a large target is the same as the number landing on a small target.
  H_1: The number of mosquitoes landing on a large target is greater than the number landing on a small target.

- H_0: The number of mosquitoes landing on a target positioned vertically is the same as the number landing on a target positioned horizontally.
  H_1: The number of mosquitoes landing on a target positioned vertically is greater than the number landing on a target positioned horizontally.

- H_0: The number of mosquitoes landing on the target is the same irrespective of the proportion of the target area that is heated.
  H_1: The number of mosquitoes landing on a target with a larger heated area is greater compared to the number of mosquitoes landing on a target with a smaller heated area.

For this last point, it was further hypothesised that the effect on the landing response of a small heated area would be negligible, and therefore, no difference in landing rate was expected between the treatment where no area was heated and the treatments with small areas heated.

A 3D video tracking system was used to record flight tracks of *An. coluzzii* mosquitoes approaching the different targets. Thus, this chapter also explores flight parameters (i.e. flight speed, tortuosity, time spent in proximity of the target, and flight distance covered in proximity of the target) of host-seeking females when presented with the different targets described above. For this part of the study, the following hypotheses were tested:

- H_0: Mosquitoes display similar flight parameters (i.e. flight speed, tortuosity, time spent in proximity of the target, and flight distance covered in proximity of the target) when flying around two different types of targets. H_1: Mosquitoes display differences in their flight parameters when flying around two different types of targets.

- H_0: Mosquitoes visit different target types in an equal number of times. H_1: There is a difference in the number of times mosquitoes visit different targets.

5.2.   Materials and Methods

5.2.1. Mosquitoes

Mosquitoes were reared as described in section 3.1 and were prepared and selected for experiments following protocols presented in Chapter 4 (section 4.2). Accordingly, adult mosquitoes were kept in lightboxes that were each set to transition between light and darkness at different times throughout the day, which allowed performing assays at different times of the day using mosquitoes always in the first three hours of the scotophase. Sugar feeders were removed four hours before each assay. Each experimental group consisted of 25 females (only on rare occasions 26 females were used instead), all between four to twelve days old, all of which were randomly selected from a cage, and therefore all individuals belonged to the same age group, which was noted each time. The mosquitoes were transferred into small WHO test tubes (4.5 cm of diameter and 12 cm of height) using a mouth aspirator and were kept in darkness for one hour to allow the eyes to adapt to low levels of light (Sato, 1957; Moon *et al.*, 2014).

5.2.2. Wind tunnel

The wind tunnel and the conditions inside the flight arena are described in section 3.2. Temperature and relative humidity inside the arena were recorded before and after each assay to ensure that environmental conditions were maintained within the desired range.

5.2.3. Odour source

As results from Chapter 4 indicate that independently of the landing surface offered, in absence of host odour the landing behaviour is negligible, host odour was offered in all experiments presented here. Human body odour was collected on nylon socks and delivered as described in section 4.2.3. In addition, carbon dioxide was also dispensed using a similar method as presented in section 4.2.3, with the only difference being that the stimulus was introduced in a pulsed manner (eight seconds on and seven seconds off) which was achieved using a timed valve switcher. This was done to resemble a rhythm of four human breaths per minute, as previous studies have shown that carbon dioxide is more attractive when presented in pulses (Gillies, 1980; Geier *et al.*, 1999; Dekker *et al.*, 2001; Dekker and Carde, 2011).

5.2.4. Landing target

When not otherwise specified, the landing surfaces were placed horizontally on the wind tunnel floor and were composed of 3 mm thick IR transmitting black acrylic sheets (Southern Acrylics, UK) of different dimensions and over which rested transparent plastic bags (Polybags, UK) filled with water (Fig. 26), which were used to control the temperature of the target. The plastic bags were custom made depending on the size and shape needed and were sealed with a plastic sealer. To maintain the final depth of the target surface consistent across assays, the bags were filled using a fixed volume of water per surface unit (700 mL of water per 400 cm^2), thus the final volume of water was adjusted depending on the area of each bag giving a consistent final target thickness of approx. 3 cm. To provide the thermal cue, the plastic bags were immersed and heated in a

water bath (JB Aqua 12 Plus, Grant Instruments, UK). As results from Chapter 4 found no difference in the landing rate on targets at 35 °C and 45 °C, it was decided to heat the bag to 38 ± 2.00 °C, which allowed for the bags to cool slightly during the experiment yet remain warm enough to give a thermal cue similar to human body temperature (mean ± SEM final temperature=34.10 ± 1.00 °C). The initial and final temperatures of all target surfaces were recorded in each replicate to ensure that the desired conditions were maintained. To capture mosquitoes landing, the targets were covered with a layer of transparent adhesive film (FICSFILM, Barrettine, UK) (Fig. 26).
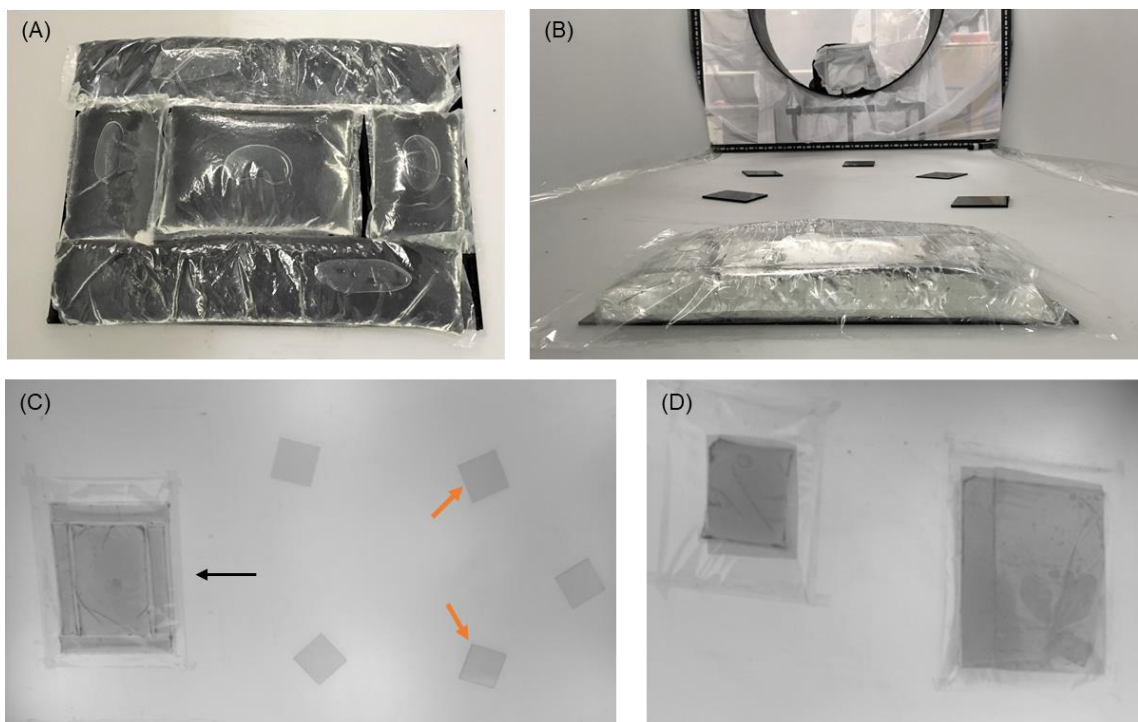
Figure 26: Landing target composed of an IR transmitting black acrylic sheet with transparent plastic bags filled with water. (A) Landing target viewed from above, (B) side view of the landing target covered with the transparent adhesive film, on the back is possible to distinguish the rest of the flight arena and the release cage. (C) Aerial view of a section of the flight arena during experimental conditions, the black arrow indicates the target while orange arrows indicate the black squares used to facilitate optomotor navigation. (D) Aerial view of the targets during a choice-assay. Note that photos (C) and (D) were taken with IR sensitive cameras with IR illumination coming from below the wind tunnel floor.

As the landing targets varied depending on the experiment, a more detailed description of each target is given below.

For Experiment 1 (Target Size): two rectangular targets of different sizes were used, one being a large target (30 cm x 40 cm, total area=1200 cm^2) and the other being a quarter of the size (15 cm x 20 cm, total area=300 cm^2). Plastic bags filled with water were created with sizes matching the two different dimensions and were paired correspondently with the same size IR transmitting black acrylic sheet. All plastic bags presented here were heated to provide uniform heat at approximately human body temperature across the area of the target.

For Experiment 2 (Target Orientation): only one rectangular target size was tested (30 cm x 40 cm) but the target could be placed either horizontally (lying flat on the wind tunnel floor) or vertically (at a 90° angle to the wind tunnel floor, with the short side in contact with the floor). While for treatments using the horizontal target the plastic bags were created by only sealing the four external sides (creating a rectangle), for treatments using the vertical target the plastic bags were further sealed multiple times across the short side of the rectangle, every ~8 cm, creating thus five roughly cylindrical cells filled with water (Fig. 27). This was done so that the entire plastic bag could be filled evenly with water, even when standing in a vertical position. All plastic bags presented here were heated uniformly to approximately human body temperature. When positioning the target in a vertical orientation, both sides of the IR transmitting black acrylic sheet were exposed, and consequently, plastic bags were added on both sides to give a uniform thermal cue across both sides of the target, creating thus a "sandwich-like" structure. To allow this structure to stand in a vertical position, a base was constructed using two inverted transparent plastic cups (10 cm of diameter) on which two incisions were made that allowed the rigid acrylic sheet to be inserted and held in place (Fig. 27).

Figure 27: Front view of the vertical target. *The three red arrows point to the cylindric-like structure filled with water, while the yellow arrows indicate the two inverted transparent plastic cups that supported the structure in a vertical position.*

<u>For Experiment 3 (Target Heat Signature)</u>: in this experiment, the size of the target was constant (30 cm x 40 cm) but the surface area of the target that was heated changed depending on the treatment, so that either all, half (hereafter ½), a quarter (hereafter ¼), an eighth (hereafter ⅛), or none of the surface was heated. Accordingly, plastic bags of the dimensions listed in Table 5 were created. For the positive control (all heated) and the test treatments (½, ¼, and ⅛) the bags covering the central surface were heated in the water bath while the bags covering the lateral surfaces, when present, were maintained at room temperature (mean ± SEM temperature=25.0 ± 1.00 °C). This gave an even surface both in height and visual cues offered, as the entire surface of the target was covered with the plastic bags. Furthermore, in the ½, ¼, and ⅛ treatments, the lateral bags help maintain the lateral surface at room temperature while the centre of the target presented the thermal cue. For the negative control (no area heated), the entire surface of the target was kept at ambient temperature, i.e. no plastic bags were warmed.

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Table 5: Different treatments and the corresponding sizes of plastic bags used to compose the target. A diagram that indicates the position of the bags in the five different treatments is presented in Fig. 28.

Treatment	Size of central (heated) surface	Sizes of lateral (room temperature) surfaces
All area heated (positive control)	30 cm x 40 cm	N/A
½ area heated	20 cm x 30 cm	Four strips of 5 cm x 30 cm
¼ area heated	15 cm x 20 cm	Two strips of 7 cm x 40 cm and two strips of 8 cm x 15 cm
⅛ area heated	10 cm x 15 cm	Two strips of 10 cm x 40 cm and two strips of 12 cm x 10 cm
No area heated (negative control)	N/A	30 cm x 40 cm

5.2.5. Experimental procedure

Experiment 1 (Target Size): consisted of a choice-assay, in which two different size targets, large and small, were presented simultaneously in the upwind end of the wind tunnel (Fig. 28). Three different assays were carried out: small vs small (two small targets presented together), large vs large (two large targets presented together), and large vs small (a large target presented alongside a small target, hereafter referred to as "competitive assay"). The small targets were placed 15 cm from the lateral walls (X-axis) while the large targets were 13 cm from the lateral walls. Independently of the size, the two targets were positioned 30 cm from the upwind net (Y-axis).
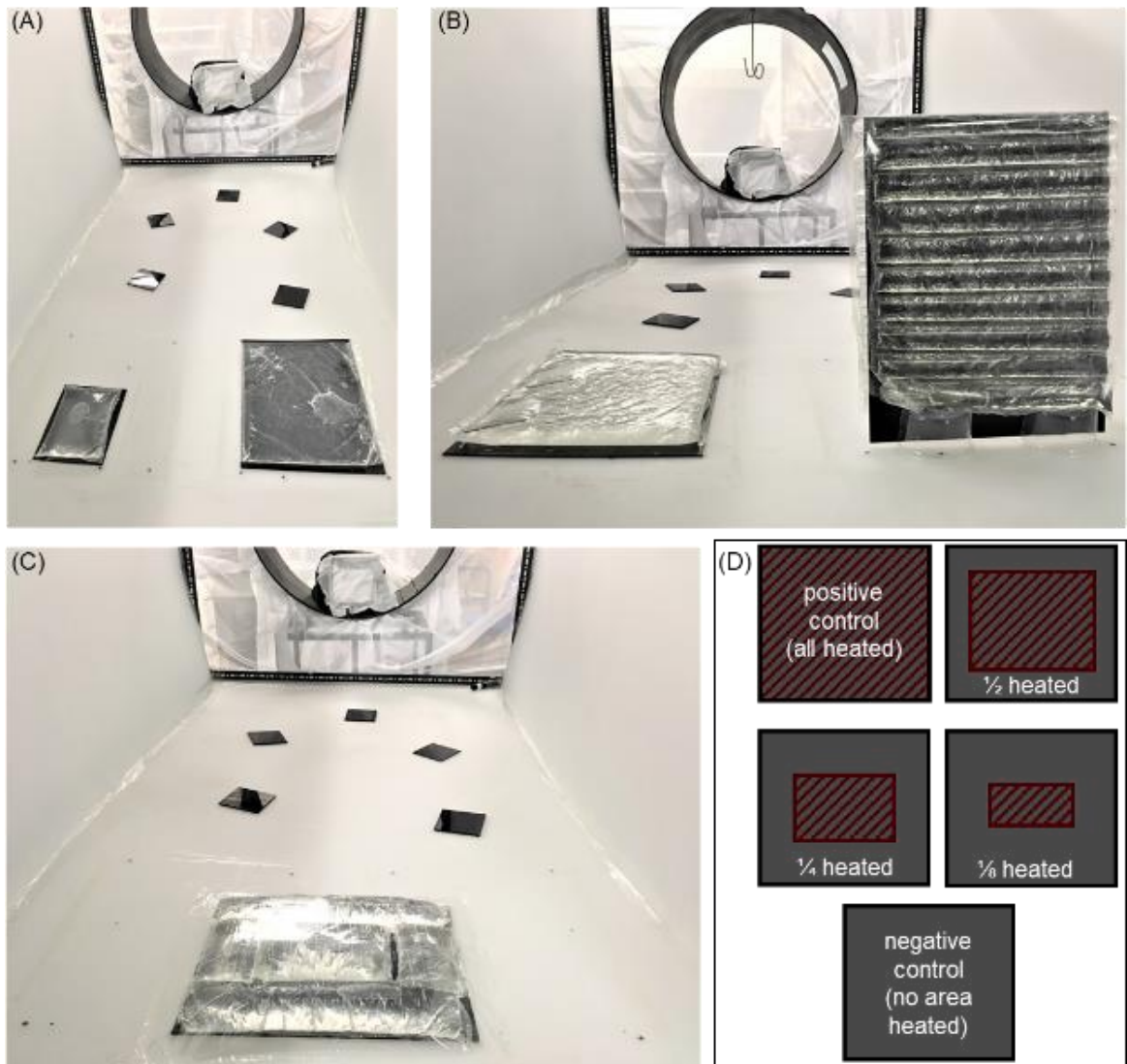
Figure 28: Overview of all three experiments. *Panel (A) shows both target types (the small on the left and the large on the right) presented together in the competitive assay in Experiment 1. Panel (B) shows both target orientation (the horizontal on the left and the vertical on the right) presented side by side in the competitive assay in Experiment 2. Panel (C) shows the target surface presented in Experiment 3, the different plastic bags composing the target can be distinguished. Panel (D) indicates the five treatments presented in Experiment 3, the red striped area shows the heated area while the remaining surface presented no thermal stimulus.*

Experiment 2 (Target Orientation): was also a choice-assay. Two targets were offered in each assay, both positioned 30 cm from the upwind net (Y-axis), with 20 cm in between them and both being located at least 13 cm from the lateral wind tunnel walls (Fig. 28). The spatial orientation was tested in three different assays: vertical vs vertical (two vertical targets presented together), horizontal vs horizontal (two horizontal targets presented together), and vertical vs

horizontal (vertical target presented alongside a horizontal target, hereafter referred to as "competitive assay").

In Experiment 3 (Target Heat Signature) a single target surface was presented horizontally in the centre of the X-axis and 30 cm from the upwind net (Y-axis) (Fig. 28). The size of the heated area (which was in all cases positioned at the centre of the target surface) varied between treatments. Five different treatments were tested: all surface heated (positive control), ½ of the surface heated, ¼ of the surface heated, ⅛ of the surface heated, and none of the surface heated (negative control).

Depending on the experiment and on the assay, prior to the commencement of the replicate, the corresponding target surfaces were created. The lights in the room were turned off, the 25 mosquitoes were then transferred into the release cage and were given five minutes to habituate to the wind tunnel conditions. After this time the odour cues were initiated, the release cage was opened remotely, and TrackIt3D started recording. The experimenter then left the room to avoid odour contamination and only returned after 15 min, when the assay was terminated. The release cage was then closed, TrackIt3D was stopped, and the number of mosquitoes recovered in different parts of the wind tunnel was counted as described in section 4.2.4. For assays in Experiment 2, where targets were presented vertically, a note was made on the number of mosquitoes landing on the surface of the downwind side of the target (facing the release cage) and on the upwind side (facing towards the incoming air and odour sources). For assays in Experiment 3, the number of mosquitoes landing on the heated area and the number of mosquitoes landing on the unheated margins of the target were also recorded separately.

The temperature and humidity inside of the flight arena at the beginning and end of each replicate were recorded. The temperature of the target surfaces was likewise checked at the beginning and end of each assay to ensure that the temperature of the thermal cue presented stayed between the desired range.

Surgical gloves were worn throughout all experiments any time the wind tunnel or experimental material needed to be handled. Wind tunnel surfaces and fabric components were washed as indicated in section 4.2.4 at the beginning of each experimental week.

Twelve replicates of Experiment 1 and ten replicates of Experiment 3 were carried out for each treatment. For Experiment 2, twelve replicates were carried out for the horizontal vs vertical assay, and the vertical vs vertical assay, while for the horizontal vs horizontal assay only one replicate was carried out per experimental day, giving a total of five replicates. This was done because the large vs large assay in Experiment 1 was the same as the horizontal vs horizontal assay of Experiment 2, and therefore the replicates from both experiments were combined into a pooled data set that consisted of a total of 17 replicates. Assays within the same experiment were tested in a quasi-randomised order, between and within days to control for the effect of testing sequence (see section 3.3). Furthermore, to exclude the effect of bias given by the position of the target within the flight arena, i.e. left or right side, the position of each target in each replicate was also quasi-randomised (see section 3.3).

5.2.6. Lighting, cameras, and 3D tracking

A set of 28, twelve-LED high-power IR lights with 90° beam angle (JC, UK) were arranged around and underneath the wind tunnel and were directed towards white cloths to create a diffused and evenly distributed IR light background. This was suitable for detecting mosquito silhouettes from above. Mosquito 3D flight tracks were obtained using TrackIt3D (version 3.0, SciTrackS GmbH, Switzerland), a software that allowed to record in real-time mosquitoes flying and that converted such information into 3D coordinates. To record the tracks, two offset 2D images of the flight arena were captured with a frequency of 50 frames per second by two high-resolution analogue cameras (acA2440 – 75 µm, Basler, Germany) fitted with HF6XA-5M lenses (Fujinon, Japan) and IR filtered (LP830 band pass, Midopt, USA), which restricted the cameras to only detect changes in illumination above 800 nm (IR light). The two cameras were suspended ~ 60

cm above the flight arena and were oriented with an angle that allowed them to have an overall field of view approximately equal to the entire flight arena (Fig. 14). Tracks were only recorded when mosquitoes were visible to both cameras simultaneously. When a mosquito flew over areas where its silhouette was not sufficiently distinguishable, the software terminated the track and a new track was created when the mosquito became visible again. Tracks were also interrupted when multiple mosquitoes intersected each other's path, and new tracks were then created as soon as the software was able to discriminate once more the individual mosquitoes. Note that as described in section 3.2, a set of warm white LED lights provided visible dim lighting (equivalent to moonlight illumination) that allowed mosquitoes to use visual cues for optomotor navigation. This light however did not influence the tracking system as the cameras could only detect IR light.

5.2.7. Post-processing of tracks

At the end of each replicate, TrackIt3D computed the 3D coordinates of mosquito flight tracks and created an Excel (.csv) file containing these at a temporal resolution of 0.02 s. Each coordinate was notated in the three Euclidean dimensions (X, Y, Z), with a time stamp. The raw Excel file was then run through TrackIt3D post-processing software which filtered erroneous data points, e.g. those beyond the arena limits; 0.9% of the total recorded points were discarded. Furthermore, the software joined individual coordinates to create continuous tracks which were smoothed using an *ad hoc* spline function created based on mosquito flight parameters, as suggested by Spitzen and Takken (2018). Where needed, the software automatically interpolated up to five consecutive missing coordinates; tracks that had more than five missing points were treated as two separate track segments. The final output was then processed using a custom-made Phyton programme (version 3.9, Python Software Foundation, 2020) (Mandelli and Carnaghi, 2022b) that further filtered out tracks that had fewer than 25 consecutive coordinates or lasted less than 0.5 s. Additionally, tracks that had a total displacement in all three axes of less than 1 cm were individually inspected and, if deemed unsatisfactory (i.e. artefacts or outliers),

were removed from the analysis. This threshold value was chosen after visually inspecting a histogram of the displacement across all tracks' during all assays (Fig. 29), where it emerged that most of the artefacts created by TrackIt3D were tracks composed of multiple points very close to each other (Fig. 30). These artefacts were caused by minor fluctuations in the IR illumination (Spitzen *et al.*, 2014) and could be clearly distinguished from tracks generated by flying mosquitoes (Fig. 30). An overview of the number of total tracks recorded in each replicate and the percentage of tracks removed from the analysis is given in Table S1 in Appendix B. Using the custom-made programme several parameters were calculated (Table 6).
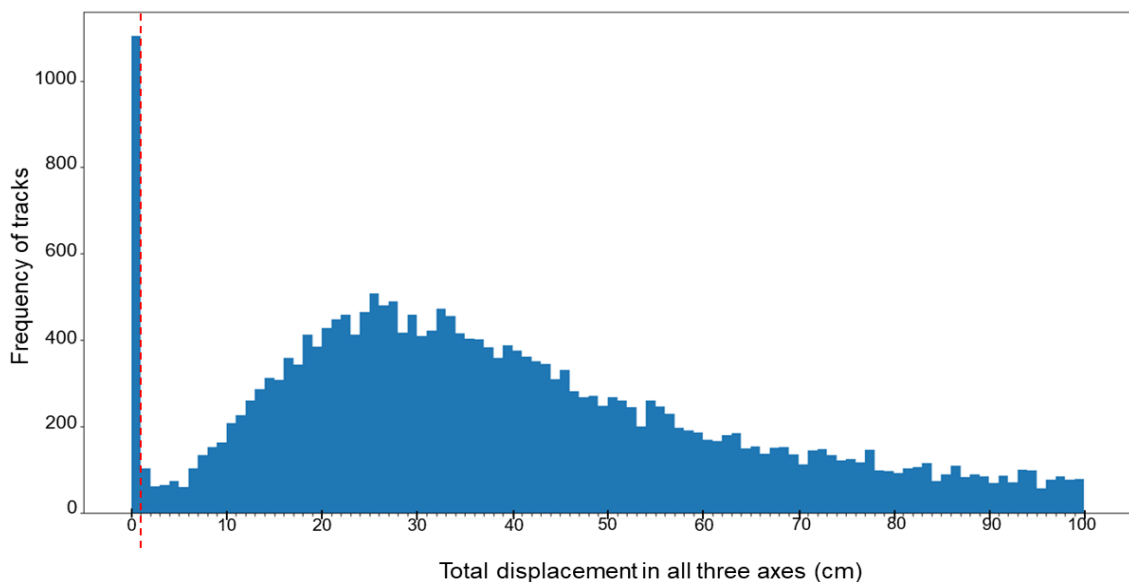
Figure 29: Frequencies of total 3D displacement (in cm) of the tracks recorded in all experiments. *The red dashed line indicates the selected threshold of 1 cm under which tracks were removed from the analysis as deemed to be artefacts.*
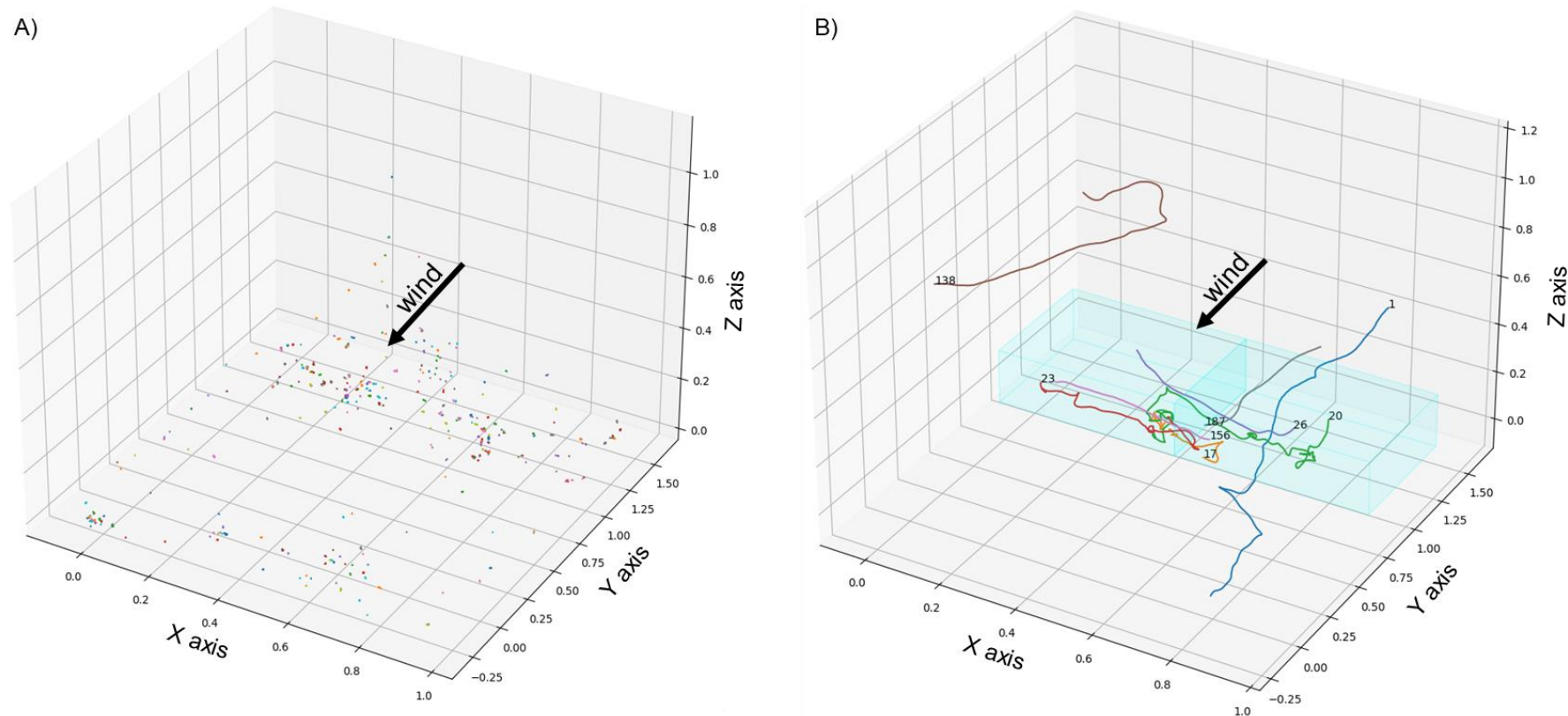
Figure 30: Visual representation of example tracks recorded by TrackIt3D in a trial. *Panel (A) shows point-type artefacts that resulted from IR illumination disturbance, while panel (B) shows tracks generated by flying mosquitoes. The black arrow in each panel indicates the wind direction. The number of each track is positioned at its end point. The two light blue cuboids (A) indicate the area of interest where the targets were positioned in dual-choice assays (see section below). Track number one and 138 were created by mosquitoes that flew outside of the areas of interest, while the other tracks were created by mosquitoes that flew inside the area of interest and landed on the targets.*

Table 6: Track parameters analysed with the custom-made programme. *Note that the notations "i" and "n" correspond to the initial data point and the final data point respectively of either the complete track or a segment of the track.*

Parameter	Definition	Equation	Unit
3D linear distance	Shorter 3D distance between the first and last data point of a track, which is created by a straight line that joins the two points. Note the number is always positive.	$\sqrt{[(x_n - x_i)^2 + (y_n - y_i)^2 + (z_n - z_i)^2]}$	m
Total 3D distance (for an individual track)	Total 3D distance between the first and last data point of a track. This is calculated by adding all the 3D linear distances of all the consecutive points of a track, thus forming the 3D path of a given track.	$\sum_{i=1}^{n-1} \sqrt{[(x_{i+1} - \chi_i)^2 + (y_{i+1} - y_i)^2 + (z_{i+1} - z_i)^2]}$	m
Total track time (for an individual track)	Time between the first and last data point of a track or segment of a track.	$t_n - t_i$	s

Parameter	Definition	Equation	Unit
3D linear speed	Total 3D distance covered per time frame. This is calculated by dividing the total 3D distance by the total time elapsed between the points under consideration.	$$\dfrac{\sum_{i=1}^{n-1}\sqrt{[(x_{i+1}-\chi_i)^2+(y_{i+1}-y_i)^2+(z_{i+1}-z_i)^2]}}{t_n-t_i}$$	m/s
Tortuosity index	Index that indicates the convolutedness of a track. It is calculated as the ratio between the 3D linear distance and the total 3D distance. The values can vary from zero to one, where low values indicate a convoluted track (i.e. a 3D path that is longer compared to the shorter 3D distance between the two points), while values close to one indicate straighter tracks.	$$\dfrac{\sqrt{[(x_n-x_i)^2+(y_n-y_i)^2+(z_n-z_i)^2]}}{\sum_{i=1}^{n-1}\sqrt{[(x_{i+1}-\chi_i)^2+(y_{i+1}-y_i)^2+(z_{i+1}-z_i)^2]}}$$	N/A

Each replicate generated a vast number of tracks (Fig. 31). Tracks that were included in the analysis were those that entered specific areas of interest (Fig. 31). These were defined as cuboids that encompassed the areas where the traps were positioned plus a 10 cm buffer in the X and Y-axes to allow for small variations of trap position between replicates (Table 7). The height of the cuboids was 30 cm for all targets positioned horizontally and 60 cm for vertical targets. These heights were determined after heat maps of track densities found these areas contained a very high proportion of all tracks.
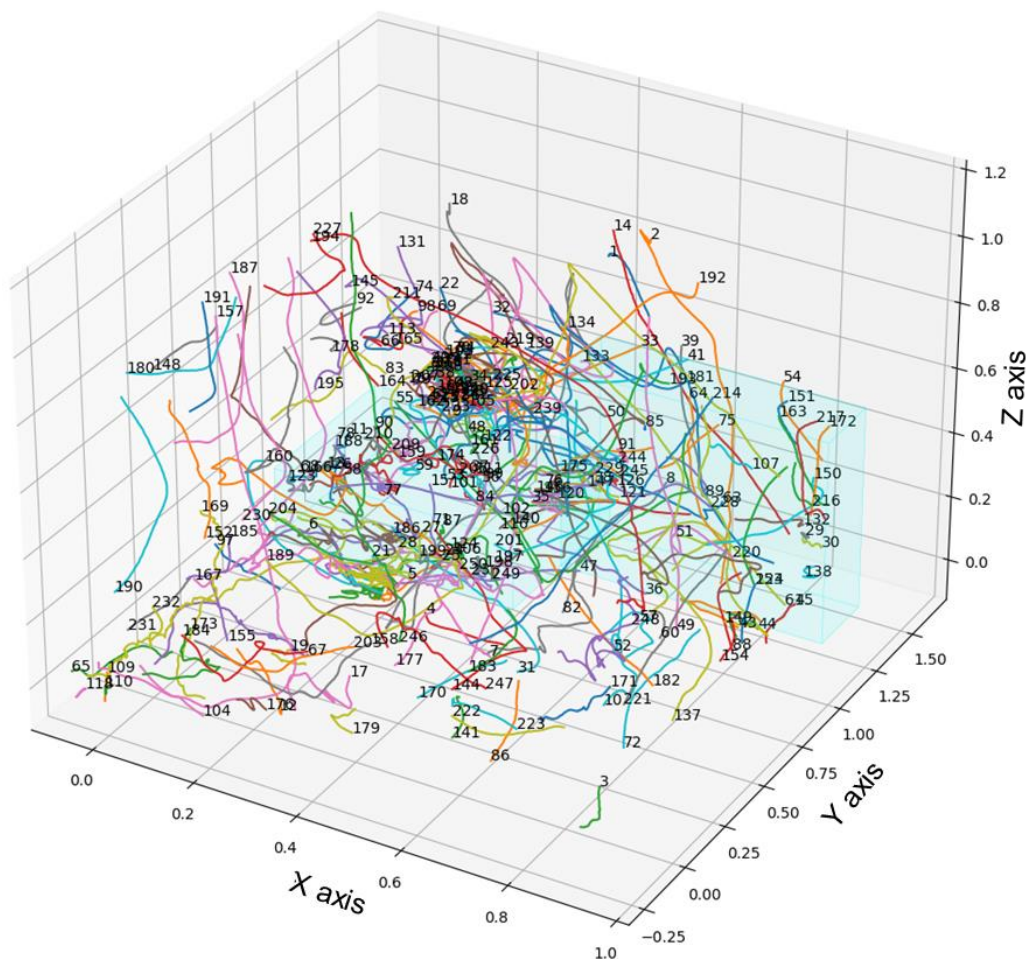
Figure 31: Example of all the filtered tracks recorded by the tracking system in a trial assay. *Areas of interest, here represented in light blue, were chosen depending on the type of targets used. In this example, the target on the left was positioned horizontally, while the target on the right was placed vertically.*

Table 7: Dimension of the areas of interest for each target type. *A detailed explanation of the reasoning behind creating the "neutral" box is presented in the paragraph below).*

Landing surface type	Dimension of target	Dimension of cuboid
Large horizontal surface (used for Experiment 1 and Experiment 3)	30 cm (X-axis) x 40 cm (Y-axis) x 3 cm (Z-axis)	50 cm (X-axis) x 60 cm (Y-axis) x 30 cm (Z-axis)
Small horizontal surface	15 cm (X-axis) x 20 cm (Y-axis) x 3 cm (Z-axis)	35 cm (X-axis) x 40 cm (Y-axis) x 30 cm (Z-axis)
Vertical surface	30 cm (X-axis) x 6 cm (Y-axis) x 40 cm (Z-axis)	50 cm (X-axis) x 45 cm (Y-axis) x 60 cm (Z-axis)
Neutral box (i.e. no target)	N/A	50 cm (X-axis) x 60 cm (Y-axis) x 30 cm (Z-axis)

For each replicate the following track parameters were analysed: total number of visits, total time of all track segments, total 3D distance of all track segments, track tortuosity, and track speed, which were calculated as per Table 6. To determine whether certain flight parameters (e.g. tortuosity, speed) were different for tracks recorded around the targets compared to other parts of the wind tunnel where no targets were provided, a box of the same dimensions as the one chosen for the large horizontal surface was created and was positioned in the downwind end of the wind tunnel, where no target was presented (Fig. 32). Hereafter this cuboid will be referred to as "neutral" box.
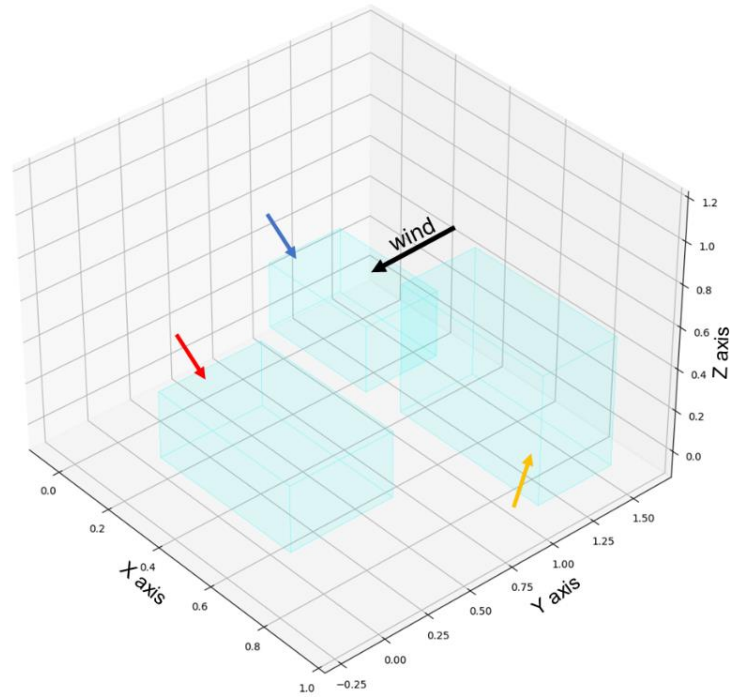
Figure 32: Position of the designated areas of interest in the wind tunnel. The blue and yellow arrows on the upwind side indicate the cuboids where the targets were positioned (in this example, the blue arrow indicates the cuboid for the small target while the yellow arrow indicates the cuboid for a vertical target). The red arrow indicates the position of the neutral cuboid, where no target was presented. The black arrow indicates the wind direction. Note that due to the perspective of the 3D representation, the Y-axis is compressed.

As multiple mosquitoes were released in each assay, it was not possible to attribute individual tracks to a specific mosquito and therefore each recorded track was treated as an independent event. Heat maps, histograms, and the 3D representation of tracks were produced with the custom-made Phyton program (version 3.9, Python Software Foundation, 2020) (Mandelli and Carnaghi, 2022b).

5.2.8. Analysis and statistics

Landing analysis

For Experiment 1 and Experiment 2, a linear mixed effects model on log-transformed data was used to analyse differences in the number of landings on different surfaces throughout all treatments and assays. This model was chosen as it allowed to control for pseudoreplication associated with the experimental design (i.e. both Experiments were a choice-test, with two targets

presented simultaneously). The treatment, i.e. the target type, intended for example as large or small, the side where the target was positioned within the wind tunnel (i.e. target presented on the left or the right), and the type of the other target presented simultaneously were introduced in the model as factors. The factor side did not have any significant effect on the number of landings for Experiment 1 nor for Experiment 2. This confirmed the symmetrical property of the wind tunnel; therefore, the factor side was removed from the model. An ANOVA was used on the linear mixed effects model and P-values were extracted using an *ad hoc* function (anomer), where the residual degrees of freedom were adjusted to allow for the design effect, calculated from the intra-cluster correlation coefficient. To compare the number of landings on the upwind surface versus the downwind surface of vertical targets in Experiment 2, a Wilcoxon signed-rank test for related samples was used. Similarly, landing preference in Experiment 1 and Experiment 2 for the assays where different target types were presented simultaneously (i.e. the competitive assays), was also determined using a Wilcoxon sign-ranked test for related samples.

Data from Experiment 3 were analysed using a GLM with negative binomial errors and log link. Differences in the number of mosquitoes found on the landing targets in different treatments were assessed using a one-way analysis of deviance, followed by multiple comparisons of means with Tukey's tests.

To standardise the number of landings on different targets independently of the area of the exposed surface, densities were calculated by dividing the raw number of mosquitoes recovered on a target by the area of the target in cm^2. This was then converted into number of landings per 100 cm^2. For Experiment 1 and 2 the differences of densities between different target surfaces were analysed using a non-parametric test for independent samples (Mann Whitney U test). In this case, the densities compared came from separate assays (e.g. densities of the small target on the small vs small assay were compared against the densities of the large target on the large vs large assay). On the other hand, to analyse the differences in densities between the non-heated margin areas and

the heated central areas in Experiment 3, a Wilcoxon signed-rank test for related samples was used.

For all three experiments, mosquito age was added to the existing statistical models as a factor and an analysis of deviance was used to determine if its introduction produced any significant change in the model. For this analysis, data from all three experiments were log-transformed. As age did not have a significant impact on the landing behaviour it was excluded from further analysis.

Analysis of 3D data

For all assays, the number and length of tracks recorded on the left side of the wind tunnel were compared with the number and length of tracks recorded on the right side of the wind tunnel. As no difference was found between the tracks recorded on either side, it was concluded that symmetry within the wind tunnel was maintained and therefore the factor side was excluded from the models.

Within each experiment, the number of times a target was visited by a mosquito (i.e. the number of tracks recorded in the area of interest) were compared between different targets and different assays using a GLM with negative binomial errors and log link, where assay and treatment (i.e. the target type) were introduced in the model as independent factors. For the analysis of the number of visits in the upwind section or downwind section of vertical targets, the factor target section was introduced in the model as an independent factor. Multiple comparisons of means were carried out using a Tukey's *post hoc* test.

The remaining parameters obtained with the tracking analysis were compared between treatments and assays from the same experiment using a two-way ANOVA for Experiment 1 and 2, and a one-way ANOVA for Experiment 3, where assay and treatment (i.e. the type of target), were introduced as the two independent variables. Data were assessed prior to the analysis to ensure that ANOVA's assumptions were respected, and for two parameters (total time spent in the area of interest, and total distance recorded in the area of interest), data were log-transformed prior to being introduced into the model. For tortuosity and linear speed, data obtained from the areas of interest were also compared with

the data obtained from the area where no visual and thermal cues were offered. To do so, the data from the neutral box (i.e. the no target box) were obtained for each replicate and then were pooled together to create a new dataset (i.e. all data deriving from the neutral box from all replicates in all assays were combined) as no difference was found in the parameters recorded in the neutral box between different assays. This was carried out separately for each experiment. The values obtained from the areas of interest were compared with the pooled neutral box dataset for each corresponding experiment.

5.3. Results

In total, 114 assays were conducted and deemed satisfactory to be included in the analysis (i.e. all conditions were maintained stable during the assays), thus a total of 2855 *An. coluzzii* females were used. On average, 25.04 ± 0.02 SEM mosquitoes were released per assay throughout all experiments.

Throughout all three experiments, the average percentage of mosquitoes activated was 68.30 ± 1.43% SEM. The proportion of mosquitoes activated was also calculated separately for each assay of each experiment. These proportions were all similar to the average proportion given above, with a difference of ± 10% (the lowest proportion was recorded in Experiment 3, in treatment ¼ heated, mean=58.70%, while the highest proportion of activated mosquitoes was recorded in Experiment 2, in the vertical vs vertical assay, mean=74.40%). Activation levels were in line with levels obtained in Chapter 4 and Hawkes and Gibson (2016).

5.3.1. Experiment 1

Both the size of the target itself and the size of the competing target had a significant effect on the landing response of *An. coluzzii* (ANOVA on linear mixed effects model, F=11.25, d.f.=1, 1, P=0.001; F=5.43, d.f.=1, 61, P=0.02, respectively). Significantly more mosquitoes landed on the large target than the ones that landed on the small target when the results of the non-competitive assays were compared, i.e. comparing the landings on the large target in the

large vs large assay with the landings on the small target in the small vs small assay (mean ± SEM number of mosquitoes recovered from a small target=2.5 ± 0.39, mean ± SEM number of mosquitoes recovered from a large target=4.7 ± 0.5; ANOVA on linear mixed effects model, F=10.38, d.f.=1, 65, P=0.002) (Fig. 33).
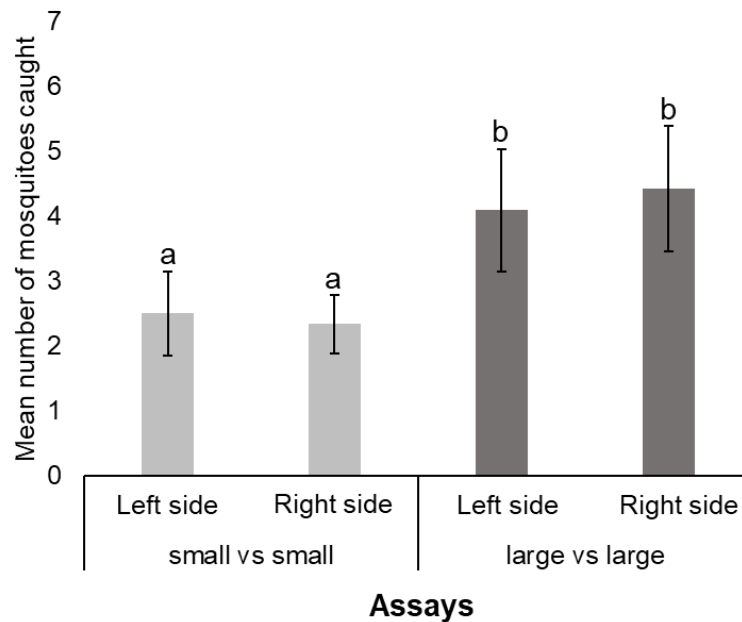
Figure 33: Mean number of mosquitoes that landed on the two target types in assays where the targets were presented alongside a similar target in Experiment 1. *Note that for ease of interpretation the bars presented here display the raw means. Statistical analysis was performed on log-transformed data. The colour of the bars indicates the target type, the error bars indicate the SEM. Different letters denote significant differences between the number of mosquitoes recovered on the targets in different assays (ANOVA on linear mixed effects model, P<0.05).*

In the competitive assay where both small and large targets were presented together, mosquitoes exhibited a strong preference for landing on the large target, with an average of 80.0 ± 4.87% SEM of the landings occurring on the large target, while only 20.0 ± 4.87% SEM of the landings were recorded on the small target (median number of mosquitoes on large target=5.00, median number of mosquitoes on small target=1.5; Wilcoxon test, n=12, W=0, P=0.002) (Fig. 34).
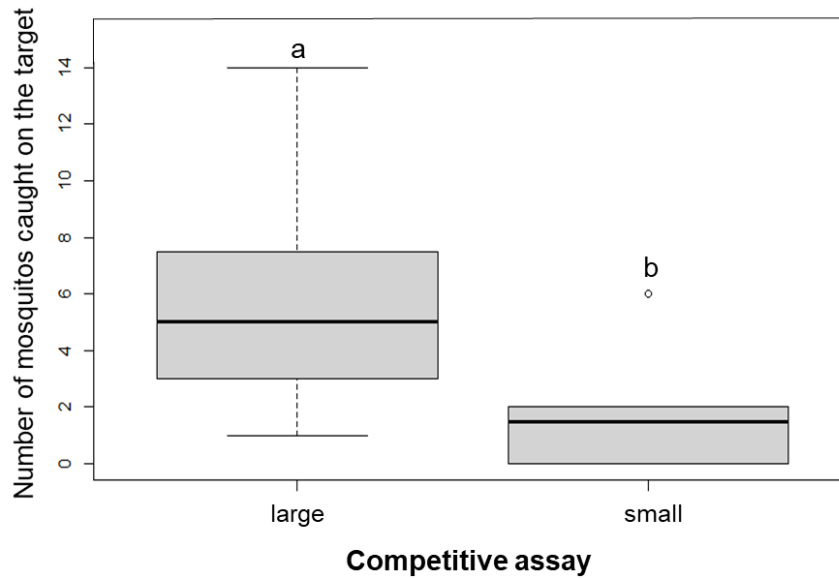
Figure 34: Number of mosquitoes that landed on the two target types in the competitive assay in Experiment 1. The black bars indicate the medians, the upper and lower limits of the boxes indicate the interquartile range. The whiskers indicate the maximum and minimum data points excluding outliers which are represented as small circles. Different letters denote a significant difference between the number of mosquitoes recovered on the different targets (Wilcoxon test, P<0.05).

On the other hand, a significantly higher density of mosquitoes (number of mosquitoes per 100 cm^2) was recovered on small targets (median=1.0) when compared to large targets (median=0.3; Mann-Whitney U test, n=24 per treatment, U=454.5, P<0.001).

3D tracking

A summary of the results from the analysis of flight parameters in Experiment 1 is given in Fig. 35.

The factor assay had a significant effect on the number of visits recorded around each target (GLM, chi-square=10.98, d.f.=2, P=0.004), while the factor treatment (i.e. target type) had no significant effect (GLM, chi-square=0.28, d.f.=1, P=0.60). Specifically, the small target in the competitive assay (i.e. large vs small) received significantly fewer visits compared to any other target (Tukey's test, with the large target in the large vs large assay: t=-2.74, P=0.03; with the large target in the competitive assay: t=-2.62, P=0.04; with the small target in the small vs small assay: t=3.42, P=0.003) (Table 8). This indicates that when presented

together, large targets drastically outcompete small targets in attracting mosquitoes. However, no difference was found in the number of times mosquitoes visited the large targets compared to the number of visits recorded around small targets when these were presented alongside targets of the same size (Tukey's test, t=0.81, P=0.85) (Fig. 35, Fig. 36), suggesting that when there is not a direct competition between target size, the small targets are found and visited as much as large targets. Thus, it appears that small targets remain highly attractive for mosquitoes, but their attractiveness is partially masked when they are presented alongside more attractive targets, i.e. larger targets.

The factor assay had a significant effect on the total time spent and the total distance covered around the different targets, while the factor treatment did not have an influence on these parameters (two-way ANOVA, for assay: F=4.41, d.f.=2, 68, P=0.02; F=3.94, d.f.=2, 68, P=0.02, respectively; for treatment: F=0, d.f.=1, 68, P=0.97; F=0.53, d.f.=1, 68, P=0.47, respectively). For both parameters, the only comparison that resulted in a significant difference was between the small target in the small vs small assay when compared to the results obtained on the small target in the competitive assay (Tukey's test, for total time: t=2.97, P=0.02; for total distance: t=2.77, P=0.04) (Table 8, Fig. 35). In specific, mosquitoes flew for longer and covered longer distances around small targets in the small vs small assay, whilst when the small target was presented alongside a large target, mosquitoes flew for less time and covered shorter distances around the small target.

Tortuosity varied significantly depending on the treatment (i.e. the target type), while the effect of the factor assay was only approaching significance (two-way ANOVA, for treatment: F=13.65, d.f.=2, 103, P<0.001; for assay: F=2.99, d.f.=2, 103, P=0.054). The tortuosity index of the tracks recorded in the proximity of the small targets in the small vs small assay was significantly lower compared to the tortuosity index of the tracks recorded in the box where no target was offered (neutral box) and also compared to the tracks recorded around the large targets, both in the large vs large assay and in the competitive assay (Tukey's test, t=-4.56, P<0.001; t=-3.74, P=0.002; t=-5.27, P<0.001, respectively) (Table 8,

Fig. 37). This indicates that when flying above or in the proximity of small targets, the flight tracks are more convoluted compared to the tracks recorded on areas where no visual or thermal cues are offered and compared to the areas where large targets are presented.

When examining the linear speed, a significant difference was detected in the analysis only when the areas of interest were compared with the neutral box, while no difference was detected between any assay (two-way ANOVA, for assay: $F=1.16$, d.f.=2, 103, $P=0.32$; for type of area, with or without the target: $F=30.41$, d.f.=2, 103, $P<0.001$) (Table 8). In particular, mosquitoes flew with a slower speed around any target type compared to the speed displayed when flying above a space without a target (Tukey's test, comparison between the large targets from the large vs large assay and the neutral box: $t=6.14$, $P<0.001$; comparison between the large target from the competitive assay and the neutral box: $t=3.54$, $P=0.005$; comparison between the small target from the competitive assay and the neutral box: $t=-4.44$, $P<0.001$; comparison between the small targets from the small vs small assay and the neutral box: $t=-6.80$, $P<0.001$) (Fig. 38). This indicates that the presence of either target elicits a slower flight, independently from the type of the target and the size of the other available target, while when flying in areas without a visual and thermal target the flight speed is higher.
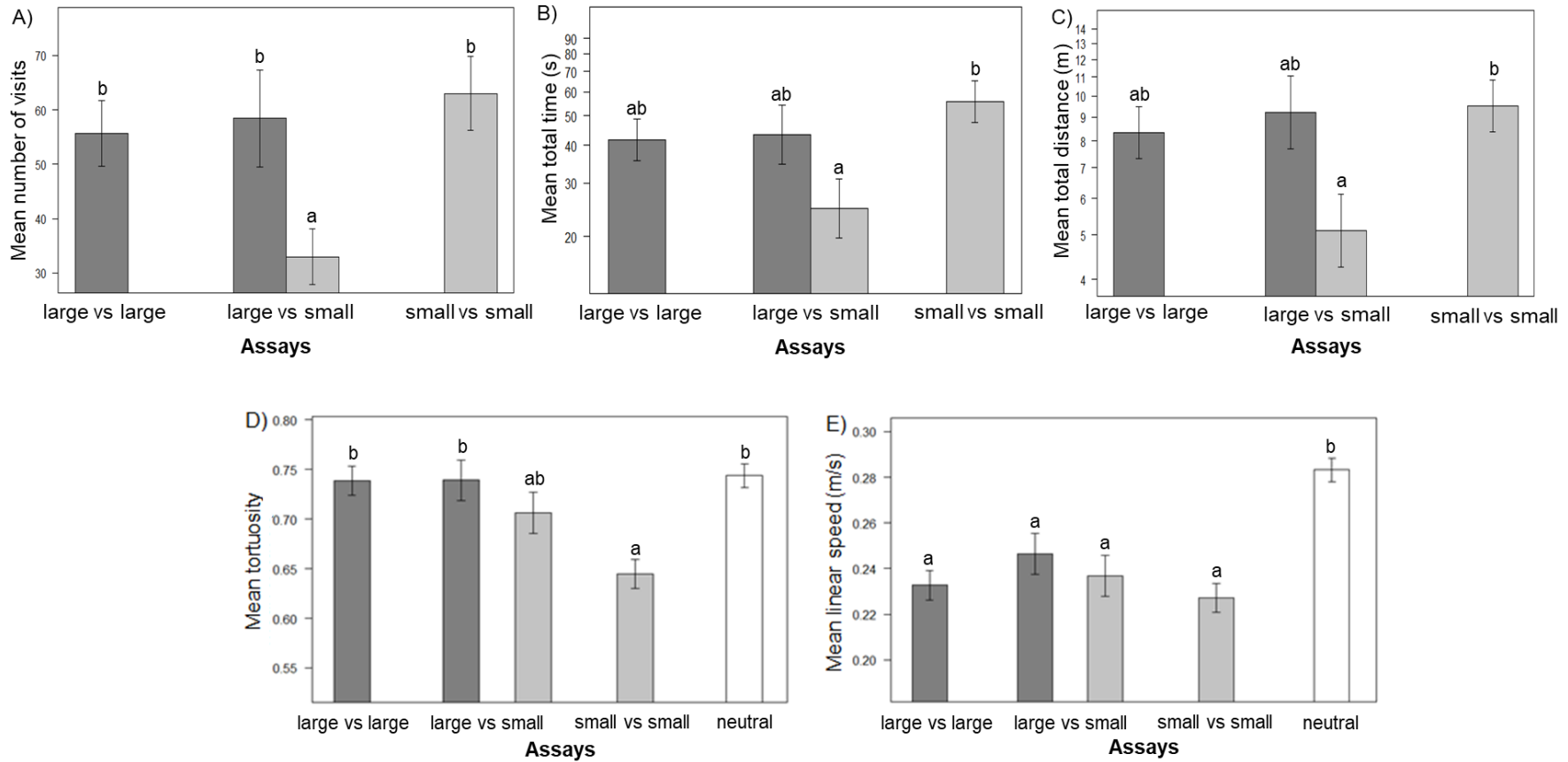
Figure 35: Comparison of track parameters recorded in Experiment 1. *(A) Mean number of visits, (B) mean total time, and (C) mean total distance. Note that the Y-axis in panels (B) and (C) is in natural logarithmic scale, and the means and SEM displayed in these two charts derive from logged data. Panels (D) and (E) show the mean tortuosity and the mean linear speed. Note that the Y-axes do not start at zero. All bar charts display means, the error bars indicate the SEM, and different letters indicate significant differences between targets (Tukey's test, P<0.05).*

Table 8: Means ± SEM of flight parameters of tracks recorded in Experiment 1 for each target type and each assay. *Note that means for the total time and total distance are de-transformed from the log scale values, and the SEM from the total time and the total distance indicate the upper and lower bound that resulted from anti-logging the transformed values. The superscript letters denote significant differences between treatments (multiple comparisons carried out with Tukey's test, P<0.05).*

Area of interest	Assay	Mean ± SEM number of visits	Mean ± SEM total time (s)	Mean ± SEM total distance (m)	Mean ± SEM tortuosity	Mean ± SEM linear speed (m/s)
Large target	Large vs large	55.71 ± 6.01 [b]	41.57 +7.14, -6.09 [ab]	8.33 +1.16, -1.01 [ab]	0.74 ± 0.01 [b]	0.23 ± 0.01 [a]
Large target	Large vs small	58.42 ± 8.89 [b]	43.41 +10.09, -8.71 [ab]	9.21 +1.85, -1.54 [ab]	0.74 ± 0.02 [b]	0.25 ± 0.01 [a]
Small target	Large vs small	33.00 ± 5.14 [a]	24.70 +6.21, -4.96 [a]	5.11 +1.04, -0.86 [a]	0.71 ± 0.02 [ab]	0.24 ± 0.01 [a]
Small target	Small vs small	63.04 ± 6.77 [b]	55.76 +9.57, -8.18 [b]	9.50 +1.32, -1.15 [b]	0.64 ± 0.01 [a]	0.23 ± 0.01 [a]
Neutral box	N/A	N/A	N/A	N/A	0.74 ± 0.01 [b]	0.28 ± 0.01 [b]
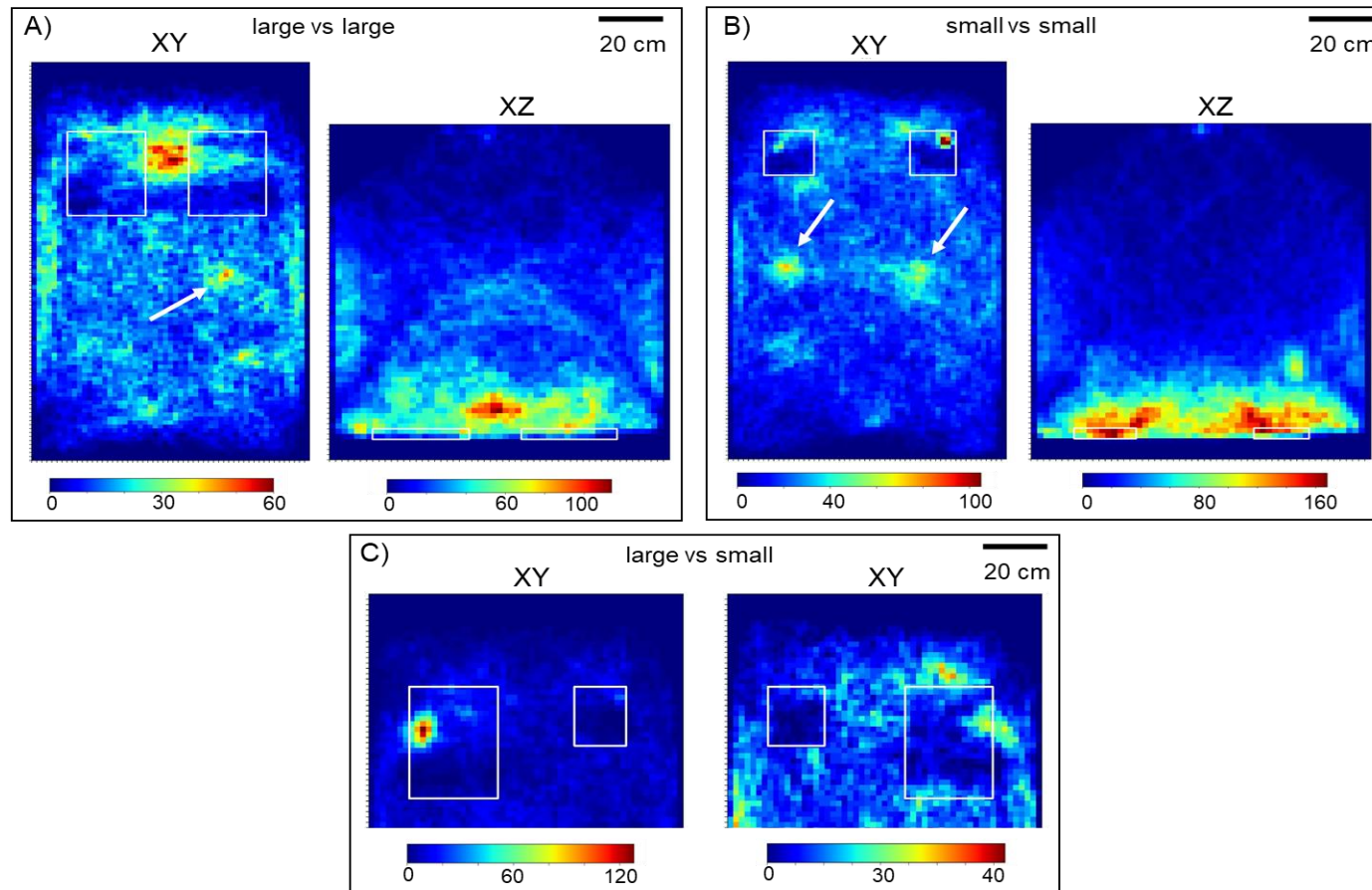
Figure 36: Heat maps showing the density of tracks recorded in Experiment 1 in different areas of the wind tunnel. *Panel (A) presents the data recorded in the large vs large assay, panel (B) the data from the small vs small assay, and panel (C) the data from the competitive assay. Each image shows a 2D plane of two axes as indicated at the top of the image. White rectangles indicate the position where the targets were placed, while the arrows highlight the position of small visual markers provided for optomotor navigation. It can be noted that mosquitoes visited more the large targets compared to the small targets when presented together.*
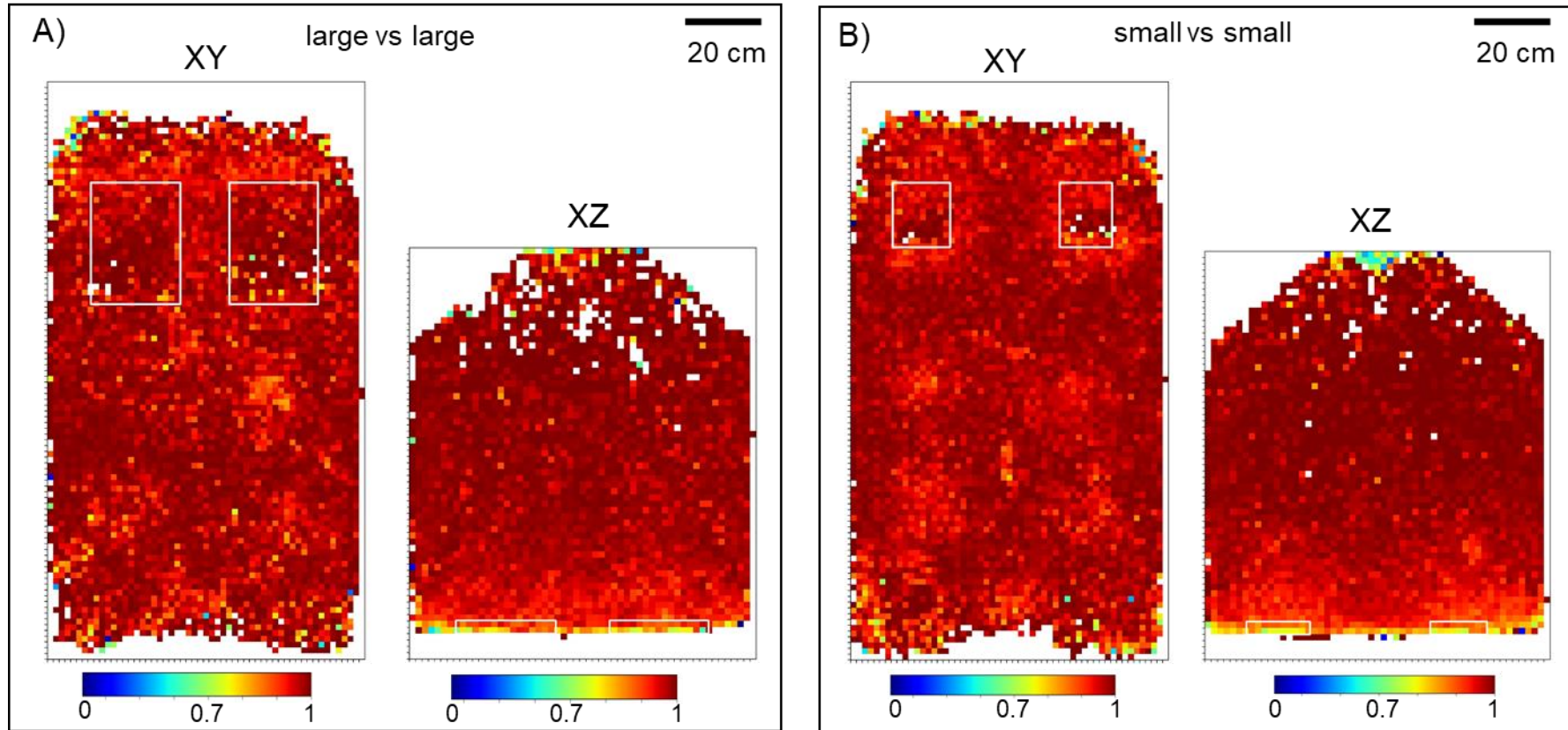
Figure 37: Heat maps showing the mean tortuosity index of the tracks recorded in Experiment 1 in different areas of the wind tunnel. *Panel (A) shows results from the large vs large assay, while panel (B) shows results from the small vs small assay. Lower values of tortuosity index indicate more convoluted tracks. Each image shows a 2D plane of two axes as indicated at the top of the image. White rectangles indicate the position where the targets were placed. It can be seen that in the areas around the small targets, tracks tend to have low values of tortuosity index, while this difference in tortuosity is not as marked around the large targets.*
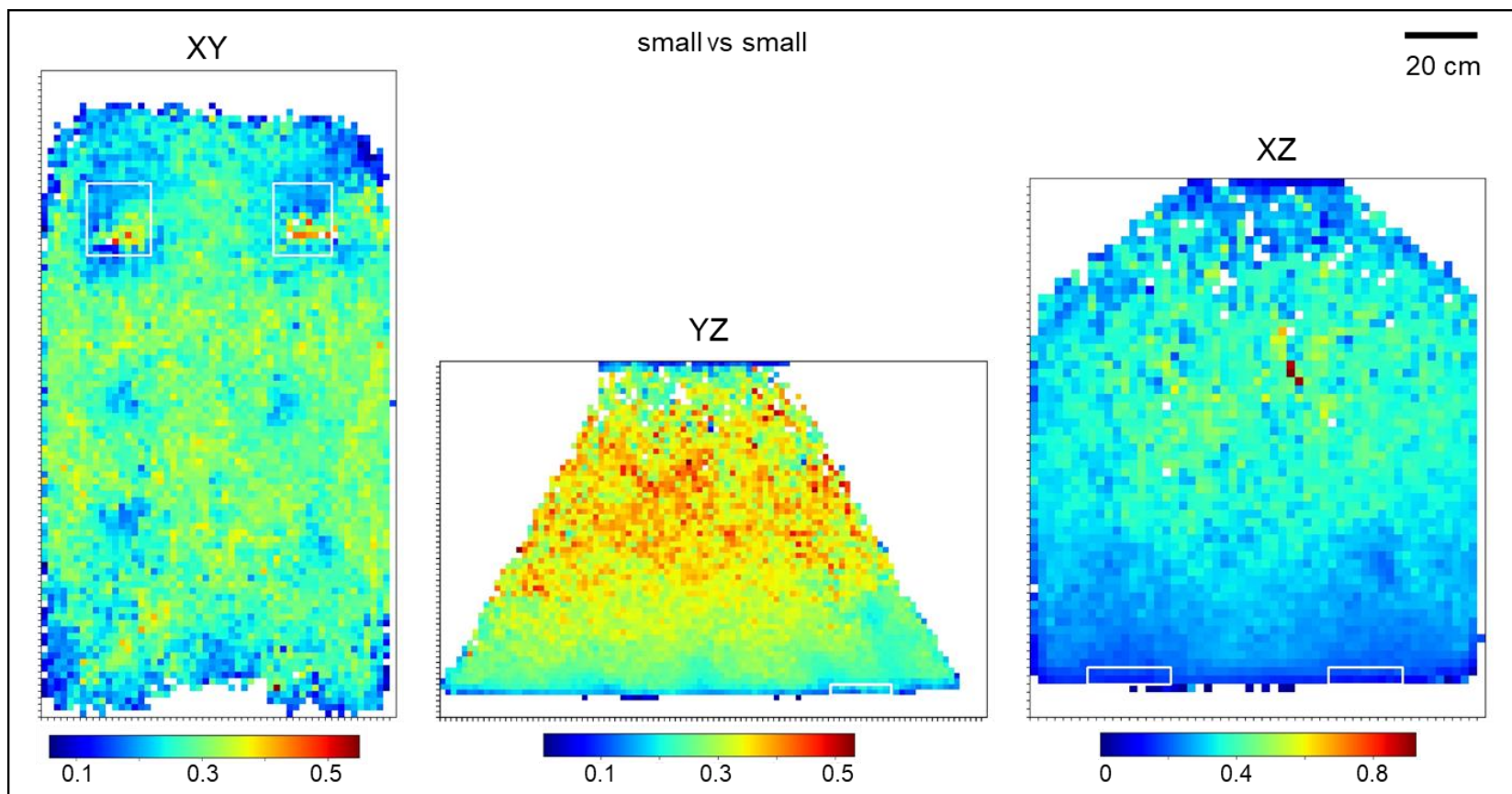
Figure 38: Heat maps showing the mean linear speed of tracks recorded in the small vs small assay in different areas of the wind tunnel. *Each image shows a 2D plane of two axes as indicated at the top of the image. White rectangles indicate the position where the targets were placed. It can be noted that tracks had a lower speed when flying close to the wind tunnel floor and when flying around the targets. The image with the YZ plane clearly shows the decrease of the space in which mosquitoes could be tracked with the increase of the elevation. This is due to the experimental setup, as the tracking system could only track mosquitoes when both cameras could detect them.*

5.3.2. Experiment 2

Neither the spatial orientation of the target itself nor the spatial orientation of the other target presented alongside had any significant effect on the landing response (ANOVA on linear mixed effects model, F=0.70, d.f.=1, 70, P=0.41; F=0.13, d.f.=1, 70, P=0.72, respectively). No significant difference was found in the number of mosquitoes landing on the vertical surface when compared to the horizontal surface in the non-competitive assays (mean ± SEM number landing on the vertical target=3.5 ± 0.37, mean ± SEM number landing on the horizontal target=3.6 ± 0.47; ANOVA on linear mixed effects model, F=0.71, d.f.=1, 73, P=0.40) (Fig. 39).
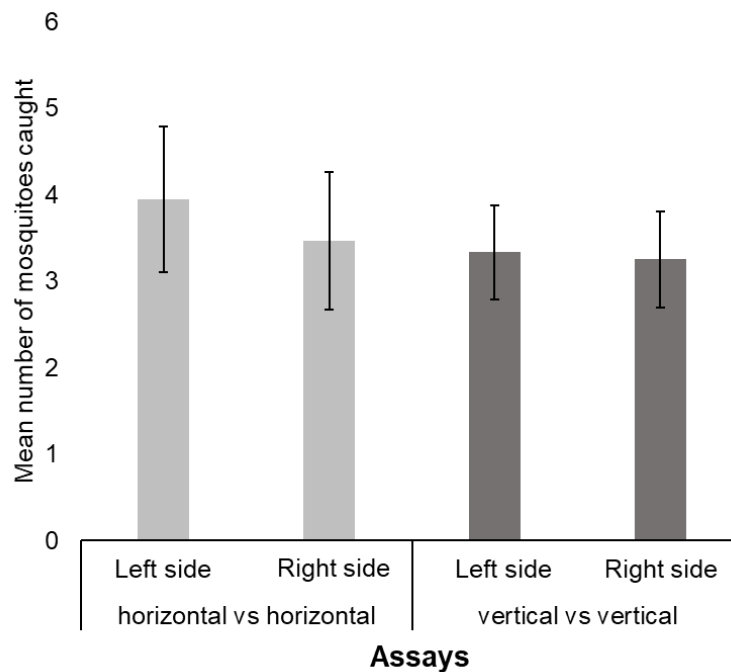
Figure 39: Mean number of mosquitoes that landed on the two target types in assays where the targets were presented alongside a similar target in Experiment 2. Note that for ease of interpretation the bars presented here display the raw means. Statistical analysis was performed on log-transformed data. The colour of the bars indicates the target type, the error bars indicate the SEM. No difference was found between the number of mosquitoes recovered on the targets in different assays (ANOVA, P=0.40).

When simultaneously presented with a target positioned vertically and a target positioned horizontally, mosquitoes did not display any preference for landing on either, as an average of 57.30 ± 7.86% SEM mosquitoes landed on the vertical

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target and 42.70 ± 7.86% SEM mosquitoes landed on the horizontal target (vertical target: median=3.5, horizontal target: median=3.0; Wilcoxon test, n=12, W=47, P=0.55) (Fig. 40).
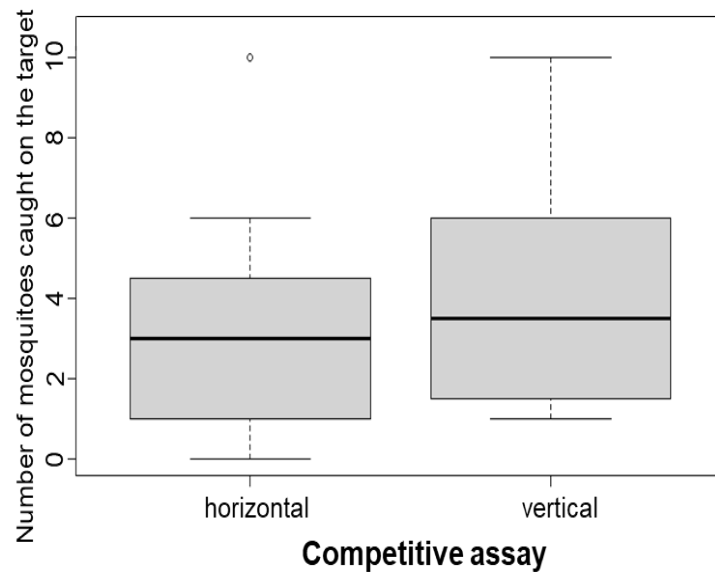
Figure 40: Number of mosquitoes that landed on the two target types in the competitive assay in Experiment 2. *The black bars indicate the medians, the upper and lower limits of the boxes indicate the interquartile range. The whiskers indicate the maximum and minimum data points excluding outliers which are represented as small circles. No difference was found in the number of mosquitoes recovered on the different targets (Wilcoxon test, P=0.55).*

The comparison of the number of mosquitoes that landed on the upwind section of the vertical target (i.e. the part facing towards the incoming air and odour sources) against the number of mosquitoes that landed on the downwind section of the target (i.e. the part facing the release cage), gave a significant difference, as almost double the number of mosquitoes were recovered from the downwind surface compared to the upwind one (upwind section: median=1.0, downwind section: median=2.0; Wilcoxon test, n=36, W=104, P=0.01).

When considering densities, it has to be noted that although the vertical target was of the same size as the horizontal target, both sides were exposed to the mosquitoes and therefore it presented twice as much target area. Thus, although the number of mosquitoes recovered on both target types was similar, the vertical target caught a significantly lower density of mosquitoes per 100 cm^2

(vertical target: median=0.1, n=24; horizontal target: median=0.3, n=34; Mann-Whitney U test, U=263, P=0.02).

3D tracking

A summary of the results on different flight parameters from tracks recorded in Experiment 2 is given in Fig. 41.

The statistical analysis determined that independently from the assay and the treatment (i.e. the target type) an equal number of tracks were recorded around the different targets throughout the entire experiment (GLM, for assay: chi-square=4.08, d.f.=2, P=0.13; for treatment: chi-square=0.11, d.f.=1, P=0.74) (Table 9, Fig. 41). Thus, no significant difference was found in the number of visits between different target types and between different assays. Heat maps (Fig. 42) suggest that when offered vertical targets alone, the majority of the flight tracks were concentrated in relatively close proximity to the wind tunnel floor, suggesting that *An. coluzzii* mosquitoes approach targets closer to the floor, independently of the height of the target. It is interesting to note however, that in the competitive assay, a high concentration of tracks was recorded at high elevations (~ 60-70 cm from wind tunnel floor). The reason behind these differences in approaching the targets are not clear and they highlight the need for future investigation.

Similarly to Experiment 1, the total time spent around the targets and the total distance covered in the areas of interest in Experiment 2 also depended solely on the factor assay, while the factor treatment had no significant effect on these parameters (two-way ANOVA, for assay: F=3.87, d.f.=2, 78, P=0.02; F=3.34, d.f.=2, 78, P=0.04, respectively; for treatment: F=2.45, d.f.=1, 78, P=0.12; F=3.26, d.f.=1, 78, P=0.07, respectively). For both parameters, the only comparison that resulted in a significant difference was between the vertical targets in the vertical vs vertical assay when compared against the parameters recorded around the horizontal target in the competitive assay (Tukey's test, for total time: t=2.89, P=0.02; for total distance: t=3.00, P=0.02) (Table 9, Fig. 41).

The tortuosity was significantly influenced by both the assay and treatment (two-way ANOVA, for assay: F=3.18, d.f.=2, 118, P=0.045; for treatment: F=21.86, d.f.=2, 118, P<0.001). In particular, tracks recorded around vertical targets in the vertical vs vertical assay had a significantly lower tortuosity index value (i.e. were more convoluted) compared to the tracks recorded around horizontal targets in the horizontal vs horizontal assay (Tukey's test, t=-3.89, P=0.002) (Fig. 43). Furthermore, tracks recorded around vertical targets in the vertical vs vertical assay, and also recorded around both the horizontal target and the vertical target in the competitive assay were significantly more convoluted compared to the tracks recorded in the neutral box, where no target was offered (Tukey's test, t=-6.28, P<0.001; t=4.17, P<0.001; and t=-3.95, P=0.001, respectively) (Table 9, Fig. 41).

As was also found in Experiment 1, in Experiment 2 the linear speed was also comparable for all target types in all assays, and a significant difference was detected only when results from the neutral box (i.e. no target area) were introduced in the analysis (two-way ANOVA, for assay: F=0.36, d.f.=2, 118, P=0.70; for type of area, with or without target: F=35.97, d.f.=2, 118, P<0.001). For all areas of interest where there was a target, the linear speed was significantly lower compared to the mean value recorded in the area where no target was presented (neutral box) (Tukey's test, comparison between the horizontal targets in the horizontal vs horizontal assay and the neutral box: t=7.72, P<0.001; comparison between the horizontal target in the competitive assay and the neutral box: t=5.75, P<0.001; comparison between the vertical target in the competitive assay and the neutral box: t=-2.77, P=0.048; comparison between the vertical targets in the vertical vs vertical assay and neutral box: t=-4.64, P<0.001) (Table 9, Fig. 41). Once again this confirms that when flying in proximity of targets, mosquitoes lower their linear speed independently of the assay or the target type.

From the track analysis carried out on flights around vertical targets alone, it was possible to determine that, overall, mosquitoes had a strong preference for the downwind surface. The factor section (i.e. upwind section or downwind section)

had a significant effect on the number of visits recorded around vertical targets (GLM, chi-square=47.22, d.f.=1, P<0.001). Significantly more visits were recorded around the downwind section of the target, and this was the case independent of the assay (Table 10, Table 11, Fig. 44). This substantial difference can be clearly observed in the heat maps (Fig. 42). Similarly, the total length of time spent around the different areas of the vertical target was significantly influenced by the section of the target (two-way ANOVA, for assay: F=3.1, d.f.=1, 68, P=0.08; for section: F=67.21, d.f.=1, 68, P<0.001). Mosquitoes spent significantly more time flying around the downwind section compared to the time spent around the upwind section (Table 10, Table 11, Fig. 44). Moreover, the total distance covered around the target was also significantly influenced by the factor section (two-ways ANOVA, for assay: F=2.61, d.f.=1, 68, P=0.11; for section: 92.47, d.f.=1, 68, P<0.001). Mosquitoes covered a significantly longer distance around the downwind section of the target compared to the distance covered around the upwind section (Table 10, Table 11, Fig. 44). Altogether, these results indicate that mosquitoes visited more frequently, spent more time, and flew longer distances around the downwind section of vertical targets compared to the upwind section, and this was found consistently irrespectively of the assay.
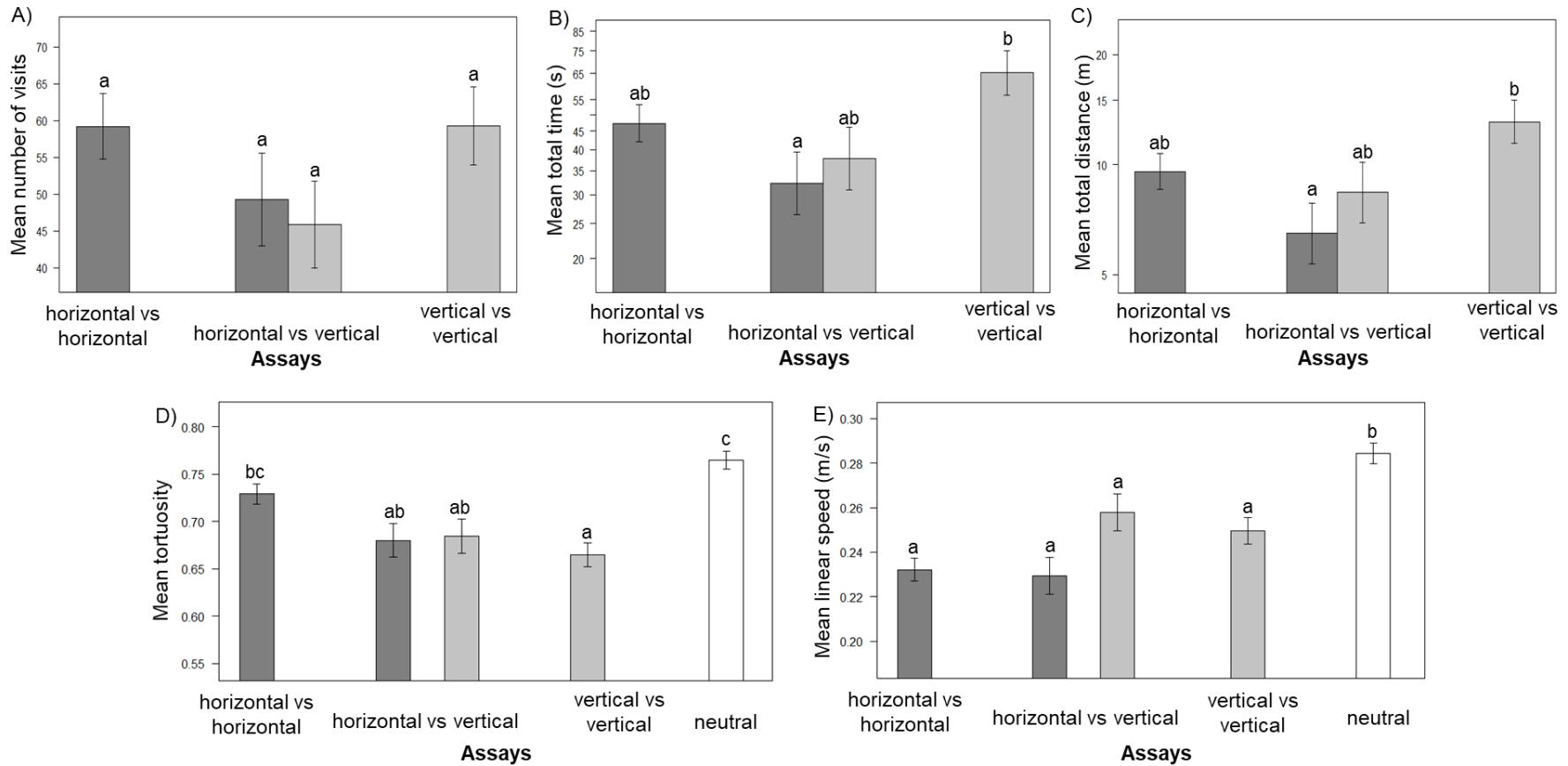
Figure 41: Comparison of track parameters recorded in Experiment 2. *(A) Mean number of visits, (B) mean total time, and (C) mean total distance. Note that the Y-axis in panels (B) and (C) is in natural logarithmic scale, and the means and SEM displayed in these two charts derive from logged data. Panels (D) and (E) show the mean tortuosity and the mean linear speed. Note that the Y-axes do not start at zero. All bar charts display means, the error bars indicate the SEM, while different letters indicate significant differences between targets (Tukey's test, P<0.05).*

Table 9: Means ± SEM of flight parameters of tracks recorded in Experiment 2 for each target type and each assay. *Note that the means for total time and total distance are de-transformed from the log scale values and the SEM for total time and total distance indicate the upper and lower bound that resulted from anti-logging the transformed values. The superscript letters denote significant differences between treatments (multiple comparisons carried out with Tukey's test, P<0.05).*

Area of interest	Assay	Mean ± SEM number of visits	Mean ± SEM total time (s)	Mean ± SEM total distance (m)	Mean ± SEM tortuosity	Mean ± SEM linear speed (m/s)
Horizontal target	Horizontal vs horizontal	59.24 ± 4.45 [a]	47.29 +5.91, -5.26 [ab]	9.57 +1.15, -1.03 [ab]	0.73 ± 0.01 [bc]	0.23 ± 0.01 [a]
Horizontal target	Vertical vs horizontal	49.33 ± 6.30 [a]	32.32 +7.10, -5.82 [a]	6.48 +1.36, -1.13 [a]	0.68 ± 0.02 [ab]	0.23 ± 0.01 [a]
Vertical target	Vertical vs horizontal	45.92 ± 5.89 [a]	37.81 +8.30, -6.81 [ab]	8.38 +1.77, -1.46 [a]	0.68 ± 0.02 [ab]	0.26 ± 0.01 [a]
Vertical target	Vertical vs vertical	59.25 ± 5.30 [a]	65.24 +9.84, -8.54 [b]	13.08 +1.89, -1.66 [b]	0.66 ± 0.01 [a]	0.25 ± 0.01 [a]
Neutral box	N/A	N/A	N/A	N/A	0.74 ± 0.01 [c]	0.28 ± 0.01 [b]
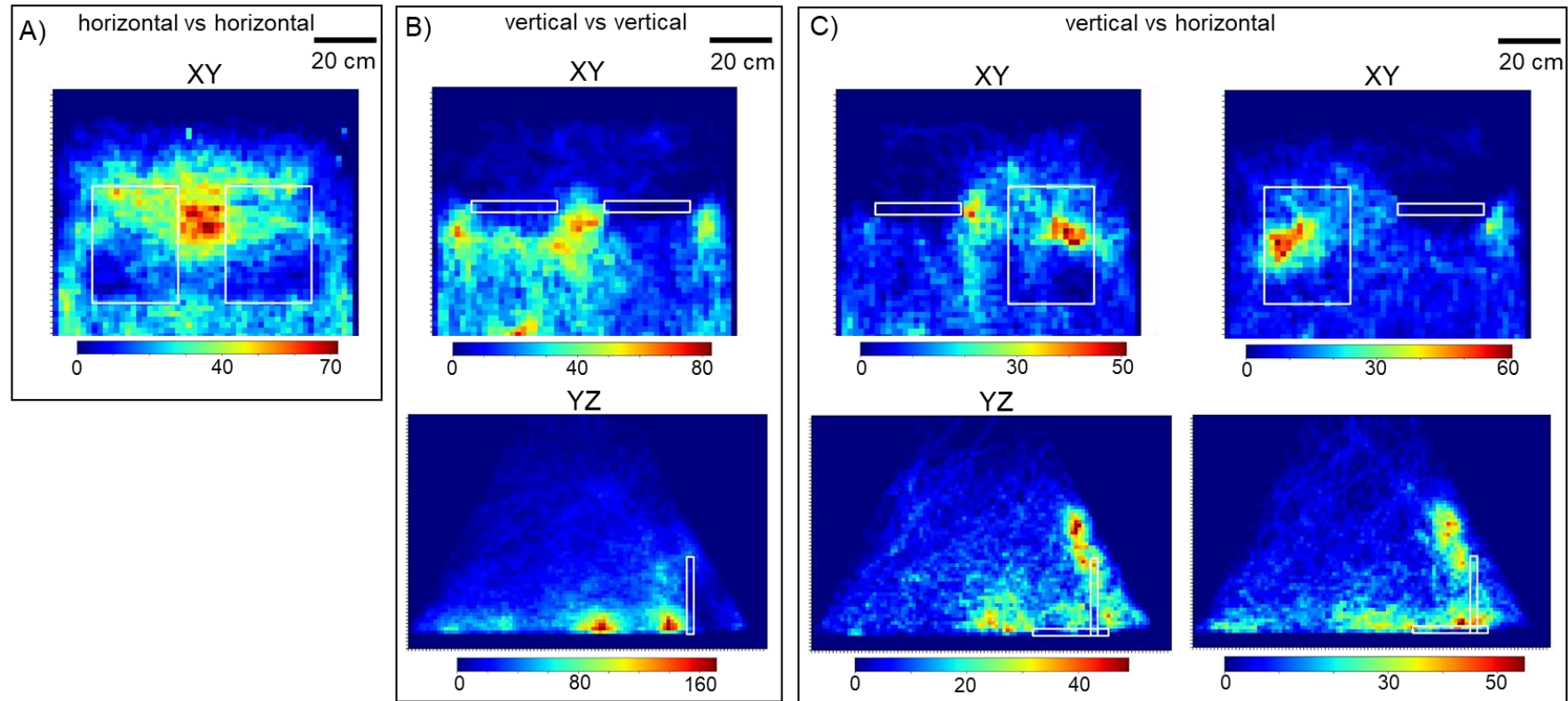
Figure 42: Heat maps showing the density of tracks recorded in Experiment 2 in different areas of the wind tunnel. *Panel (A) presents the data recorded in the horizontal vs horizontal assay, panel (B) the data from the vertical vs vertical assay, and panel (C) the data from the competitive assay. Each image shows a 2D plane of two axes as indicated at the top of the image. White rectangles indicate the position where the targets were placed. Note that the vertical targets were visited substantially more from the downwind side of the wind tunnel. Furthermore, in the vertical vs vertical assay, mosquitoes approached the target closer to the floor, while when offered the competitive assay mosquitoes visited the vertical target at high elevations (~ 60-70 cm from the floor).*
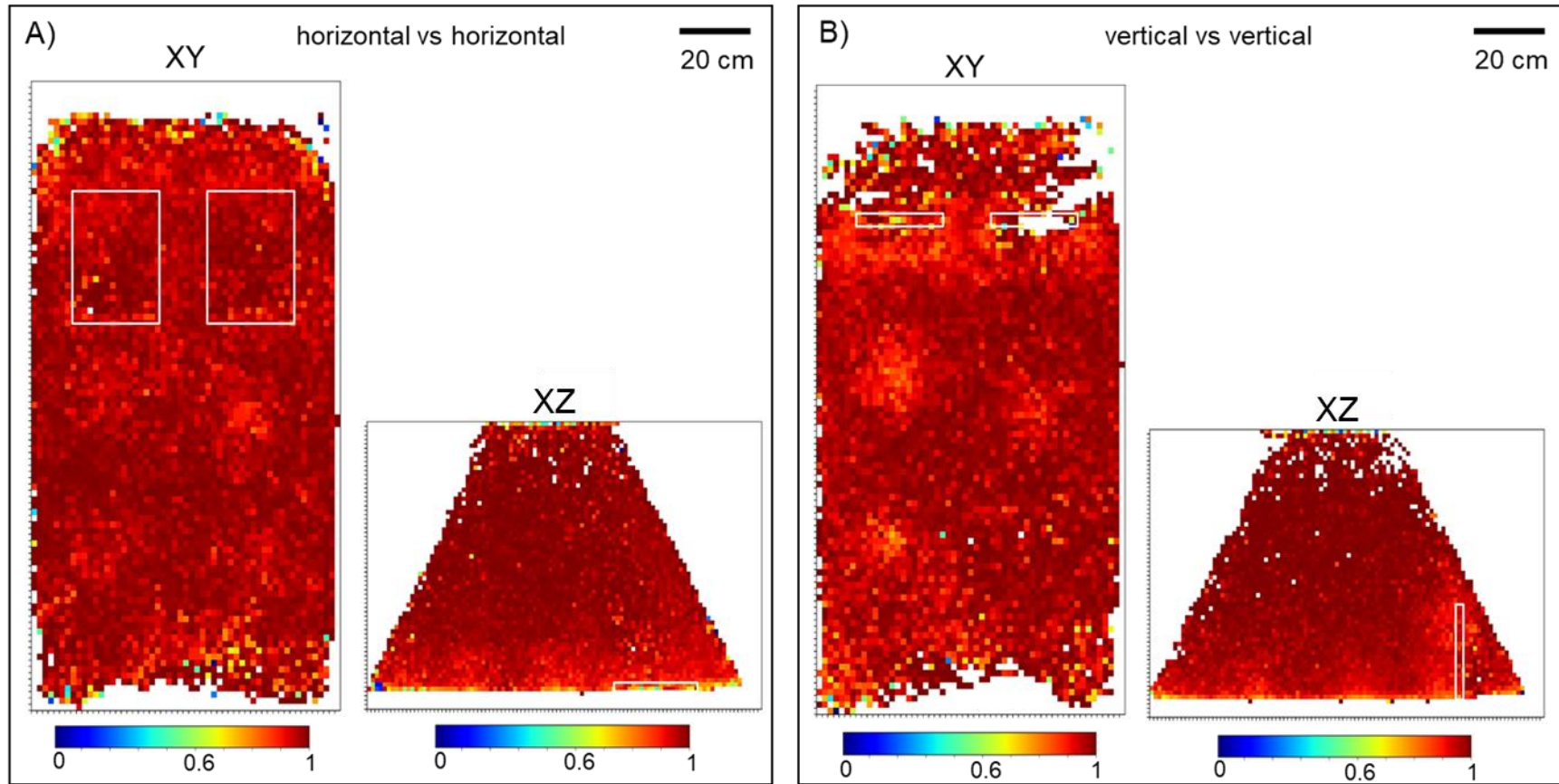
Figure 43: Heat maps showing the mean tortuosity index of the tracks recorded in Experiment 2 in different areas of the wind tunnel. *Panel (A) shows results from the horizontal vs horizontal assay, while panel (B) shows results from the vertical vs vertical assay. Lower values of tortuosity index indicate more convoluted tracks. Each image shows a 2D plane of two axes as indicated at the top of the image. White rectangles indicate the position where the targets were placed. The heat maps clearly show that around vertical targets the tracks are more convoluted.*

Table 10: Means ± SEM of flight parameters of tracks recorded in the upwind and the downwind sections of vertical targets. *Note that the means for total time and total distance are de-transformed from the log scale values and the SEM for total time and total distance indicate the upper and lower bound that resulted from anti-logging the transformed values. The superscript letters denote significant differences between sections (multiple comparisons carried out with Tukey's test, P<0.05).*

Area of interest	Assay	Mean ± SEM number of visits	Mean ± SEM total time (s)	Mean ± SEM total distance (m)
Downwind section	Vertical vs horizontal	37.25 ± 5.42 [b]	27.49 +7.08, -5.63 [b]	6.49 +1.57, -1.26 [b]
Downwind section	Vertical vs vertical	53.92 ± 5.46 [b]	52.79 +9.28, -7.90 [b]	10.81 +1.78, -1.53 [b]
Upwind section	Vertical vs horizontal	20.50 ± 3.11 [a]	8.89 +2.29, -1.82 [a]	1.57 +0.38, -0.30 [a]
Upwind section	Vertical vs vertical	21.21 ± 2.27 [a]	9.31 +1.63, -1.39 [a]	1.73 +0.28, -0.25 [a]

Table 11: Results from Tukey's test between the different sections of vertical targets for the three flight track parameters analysed. The asterisk indicates a significant difference between the terms compared at a level of at least P<0.05.

Two sections under comparison		Number of visits		Total time spent on the area of interest		Total distance covered in the area of interest	
Section 1	Section 2	t-value	P-value	t-value	P-value	t-value	P-value
Downwind section on the vertical vs vertical assay	Downwind section on the competitive assay	2.09	0.16	2.33	0.10	1.93	0.22
Upwind section on the competitive assay	Downwind section on the competitive assay	-2.84	0.02*	-3.49	0.004*	-4.64	<0.001*
Upwind section on the vertical vs vertical assay	Downwind section on the competitive assay	-3.12	0.01*	-3.86	0.001*	-5.01	<0.001*
Upwind section on the competitive assay	Downwind section on the vertical vs vertical assay	-5.30	<0.001*	-6.35	<0.001*	-7.29	<0.001*
Upwind section on the vertical vs vertical assay	Downwind section on the vertical vs vertical assay	0.15	<0.001*	-7.58	<0.001*	-8.49	<0.001*
Upwind section on the vertical vs vertical assay	Upwind section on the competitive assay	0.18	1.00*	0.165	1.00	0.36	0.98
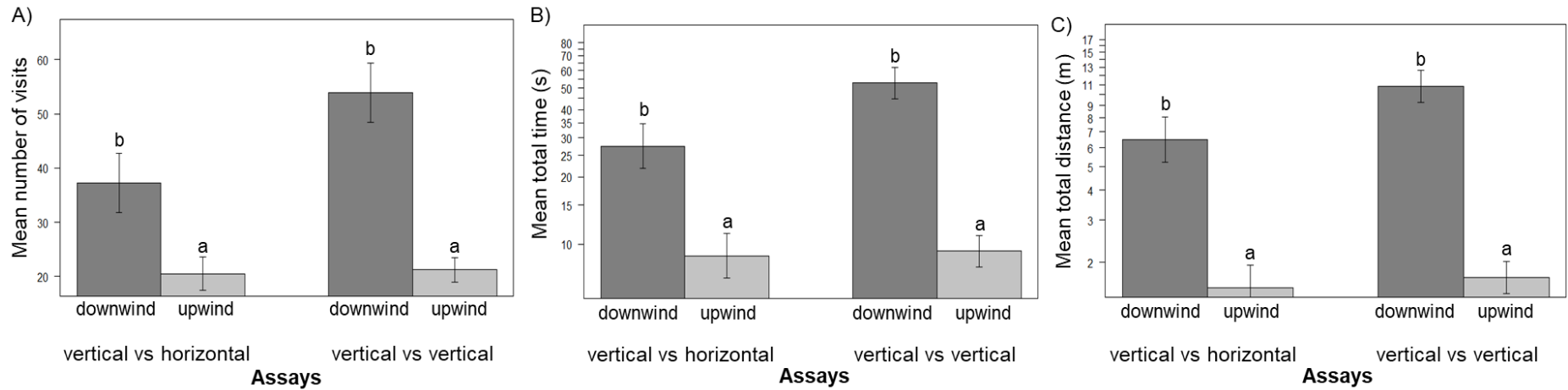
Figure 44: Comparison of track parameters recorded downwind and upwind around vertical targets in Experiment 2. *(A) Mean number of visits, (B) mean total time, and (C) mean total distance. Note that the Y-axis in panels (B) and (C) is in natural logarithmic scale, and the means and SEM displayed in these two charts derive from logged data. Note that the Y-axes do not start at zero. All bar charts display means, the error bars indicate the SEM, while the letters indicate a significant difference between targets (Tukey's test, P<0.05).*

5.3.3. Experiment 3

A summary of the mean number of mosquitoes recovered on the target surface for each treatment is shown in Fig. 45.
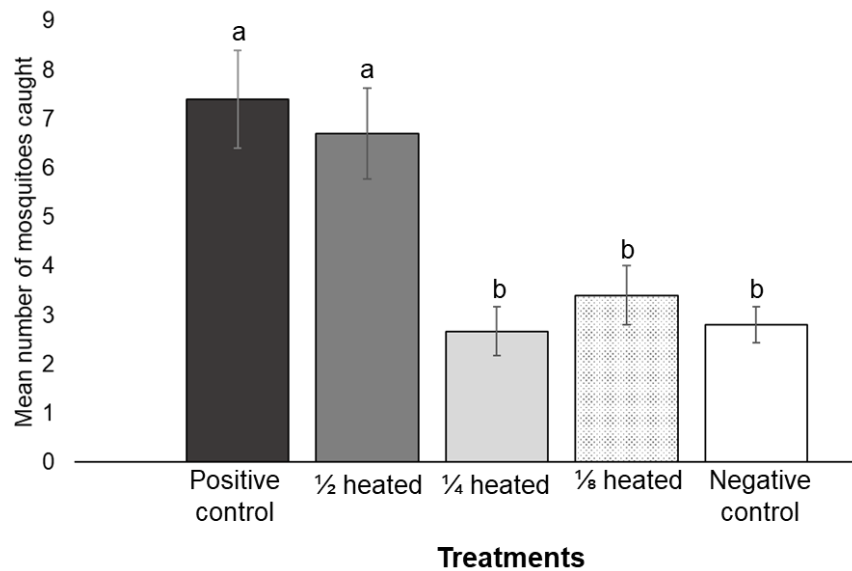
Figure 45: Mean number of mosquitoes that landed on the different targets in Experiment 3. *The colour of the bars indicates the treatment, the error bars indicate the SEM. Different letters denote significant differences between treatments (Tukey's test, P<0.05).*

The different treatments had a significant effect on the landing behaviour of *An. coluzzii* (GLM, chi-square=42.79, d.f.=4, P<0.001). Results of the multiple comparisons are presented in Table 12. Results cluster in two distinct groups: the positive control and the target where only ½ of the surface was heated caught a similar number of mosquitoes, which was significantly higher when compared to the rest of the treatments (¼ heated, ⅛ heated, and the negative control).

Table 12: Pairwise comparisons of mean ± SEM number of mosquitoes caught on the target surface for each treatment in Experiment 3. Positive control refers to the treatment where all the surface was heated whilst in the negative control no surface was heated. The asterisk indicates significant difference at a level of at least P<0.05 (Tukey's test).

Treatment 1	Mean ± SEM	Treatment 2	Mean ± SEM	t-value	P-value
Positive control	7.40 ± 0.99	½ heated	6.70 ± 0.93	0.59	0.98
Positive control	7.40 ± 0.99	¼ heated	2.67 ± 0.50	4.34	<0.001*
Positive control	7.40 ± 0.99	⅛ heated	3.40 ± 0.60	3.74	0.002*
Positive control	7.40 ± 0.99	Negative control	2.80 ± 0.36	4.37	<0.001*
½ heated	6.70 ± 0.93	¼ heated	2.67 ± 0.50	-3.86	<0.001*
½ heated	6.70 ± 0.93	⅛ heated	3.40 ± 0.60	-3.21	0.01*
¼ heated	2.67 ± 0.50	⅛ heated	3.40 ± 0.60	0.91	0.89
Negative control	2.80 ± 0.36	½ heated	6.70 ± 0.93	-3.87	0.001*
Negative control	2.80 ± 0.36	¼ heated	2.67 ± 0.50	0.175	1.00
Negative control	2.80 ± 0.36	⅛ heated	3.40 ± 0.60	-0.76	0.94

As different sized areas were heated and unheated in three treatments (½ heated, ¼ heated, and ⅛ heated), the comparisons of the landing rates between different areas of the target surface were made using densities, i.e. using standardised measures (Table 13). No significant difference was found when comparing the densities of mosquitoes that landed on the central heated part with the densities recovered on the unheated margins for the ½ heated target (median for central area=0.7, median for margin area=0.4; Wilcoxon test, n=10, W=36, P=0.12) and the ¼ heated target (median for central area=0.3, median for margin area=0.1; Wilcoxon test, n=9, W=37.5, P=0.09). In contrast, this

comparison gave a significant difference for the target where only ⅛ of the surface was heated (median for central area=1.0, median for margin area=0.1; Wilcoxon test, n=10, W=48, P=0.04). It is interesting to note that in the three treatments that cluster together with the lowest landing density (¼ heated, ⅛ heated, and the negative control) the mean density of mosquitoes recovered on the unheated margins was comparable (approx. mean ± SEM density=0.20 ± 0.05 in all three treatments) although these areas were of considerably different sizes.

Table 13: Mean ± SEM densities per 100 cm^2 of mosquitoes recovered on heated and unheated areas of targets.

Treatment	Mean ± SEM density of mosquitoes on the whole target	Mean ± SEM density of mosquitoes on the heated area	Mean ± SEM density of mosquitoes on the unheated area
Positive control	0.62 ± 0.08	0.62 ± 0.08	N/A
½ heated	0.56 ± 0.08	0.70 ± 0.12	0.42 ± 0.09
¼ heated	0.30 ± 0.09	0.63 ± 0.21	0.19 ± 0.06
⅛ heated	0.28 ± 0.05	0.80 ± 0.19	0.21 ± 0.05
Negative control	0.23 ± 0.03	N/A	0.23 ± 0.03

This result was substantiated when looking at the number of mosquitoes that landed in different parts of the target that had both a heated and unheated area (treatments: ½ heated, ¼ heated, ⅛ heated) (Fig. 46). Overall, the number of mosquitoes landing on the centre of the target (i.e. heated area) strongly depended on the target type, while the number of mosquitoes landing on the margins of the target (i.e. unheated area) did not differ significantly (ANOVA on linear mixed effects model, interaction term between treatment and part of the

target: F=4.40, d.f.=2, 44, P=0.02). Thus, it appears that the size of the unheated area does not affect the number of mosquitoes that land on it, while the size of the heated area plays an important role in increasing the number of landings.
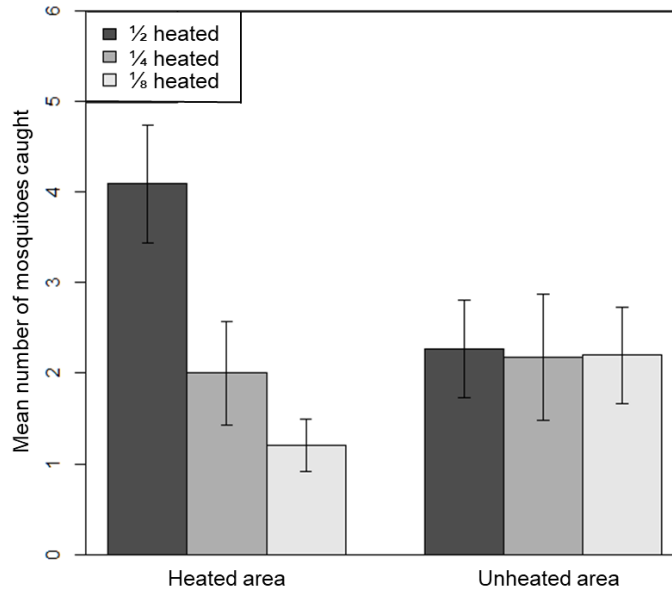
Figure 46: Mean number of mosquitoes recovered from the different parts of the targets that presented both a heated and unheated area. The colour of the bars indicates the treatment, the error bars indicate the SEM.

The mean densities recovered from whole targets provide an estimate on the efficiency of the targets. Taking into account that the number of mosquitoes released and activated was similar in all treatments, and comparing the mean densities of mosquitoes recovered on whole targets (Table 13), it arises that the positive control and the treatment with ½ heated area are on average approximately twice as efficient in trapping mosquitoes compared to the targets where only ¼ and ⅛ of the surface was heated, while also being on average approximately 2.5 times more efficient compared to the negative control.

A clear linear relationship was found between the mean number of mosquitoes caught on the heated areas of the targets and the area sizes of the thermal signature (Fig. 47). Furthermore, Fig. 47 also indicates that the number of mosquitoes caught on the heated area is directly proportional with the area size of the thermal signature, thus suggesting that by doubling the area of the thermal

cue offered on a target one would recover the double number of mosquitoes from the surface.
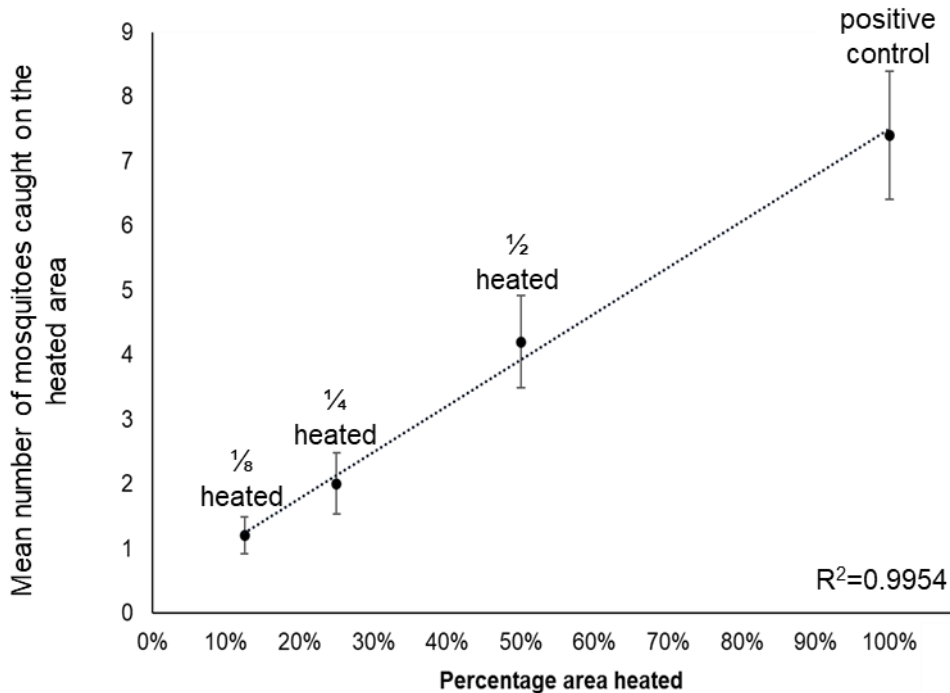
Figure 47: Mean number of mosquitoes caught on the heated area on the landing target. *The points indicate the mean numbers of mosquitoes recovered while the error bars indicate the SEM.*

3D tracking

Results from analysis on the flight tracks recorded in Experiment 3 are summarised in Fig. 48.

The factor treatment had a significant effect on the number of visits that were recorded across all targets (GLM, chi-square=52.26, d.f.=4, P=0.02). Significantly more visits were recorded around the target where no surface was heated compared to the target where all the surface was heated (Tukey's test, t=-2.81, P=0.04), while no difference was detected in comparisons with any of the other treatments (Table 14, Fig. 48). From the heat maps it can be observed that all targets received a high number of visits and that these were mostly concentrated in the central and slightly upwind area of the target (Fig. 49). Furthermore, in all treatments, independently of the proportion of the target area

that was heated, a high concentration of tracks was recorded in the upwind area of the wind tunnel, roughly above the target at a height between ~30-40 cm from the floor, which corresponds to the height at which the host odour stimulus was released (Fig. 49).

No difference was detected in the mean total time mosquitoes spent flying around the different targets (one-way ANOVA, F=1.70, d.f.=4, 44, P=0.17) nor in the mean total distance mosquitoes covered flying around the different targets (one-way ANOVA, F=2.13, d.f.=4, 44, P=0.09) (Table 14, Fig. 48). However, it should also be considered that as significantly more mosquitoes were caught on the fully heated target and the ½ heated target, fewer mosquitoes could be expected to be flying around these areas of interest. Thus, the fact that the targets were investigated for equal length of time and equal distance by ultimately fewer mosquitoes would suggest that targets with larger heated areas might have had a higher capability to draw and retain mosquitoes in their proximities, although no clear conclusions can be extrapolated from this dataset and further studies must be carried out to verify this hypothesis.

No difference was detected in the tortuosity of the tracks recorded around different targets when only the areas with the targets were considered, and a significant difference was found in the analysis of tortuosity only when the results from the neutral box were introduced in the model (one-way ANOVA, F=9.72, d.f.=5, 92, P<0.001). In particular, tracks recorded around the targets that were only ½ heated, ¼ heated, ⅛ heated, and not heated at all, were significantly more convoluted compared to the tracks recorded where no target was presented (Tukey's test, for the ½ heated target: t=5.19, P<0.001; for the ¼ heated target: t=3.87, P=0.003; for the ⅛ heated target: t=3.07, P=0.03; for the negative control target: t=4.28, P<0.001) (Table 14, Fig. 48). The comparison between the tortuosity index of the tracks recorded around a fully heated target and the neutral box gave a non significant result (Tukey's test, t=-2.73, P=0.07) (Table 14, Fig. 48).

No difference was detected in the linear speed of tracks recorded around the different targets, and a significant difference was found only when the results

from the neutral box were included in the analysis (one-way ANOVA, F=7.09, d.f.=5, 92, P<0.001). For all treatments, the linear speed of the tracks recorded around the targets was significantly lower compared to the linear speed of the tracks recorded in the area where no target was provided (neutral box) (Tukey's test, for the positive control: t=-3.80, P=0.003; for the ½ heated target: t=2.98, P=0.04; for the ¼ heated target: t=2.91, P=0.048; for the ⅛ heated target: t=3.58, P=0.01; and for the negative control: t=3.73, P=0.004) (Table 14, Fig. 48). This is in accordance with results obtained in the two previous experiments, where tracks around the targets also had a lower linear speed compared to tracks recorded in the area where there was no target.
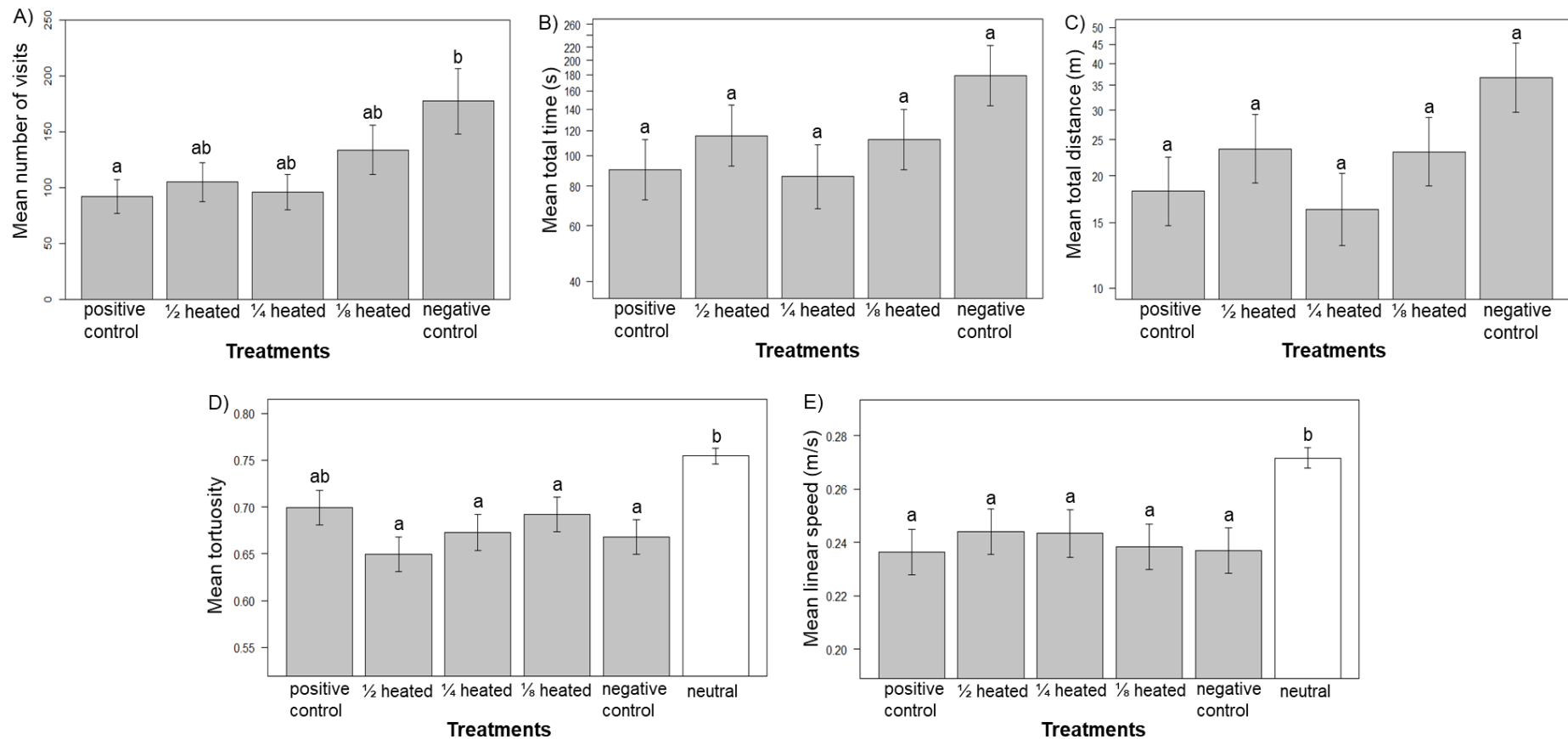
Figure 48: Comparison of track parameters recorded in Experiment 3. *(A) Mean number of visits, (B) mean total time, and (C) mean total distance. Note that the Y-axis in panels (B) and (C) is in natural logarithmic scale, and the means and SEM displayed in these two charts derive from logged data. Panels (D) and (E) show the mean tortuosity and the mean linear speed of the tracks recorded in the areas of interest. Note that the Y-axes do not start at zero. All bar charts display means, the error bars indicate the SEM, while different letters indicate significant differences between targets (Tukey's test, P<0.05).*

Table 14: Means ± SEM of flight parameters of tracks recorded around each target type in Experiment 3. *Note that the means for total time and total distance are de-transformed from the log scale values, and the SEM from total time and total distance indicate the upper and lower bound that resulted from anti-logging the transformed values. The superscript letters denote significant differences between treatments (multiple comparisons carried out with Tukey's test, P<0.05).*

Area of interest	Mean ± SEM number of visits	Mean ± SEM total time (s)	Mean ± SEM total distance (m)	Mean ± SEM tortuosity	Mean ± SEM linear speed (m/s)
Positive control	92.10 ± 15.28 [a]	90.33 +22.23, -17.84 [a]	18.18 +4.28, -3.46 [a]	0.70 ± 0.01 [ab]	0.24 ± 0.01 [a]
½ heated target	105.27 ± 17.41 [ab]	115.67 +28.46, -22.84 [a]	23.63 +5.57, -4.50 [a]	0.65 ± 0.02 [a]	0.24 ± 0.01 [a]
¼ heated target	96.20 ± 15.94 [ab]	85.81 +22.39, -17.86 [a]	16.28 +4.06, -3.26 [a]	0.67 ± 0.02 [a]	0.24 ± 0.01 [a]
⅛ heated target	133.91 ± 22.07 [ab]	112.46 +27.67, -22.21 [a]	23.20 +5.46, -4.42 [a]	0.69 ± 0.01 [a]	0.24 ± 0.01 [a]
Negative control	177.52 ± 29.16 [b]	178.82 +44.00, -35.31 [a]	36.64 +8.63, -6.98 [a]	0.67 ± 0.02 [a]	0.24 ± 0.01 [a]
Neutral box	N/A	N/A	N/A	0.75 ± 0.01 [b]	0.27 ± 0.01 [b]
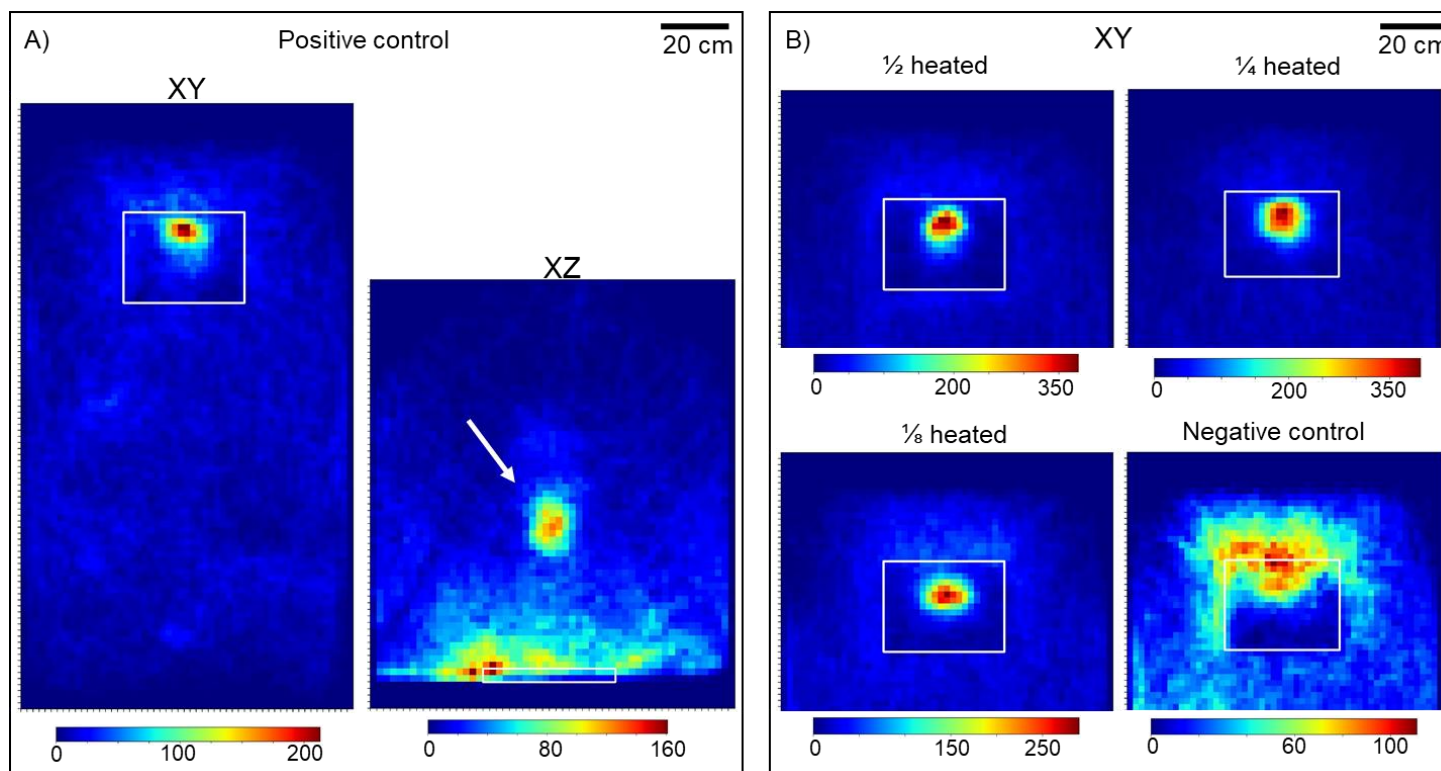
Figure 49: Heat maps showing the density of tracks recorded in Experiment 3 in different areas of the wind tunnel. *Panel (A) presents the data recorded in the positive control, both in the XY and XZ plane, while panel (B) shows the aerial view (plane XY) of the tracks recorded around the target in the remaining treatments. Each image shows a 2D plane of two axes as indicated at the top of the image. White rectangles indicate the position where the targets were placed. The white arrow in panel (A) indicates the point where a high concentration of tracks was recorded, ~30-40 cm above the target. This position corresponds to the point where the host odour stimulus was released. The plane XZ is presented only for the positive control for example purposes, as the concentration of tracks close to the odour release point occurred in all treatments.*

5.3.4. Comparison of landing numbers according to exposed target surface

The large target in Experiment 1 and the positive control in Experiment 3 presented the same characteristics (identical colour, size, and temperature, which was homogeneously distributed across all its surface), thus they can be considered equivalent treatments. It is interesting to note that when combining the number of mosquitoes recovered on both large targets that were presented simultaneously in the large vs large assay (Experiment 1), the resulting mean was similar to the mean number of landings recovered on the single surface in the positive control treatment of Experiment 3 (mean ± SEM sum of landed mosquitoes on two large targets in Experiment 1=8.5 ± 1.45, mean ± SEM number of landings on the positive control target in Experiment 3=7.4 ± 0.99). This implies that independently of the number of targets presented, and thus, independently of the area of the trapping surface exposed, a similar proportion of mosquitoes responded to the cues and landed on the target/targets, and a similar number of mosquitoes remained unresponsive. Specifically, doubling the number of large targets saw no noticeable increase in the number of mosquitoes caught relative to one target. This means that an increase in the number of targets or greater target surface area does not necessarily lead to an increase in the number of mosquitoes caught. Results from Experiment 1 and Experiment 2 further support this. For instance, in Experiment 1, large targets caught on average only double the number of mosquitoes compared to small targets, although they presented four times the trapping area of the latter (mean ± SEM number of mosquitoes on a large target=4.7 ± 0.50, mean ± SEM number of mosquitoes on a small target=2.5 ± 0.39). Similarly, in Experiment 2, the target positioned vertically caught a similar number of mosquitoes compared to the horizontal surface, although the vertical target presented double the area exposed (mean ± SEM mosquitoes on vertical target=4.0 ± 0.81, mean ± SEM mosquitoes on horizontal target=3.3. ± 0.80).

5.4. Discussion

The results reported here demonstrate that the physical characteristics of a target significantly influence the flight and landing responses of host-seeking *An. coluzzii* females. In specific, mosquitoes landed more on large targets and visited these targets more often when they were presented alongside small targets, whilst no effect was detected in the landing rate or flight parameters for the two spatial orientations tested here. Thus, for *An. coluzzii* mosquitoes, the size of the target plays a more important role than the orientation when considering the landing phase. Furthermore, Experiment 3 demonstrated that is not only the presence or absence of the thermal cue but also the size in which this is presented that conveys important information that drives the landing phase.

Mosquitoes visited fewer times the targets that had their entire surface heated (positive control) compared to targets that had no surface heated (negative control). From this, one could be tempted to deduce that targets that had no surface heated were more attractive to mosquitoes, as they kept drawing mosquitoes in their proximities. However, it is important to consider that the positive control caught significantly more mosquitoes, and once landed, the mosquitoes could not reinstate their flight. Thus, it is likely that the result of fewer visits recorded for the positive control is partly an artefact of the experimental design, where fewer mosquitoes were available to repeatedly visit the target. Similarly, given that different treatments resulted in different landing rates, the fact that flight tracks had comparable duration and distance across different treatments would suggest that, in treatments where more mosquitoes landed, the targets might have had a higher capability to draw mosquitoes close to them and keep the mosquitoes in their proximities, thus offsetting the fact that fewer mosquitoes were free and able to visit the targets. However, further studies with an experimental design that allows mosquitoes to land and reinstate their flight are needed in order to confirm this. It is also to be noted that although targets with a small heated area did not stimulate as many landings as those elicited by targets with at least half of the area heated, mosquitoes were still highly attracted to them, as demonstrated by the fact that mosquitoes repetitively visited the

target area and remained in their proximity for a considerable length of time. Therefore, it can be concluded that although the targets with small heated areas were missing the cues that stimulate a landing response, they were still sufficiently attractive to lure mosquitoes within a short distance from their surface.

The most important result from Experiment 3 indicates that when the thermal cue was presented below a certain area size, only a small number of mosquitoes landed on the target. Thus, it is clear that below a certain area, the thermal cue is insufficient to trigger landing. As thermal cues act in the mid- to close-distance range, it can be expected that the likelihood that mosquitoes would fly close enough to small heated areas might be quite small. Inversely, mosquitoes can be expected to encounter more easily larger heated areas. This is perhaps the most salient outcome of the study, and two main implications can be extrapolated from these findings. First, the study presented here demonstrated that the thermal cue significantly increases landing only when presented across an area that exceeded a threshold value in size. From a biological perspective, this could be explained by the fact that mosquitoes might encounter several visually conspicuous objects that after being warmed by the sun, retain a certain degree of thermal energy. The size of the heated area might add an extra level of fidelity that indicates a host, as by exclusion, small heated surfaces might probably not signal the presence of a human being. However, it is interesting to note that a previous study recorded abundant landing responses in *Ae. aegypti* on small Peltier plates (6 cm x 9 cm) when exposed to carbon dioxide (McMeniman *et al.*, 2014). The discrepancies in the results could be due to either species differences, or differences in the experimental area that was given to the mosquitoes, as in the study presented here the stimulus was offered in a large wind tunnel, while the experiment with *Ae. aegypti* mosquitoes used a smaller cage (30 cm x 30 cm x 30 cm), which might have promoted mosquitoes to be more in contact with the thermal gradient.

The second important implication that arises from this experiment is that it was demonstrated that the targets used caught similar numbers of mosquitoes

irrespective of whether only half or the whole surface area was heated. From an application point of view, these results provide the basis for key trap optimisation developments. Several studies have demonstrated how the inclusion of a thermal cue augments trap catches (Kline and Lemire, 1995; Hawkes *et al.*, 2017a; Cribellier *et al.*, 2020). However, the inclusion of thermal cues can prove difficult and/or expensive in the field as it requires energy either to warm large volumes of water or heat the device electrically (Cribellier *et al.*, 2020). Thus, halving the area of a trapping device that needs to present the thermal cue could drastically reduce the costs associated with producing this cue without any significant reduction in capture rates. At last, considering the unheated margins of the target (which size varied depending on the treatment), the fact that independently from their area sizes all unheated surfaces caught similar numbers of mosquitoes suggests that the size of the target, if not accompanied with adequate thermal cues, may not be the only dominant factor that defines the number of catches. Thus, to create effective targets it might be important to consider not only the final size of the target but specifically, the size of the areas that deliver the specific host cues.

In Experiment 1, consistently more mosquitoes landed on large targets compared to small targets. This preference was particularly accentuated when the two different target types were presented together, as in this case mosquito flight activity considerably concentrated around large targets. However, no difference was found in any of the flight parameters when comparing the two target types when presented alongside similar targets, i.e. comparing the flight activity around the small targets in the small vs small assay with the flight activity around the large targets in the large vs large assay. Thus, it appears that the strong difference in attraction and flight behaviour only occurs when the two targets are in direct competition. This raises important points with regards to the attraction level that different targets elicit and the distance at which different cues affect mosquito behaviour. Assuming a dark-adapted eye, with a resolution angle of approx. 40 ° (Land *et al.*, 1999), the estimated distance at which *Anopheles* mosquitoes could resolve the large target is ~ 41 cm, whilst the small target could only be resolved ~ 21 cm away. As visual cues are known to be attractants (Van

Breugel *et al.*, 2015; Hawkes and Gibson, 2016), the large target could have elicited a stronger attraction from further distance, which could explain the reason of the higher flight activity around the large target, along with its higher capture rate. From an application point of view, this suggests that the presence of competitor sources of attraction that present combinations of host cues could diminish the effectiveness of targets, especially if these are of a smaller size compared to the competitor source. Thus, care must be taken when evaluating where to position targets. This could be of importance for example if targets are to be placed in proximity of a sleeping person.

In contrast to the results presented here, a field study conducted in The Gambia region found no evidence of competition between multiple counter-flow traps that were placed in each of the four corners of an experimental hut, since all traps collected similar numbers of *An. gambiae s.l.* mosquitoes (Jawara *et al.*, 2009). It has to be noted however that in the field study all traps were identical, so all traps elicited the same levels of attraction, whilst in the study presented here the targets were purposely different, thus they emanated different levels of attraction. These differences in the experimental set-ups could, at least in part, explain the discrepancies reported in the two studies. Previous research found that an important factor that defines source competition is the physical distance at which the sources are placed (Okumu *et al.*, 2010b). Whilst in the field study the traps were positioned with metres between each other, in the study presented here the targets were separated by <1 m. Given that some factors of a target can influence the distance at which it is detected by mosquitoes (e.g. the size of a visual target influences the distance at which mosquitoes can see it), it follows that the gap space between two targets presented simultaneously could play an important role in target competition. Therefore, it would be interesting to investigate whether the highest attraction levels reported for large targets are maintained even when the two objects are positioned with a greater distance between them.

Throughout the different treatments in Experiment 1, the tracks recorded in the proximity of small targets were more convoluted compared to those recorded in

the neutral area and around large targets. This could be explained by considering the physical properties of a flight track that is restricted to travel above a small region. In this case, to keep pivoting above the region, the tracks have to necessarily keep turning, thus increasing the mean tortuosity. This suggests that although small targets resulted in fewer total landings, they still elicited high levels of overall attraction towards the vicinity of the target as mosquitoes repeatedly hovered above them, indicating intensive exploratory behaviour which is associated with the assessment of the stimulus (Spitzen *et al.*, 2013). Similar exploratory behaviours were reported in *An. coluzzii* (Hawkes and Gibson, 2016) and *Ae. aegypti* (Van Breugel *et al.*, 2015) exposed to odour cues, as they were described to approach very closely, without alighting on, small visually conspicuous objects of dimensions comparable to or smaller than the small target used here. This, taken together with the results reported in Experiment 3 suggests that large thermal, and to a lesser degree, visual cues are important in eliciting landing, whilst small visual and thermal cues are still sufficiently attractive to lure mosquitoes into proximity of the target. This could have important implications for the design of control tools, as it suggests that tools that require physical contact (e.g. sticky traps, or surfaces that deliver lethal substances) should incorporate large thermal and visual cues, while for tools that only require mosquitoes to fly in the proximity of the capture mechanism (e.g. suction traps), small visual and thermal cues would suffice as long as they can attract mosquitoes to fly close to the capture mechanism.

When considering the tortuosity, it is also to be noted that in Experiment 2 tracks in the proximity of vertical targets were more convoluted compared to the ones recorded around horizontal targets. This could be caused by the physical characteristics of vertical targets, as they create a physical barrier that forces mosquitoes to drastically change their path to avoid collision (Nakata *et al.*, 2020).

The spatial orientation of an object can drastically alter how it is perceived by the organisms around it, and therefore the response that it elicits (Kennedy, 1940). For example, the orientation of an object could change how it is seen by

mosquitoes by changing the type and portion of the mosquito's eyes that is filled with the image of the object. Specifically, vertical objects would largely occupy frontal ommatidia, while horizontal objects would mostly occupy ventral ommatidia. The spatial orientation also determines the distance at which the object can be detected and the type of response elicited in the insect, as for some mosquito species vertical objects might act as attractants, whilst other mosquito species might actively avoid them (Bidlingmayer and Evans, 1987). As a previous study reported that *An. coluzzii* females were highly attracted to a 20 cm x 20 cm black plastic tile that was held in a vertical position and was presented with host odour (Hawkes and Gibson, 2016), in the study presented here it was expected to find a stronger attraction response around vertical targets. However, as the target offered here did not only present a visual cue, other factors need to be taken into account before drawing any conclusion. For example, the spatial orientation also impacts how a thermal cue is detected (Lazzari, 2019).

Convection currents rise from the surface of a warm object and mostly move upwards (Zermoglio *et al.,* 2017). Thus, convection currents emitted by the horizontal target would occupy a larger volume of the air column compared to those originating from the comparatively slim 'footprint' of the vertical target. Previous research has proven that host-seeking mosquitoes are strongly attracted by convection currents (Peterson and Brown, 1951; Khan *et al.,* 1968; Dekker *et al.,* 1998; Zermoglio *et al.,* 2017), therefore, it would be expected to find a stronger attraction response for horizontal targets. The results presented here found neither a preference for landing on either vertical or horizontal targets, nor any differences in the number of visits, the total time, nor the total distance covered around the targets. Yet, when considering the standardised measurement of catches per 100 cm^2, horizontal targets were between two to three times more efficient in catching mosquitoes. The reduced capture rate per surface area of vertical targets might suggest that convection currents, which occupy a larger area on horizontal targets, play a crucial role in eliciting landing. This is consistent with a previous study that reported that convection currents, and specifically, the area size that they occupy, play an important role in eliciting

landing responses (Wright and Kellogg, 1964). Furthermore, a recent investigation noted that *Aedes albopictus* (Skuse, 1895) displayed lower landing rates on a feeding substrate kept at 37 °C if this was positioned vertically rather than horizontally on the floor (Hol *et al.*, 2020). It is interesting to note that several studies also reported that *Anopheles spp.* mosquitoes mostly approached and landed on the top section of a bed net when this was baited with a human (Lynd and Mccall, 2013; Sutcliffe and Yin, 2014; Parker *et al.*, 2015). In the mentioned studies the authors also described an increased flight activity on the top part of the net. The concentration on the top part of the net could also be a construct that results from the natural response of *Anopheles* mosquitoes to a vertical barrier, as it has been demonstrated that these mosquitoes flight upwards when they arrive in the proximity of an obstacle (Hawkes and Gibson, 2016; Cribellier *et al.*, 2018). Overall, these results substantiate the hypothesis for a preference towards horizontal targets. On the other hand, a different study reported that *Ae. aegypti* landed nearly ten times more on a purple unheated target when it was oriented vertically compared to when it was placed horizontally (Smith *et al.*, 2020a). The remarkable difference between Smith *et al.* (2020a) findings and the results reported here could be partially attributed to differences in species behaviour, as it has been reported that different species present radically different responses to traps positioned with different orientations (Browne and Bennett, 1981; Bidlingmayer and Evans, 1987). For example, as *Ae. aegypti* originated by tree-hole breeding ancestors (Powell and Tabachnick, 2013), it could be expected that these mosquitoes positively respond to vertical targets as they might resemble trees, a trait that might not be present in *An. coluzzii* mosquitoes.

Finally, there is another aspect that needs to be considered when comparing the capture rate on vertical and horizontal targets. Vertical targets presented double the surface, yet half of it (the upwind side) was mostly ignored by mosquitoes. Thus, the striking difference in the density of mosquitoes caught per area unit could be the result of the overall avoidance of the upwind surface. This could have been caused by the position of the target, as it was placed perpendicular to the wind direction. It would be interesting to conduct further studies with

vertical targets positioned parallel to the wind direction to examine whether the preference for one side over the other remains. Future work may also investigate the effect of other spatial orientations and variations of these. For example, it would be interesting to determine whether the height at which horizontal targets are presented has an impact on mosquitoes' landing response.

When examining solely vertical targets, it is clear that mosquitoes had a strong preference for the downwind side, as evidenced by both their landing response and flight activity. These results corroborate the findings of Bidlingmayer and Evans (1987), as they also reported a greater capture rate on the downwind side of a vertical barrier. In their analysis, the authors attributed this outcome to the wind speed, as high wind velocities were recorded on the upwind side, which might have interfered with normal host-seeking behaviour, as for example, fast gusts of wind might have deterred mosquitoes from flying on the upwind side of the target. Here, an attempt was made to visualise air movement in the wind tunnel arena in presence of the two vertical targets using a smoke pellet (13g white pellet, Rothenberger, Germany). With this qualitative technique it was possible to observe how vertical targets created a barrier that broke down the air stream and created multiple eddies on the downwind side. It can be supposed that the odour plume was also scattered and fragmented after encountering the vertical targets. However, here the airspeed was not measured. Further studies are needed in order to elucidate whether the strong preference here recorded was attributed to differences in airspeed, or if other factors played a role in driving more mosquitoes to land on the downwind side of the target.

The recorded flight speed of mosquitoes approaching the target was similar for all treatments and was comparable in magnitude with the speed of approaching tracks recorded in previous studies that used different mosquito species exposed to different host cues (~0.24 ± 0.14 m/s) (Beeuwkes *et al.*, 2008; Lacey and Cardé, 2011; Smith *et al.*, 2020a). Furthermore, throughout all experiments, the flight speed of the tracks recorded around the targets was consistently lower compared to the flight speed of the tracks recorded in the area where no target was offered. Thus, is clear that the presence of a range of host cues induced

slower flights in mosquitoes. This is similar to what was reported by Beeuwkes *et al.* (2008) and Spitzen *et al.* (2013), who found that *An. gambiae* mosquitoes significantly decreased their flight speed and increased the tortuosity of their tracks when approaching the source of either an odour cue alone or combined with a thermal cue. Thus, it appears that the reduction in flight speed is a common trait associated with the approach towards a target, which might be explained by these possible mechanisms: a) reducing flight speed allows mosquitoes to thoroughly scan the environment (Spitzen *et al.*, 2013), or b) preparation for the landing phase requires a lower speed (Healy *et al.*, 2002; Beeuwkes *et al.*, 2008). As a recent study demonstrated that *Aedes* mosquitoes do not prepare for landing like other insects, in that they do not extend the legs or orient their body at a specific angle but instead just bounce on the surface after contact (Smith *et al.*, 2020a), it is more likely that the reduction in flight speed is not linked with the preparation for landing, but rather is an adaptative strategy that facilitates a more thorough search (Spitzen *et al.*, 2013).

By reducing speed, mosquitoes might better detect the thermal cue, as the reduction in flight speed might lead for example to an increase in the time spent in proximity of the cue which would enhance the chances of contacting convection currents. Ultimately, this would enable mosquitoes to localise more accurately the target (Beeuwkes *et al.*, 2008; Spitzen *et al.*, 2013). Alternatively, it is also possible that the change in speed might be a response to the change of airflow that might be found around a target. This change of airflow could be caused by the target's physical structure obstructing the airflow and/or by convection currents rising from it. It is interesting to note that Spitzen *et al.* (2013) attributed the cause of the reduction in flight speed mainly to the thermal cue, while in the study presented here flight speed was still reduced even when flying above a visually conspicuous but unheated target (i.e. negative control treatment in Experiment 3). Thus, it would appear that flight speed is not only reduced as a response to heat, but other host cues (e.g. visual cues) might be sufficient to elicit this response. This is supported by findings reported in a previous study conducted on *An. coluzzii* mosquitoes, in which it was demonstrated that when approaching a black tile, in presence of host odour, host-seeking mosquitoes

rapidly decreased their ground speed (Hawkes and Gibson, 2016). Thus, a visual cue combined with host odour might be sufficient to elicit the reduction in flight speed.

Overall, results from the three experiments suggest that independently from the number of targets presented or the surface area exposed, the proportion of mosquitoes that responded with a landing outcome did not increase in a manner that corresponded with the increment of the surface exposed. For example, vertical targets caught a similar number of mosquitoes compared to horizontal targets, even though their surface was double the area presented by the horizontal targets. In practical terms, this might have some significant implications, as it suggests that by doubling the number of targets in an area (or by doubling the size of the target) one will not necessarily catch double the number of mosquitoes. For field applications, more work is required to better understand what modifications of the targets could be implemented in order to elicit a response in the mosquitoes that remained unresponsive under the conditions presented here. For example, as humidity was previously demonstrated to be an important cue mediating short-range and alighting behaviour (Wright and Kellogg, 1962; Khan and Maibach, 1966; Eiras and Jepson, 1994), it would be interesting to determine whether the addition of water vapour could improve landing rates.

Another important point that arises from this finding is that, as not all the mosquitoes in a subpopulation might respond to the target, when considering the intensification of control efforts the increase in the number of targets or the trapping surface might not be the best cost-effective approach, as this might not lead to a drastic increase in the capture rate. Thus, to intensify control efforts it is not enough to simply increase the trapping surface, and several factors should be taken into account when planning the intensification of vector control measurements. The last consideration that arises from the disparity between the catching rates and the target area exposed is the fact that different target sizes could be used for different purposes. For example, as larger targets caught a greater number of mosquitoes, it might appear that they would be more suitable

for vector control mass-trapping techniques, as for this purpose traps need to catch a substantial number of mosquitoes each night if they are to reduce the mosquito population in the area. On the other hand, smaller targets could be better used for discreet vector-surveillance points, as their smaller size might render them more suitable to be placed close to human dwellings. Furthermore, a smaller size might translate to a cheaper cost of production and/or operation. Finally, when considering the density of mosquitoes caught it becomes evident that small targets were three times more efficient than large targets. This emphasizes that several factors, and not only the "raw number of mosquitoes caught" should be taken into account before drawing conclusions on the uses of different sizes. Future research that includes an in-depth cost-effectiveness study similar to that carried out by Lindh *et al.* (2009) for tsetse traps is recommended to identify the advantages and disadvantages of using targets of different sizes, and therefore, to determine the optimal sizes to be used for different entomological applications.

Although landing is a necessary part of any flight displacement (Shen and Sun, 2017) and is one of the most critical parts of flight behaviour (van Breugel and Dickinson, 2012), to date it remains vastly unexplored in mosquitoes. Several studies have extensively reported the mechanisms that control the landing stage, along with the in-depth characterisation of flight manoeuvres that take place during landing in numerous flying insects, including stingless bees (Tichit *et al.*, 2020), honey bees (Srinivasan *et al.*, 2000; Evangelista *et al.*, 2010; Baird *et al.*, 2013; Zhao *et al.*, 2017), houseflies (Wagner, 1982; Balebail *et al.*, 2019), and fruit flies (Tammero and Dickinson, 2002; van Breugel and Dickinson, 2012; Shen and Sun, 2017). Yet, little is known on what are the specific cues that induce landing in mosquitoes (Lacey and Cardé, 2012), and what are the mechanisms that control this phase. To the author's best knowledge, only one study characterised the landing phase in mosquitoes through recordings obtained using high-speed cameras (Smith *et al.*, 2020a). They reported that *Ae. aegypti* mosquitoes do not prepare for landing like other insects do (i.e. no visible body pitching or legs adjustment), but rather they adopt a passive strategy, bouncing on the surface several times as a mean to dissipate kinetic energy.

Although this study shone some light on this critical behaviour, it did not examine the immediately preceding flight approach toward the target, and aside from measuring the flight speed, no other characterisation was carried out for the pre-landing moments. A previous study carried out on *Culex quinquefasciatus* (Say, 1823) attempted to characterise the flight approach prior to landing on black beads that were coated with host odour (Lacey and Cardé, 2011). However, the authors found no stereotypical approach, as they described that some mosquitoes descended right from above and landed directly on the target, others hovered around, while other mosquitoes landed downwind and arrived secondarily via short flights or hops. It is to be noted that in this study the thermal cue was missing, thus it might be possible that the lack of a uniform response was caused by the absence of this crucial landing cue. It is important to note that the two studies mentioned above were carried out on mosquito species that present striking physiological and behavioural differences compared to anopheline species.

A study conducted by Spitzen *et al.* (2013) endeavoured to describe the dynamics of the flight tracks that *An. gambiae* generated when approaching different host cues. In their experiment, mosquitoes were offered different treatments which were composed of combinations of olfactory cue and heat. The authors concluded that in the presence of host odour, mosquitoes created longer and more convoluted tracks, and this was not replicated when only heat was presented, although the addition of a thermal cue in combination with the odour cue elicited a reduction in the flight speed. It should be noted however that in their experimental design the heated object was positioned outside of the flight arena, thus the effect of convection current was excluded from the design. Although this study set the basis for describing flight tracks approaching a target, the authors did not characterise the sequence of events that took mosquitoes from flying to landing, and the effect that convection currents might have on track dynamics are still unknown. Thus, much is still left to explore in these respects. The study reported here demonstrated the feasibility of carrying out landing assay using different target surfaces, along with recordings of flight tracks in 3D. This approach opens new avenues for exploration of the short-range and landing

phase in host-seeking *Anopheles* females, as it combines the quantification of landing with the characterisation of pre-landing movements. Together, both aspects provide a more comprehensive understanding of how mosquitoes respond to different host-emitted cues (Lacey and Cardé, 2011). As previously demonstrated by Cribellier *et al.* (2018, 2020), understanding the events that take place in close proximity of a target may help when devising improvements for current vector control methods. Thus, further studies focussed on elucidating the modalities by which *Anopheles* mosquitoes respond to different landing targets might help direct future improvements of traps.

Four main limitations have to be taken into account when considering the results presented in this study. Firstly, the study was conducted in a wind tunnel, and although this is considered to be a large wind tunnel that favours free flight behaviour within the arena, it might have restricted some of the behaviours that mosquitoes display in the wild or it might have provided distorted cues due to proximity of bounding walls (see section 3.2) (Daykin and Kellogg, 1965; Kennedy, 1977, 1978). Secondly, the analysis of the 3D tracks was restricted solely to the areas where the target was placed plus the buffer areas around the target (approx. 10 cm of buffer on the X and Y-axes, and ~ 27 cm of buffer in the Z-axis). Therefore, the analysis does not show the full sequence of behaviour from take-off, through upwind flight, to landing. Thirdly, the analysis of the flight parameters was carried out on mean values that were obtained by averaging the values of all the tracks recorded in the area. As highlighted by Spitzen and Takken (2018), some flight parameters might vary drastically in a short distance, depending for example on the entrance/exit of an odour plume or the distance from a target. Consequently, more information might have been extrapolated by analysing the tracks within sections that incremented in distance from the target. Lastly, the experimental design included the release of 25 mosquitoes in each assay. This guaranteed a high rate of successful replicates throughout the three experiments, however, it rendered it unfeasible to determine the order of visitation of the targets, as it was not possible to attribute each track to an individual mosquito. Accordingly, further research using fewer or single individuals might provide insights on the order of visitation, which, linked with the

landing numbers, might offer a comprehensive picture of the sequence of events that take place between the response to attractive cues and the landing. Focus should also be put on developing an assay where it would be possible to record the landing rates while allowing mosquitoes to continue moving after landing (e.g. without using sticky targets). Results from these studies might elucidate whether behaviours observed here differ when mosquitoes are free to continue flying and making repeated landings. Despite these limitations, the analysis fully satisfied the testing of the outlined hypotheses, and also provided a general baseline understanding on mosquito approach to different targets. Further work that takes into consideration the limitations mentioned above and that builds on the results presented here might further our understanding on the landing phase in *An. coluzzii* mosquitoes.

5.5. Conclusions

The results reported here indicate that different target characteristics elicited different landings rates and flight behaviour in *An. coluzzii* host-seeking females. While no difference was detected in the flight and landing response of mosquitoes exposed to targets positioned horizontally or vertically, a strong difference was found when comparing the target size. Mosquitoes landed in greater numbers on large targets, which were also visited more compared to small targets when both target types were presented together. On the other hand, small targets caught a higher density of mosquitoes. Furthermore, the results presented here reinforce findings described in Chapter 4, as it was further demonstrated the importance of the thermal cue, and in specific, it was described for the first time the crucial role that the area of the thermal cue plays in eliciting landing. Targets with only half of their area heated caught an equal number of mosquitoes compared to those where the entire area was heated. Altogether this study unpicks the relative effects that single physical characteristics of a target can have on mosquito behaviour, and how these play specific roles in attracting and subsequently eliciting landing in *An. coluzzii* females. Not only does this further our understanding of this neglected behaviour, but it also demonstrates much potential for optimisation of traps and targets by improving their

effectiveness (e.g. catching rates) while also reducing their cost. Moreover, it further demonstrates the importance of selecting the appropriate physical characteristics of traps or targets, as minor variations might cause drastically different behavioural responses, which could translate into the collection of different species compositions or different abundances (Browne and Bennett, 1981; Bidlingmayer and Evans, 1987). Therefore, the physical characteristics of a target should be chosen depending on its main purpose, as targets for vector control or vector surveillance might have different requirements.

6. STUDY OF CLOSE-RANGE, LANDING, AND POST-LANDING BEHAVIOURAL RESPONSES TO BLOOD MEALS AT DIFFERENT TEMPERATURES

6.1. Introduction

The endpoint of a successful host-seeking journey for *Anopheles* mosquitoes culminates with the intake of host's blood, which is then digested and the nutrients deriving from it are used for egg development. Thus, after having detected and having landed on a host, mosquitoes must proceed with the sequences of behaviours that will take them to imbibe the host's blood. These behaviours have evolved under the strict selective pressure of reducing the duration of contact with the host to a minimum, whilst still achieving sufficient blood intake (Gillett, 1967; Pereira *et al.*, 2017). As blood-feeding is an extremely high risk event (Edman *et al.*, 1972, as cited in Vinauger *et al.*, 2018), it can be expected for these behaviours to be the result of a finely tuned response to the range of host cues received. Although Chapter 4 provided valuable information on the host cues that drive landing, due to the experimental set-up, it was not possible to draw any conclusions on the behaviours adopted by the mosquito immediately before and after landing had occurred, thus the pre-feeding and feeding phases remained unclear.

A search in the literature also suggests that little has previously been reported on the moment-to-moment behaviours that take a female mosquito to successfully blood feed after it has landed on a host (Hol *et al.*, 2020), Additionally, the existing reports have focussed on *Aedes* mosquitoes (Choumet *et al.*, 2012) and are several decades old (see for example reports by Gordon and Lumsden, 1939; Christophers, 1960; Jones and Pilitt, 1973). Very few studies examined blood-feeding behaviour as a concert of multiple behaviours, and the majority of the existing reports investigated specific aspects of either blood foraging or feeding. For example, some studies have focussed on probing and foraging behaviour, which was described for different mosquitoes' species in relation to salivary gland function (Ribeiro *et al.*, 1985; Ribeiro, 2000), mosquito's infection with biocontrol agents (Moreira *et al.*, 2009) or with *Plasmodium* parasite (Ponnudurai *et al.*, 1991; Choumet *et al.*, 2012), presence of insecticides on the feeder's surface (Moritomo *et al.*, 2021), and different attractiveness to host odour

(Geier and Boeckh, 1999). Other studies have examined feeding propensity and nutrient utilisation (i.e. egg formation) in mosquitoes exposed to different blood sources, blood components, or blood with different erythrocytes densities (Emami *et al.*, 2013; Phasomkusolsil *et al.*, 2013; Mamai *et al.*, 2017; Harrison *et al.*, 2021). All the studies mentioned above limited their research on a restricted range of behavioural aspects and, for this reason, an exhaustive characterisation of blood-feeding behaviour has not yet been achieved (Hol *et al.*, 2020). In addition, only few studies have reported feeding responses when mosquitoes were subjected to blood kept at different temperatures. For example, Greppi et al. (2020), McMeniman et al. (2014), and Raji et al. (2019) examined the role that different host cues had in driving *An. gambiae* and *Ae. aegypti* females to feed on blood kept at either room temperature or host temperature. In each study, mutant mosquitoes that had a specific sensory receptor silenced (respectively a heat seeking mediator, a carbon dioxide receptor, and a lactic acid receptor) were created. By comparing the feeding responses between wildtype mosquitoes and mutant mosquitoes the authors were able to conclude that blood-feeding is enhanced by each one of the host cue studied (heat, carbon dioxide, and host odour), as all mutant mosquitoes exhibited reduced feeding behaviour. However, it is important to highlight that the three above-mentioned studies exposed mosquitoes to only two temperatures, thus little is known on mosquitoes response when exposed to a wide range of temperatures.

Only two studies have examined several mosquito behavioural traits in relationship with different blood temperatures. Grossman and Pappas (1991) recorded the time spent by *Ae. aegypti* females on pre-foraging, foraging, probing, and feeding behaviour when exposed to an arm kept at a range of temperatures (from 29 °C to 36.2 °C). The authors found no difference in the behaviours that preceded blood intake but recorded a significantly shorter time of engorgement for treatments at higher temperatures. This shorter feeding time translated into a faster feeding rate (i.e. increased intake volume per time frame), which led the authors to conclude that the feeding efficiency of *Ae. aegypti* increased when left to feed on an arm that presented normal host temperature (around 36.2 °C). The second study was conducted by Cosgrove and Wood (1995) on three mosquito species (*Ae. aegypti*, *An. stephensi*, *An. arabiensis*) which were exposed to artificial meal formulations set at temperatures ranging from 28 °C to 40 °C. The authors investigated the number of mosquitoes

probing and feeding at different time intervals, as well as the time needed for 50% of the mosquitoes to be fully fed. They found that *Ae. aegypti* had the highest probing and feeding propensity across all investigated temperatures if compared to the two *Anopheles* species, and they also found that *Ae. aegypti* engorgement rate was faster for temperatures between 36 °C to 40 °C, while *An. arabiensis* showed a consistently low probing and feeding response for all blood temperatures, although a non-significant increase of the two behaviours was obtained at the highest temperature. *Anopheles stephensi* showed an in-between behaviour in relation to the two other species and had a faster engorgement rate when presented with blood between 32 °C to 40 °C. It is to be noted however that both studies only examined a few behavioural traits, thus they provide the basis of a general overview of mosquitoes approach and feeding behaviour, whilst a detailed moment-to-moment description of mosquitoes feeding behaviour is still absent. Furthermore, it is to be noted that in all the previously mentioned studies the hottest tested temperature was 40 °C. While other studies carried out on different hematophagous insects (kissing bugs, bed bugs, and tsetse flies) considered the feeding response (i.e. propensity to feed) of these animals when exposed to blood at high temperatures (42 °C and 48 °C) (Lahondère and Lazzari, 2015; DeVries *et al.*, 2016; Lahondère *et al.*, 2017), the effects that hot blood might have on *An. coluzzii* behaviour and physiology, are still unknown.

Aside from the behavioural aspects, it is also important to look at the physiological changes that occur in the mosquito's body when considering a blood-feeding event. Being ectotherms that feed on warm-blooded hosts, mosquitoes are exposed to considerable risks every time they need to acquire a new blood meal to form eggs (Benoit and Denlinger, 2017; Vinauger *et al.*, 2018). Aside from the risk of being killed by the defensive behaviours of the host (Edman and Scott, 1987), there are several other physiological challenges that female mosquitoes have to endure when blood-feeding, such as the detoxification of amino acids excess and heme group ingested with the blood, excretion of large volumes of liquid, osmotic stress resulted from the ingestion of ions, and the increase of bacteria population in their gut (Benoit and Denlinger, 2017; Benoit *et al.*, 2019). Among the many risks, the thermal stress associated with the large ingestion of warm blood is often overlooked (Benoit *et al.*, 2011; Lahondère and Lazzari, 2015; Benoit and Denlinger, 2017), although it poses a serious threat to the physiological state of the blood-sucking insects (Lahondère *et al.*,

2017) and the microorganisms and parasites associated with them (Lahondère and Lazzari, 2012, 2013; Benoit *et al.*, 2019). Previous studies have examined how different mosquito species respond and mitigate the thermal damage that is associated with imbibing warm blood. Lahondère and Lazzari (2012) showed that by maintaining droplets of excretion at the end of their abdomen, the body temperature of *An. stephensi* females decreases during a blood-feeding event. This mechanism, known as evaporative cooling, allows anopheline mosquitoes to thermoregulate when intaking warm blood. This has also been described in *Cx. quinquefasciatus* (Reinhold *et al.*, 2021). On the other hand, Benoit *et al.* (2011) showed how the production of Heat Shock Proteins (HSPs) in *Ae. aegypti* is essential to tolerate the rapid body temperature change, which follows an increase of up to 10 °C in just one minute when imbibing blood. Heat Shock Proteins are molecular chaperones that help preserve enzymatic function and protein structure when these are subjected to critical temperatures. By suppressing the expression of HSPs, the authors demonstrated that blood digestion was impaired and egg production was reduced.

Although mosquitoes have evolved different strategies to mitigate thermal damage, it is not known if these strategies are still effective when mosquitoes imbibe blood at high temperatures (e.g. from a person with fever). Given that fever is one of the main malaria symptoms (Nacher, 2005), it would be of epidemiological interest to assess whether mosquitoes respond in similar ways to blood at host temperature and blood at host-fever temperature. Moreover, it can be expected that thermal cues that are below or above the accepted temperature range might either not elicit a response or even elicit an avoidance behaviour. Even if considering only host temperature, much is still unknown on the moment-to-moment behaviour that takes *Anopheles* females to feed after they have alighted on the host (Choumet *et al.*, 2012; Hol *et al.*, 2020). Altogether, these considerations point at the need of further exploring this topic, given that is during the mosquito-host skin interaction that pathogens and parasites are transmitted (Choumet *et al.*, 2012).

Understanding the behavioural details that precede feeding and the physiological chain of events that follows it might result in identifying aspects that could be targeted as possible vector control approaches. Thus, the study presented here investigated the behavioural and physiological responses of *An. coluzzii* females when offered

blood at a range of temperatures (30 °C, 36 °C, 42 °C, 48 °C). As *Anopheles* mosquitoes are anautogenous (i.e. they need to acquire blood to produce eggs), the measurement of reproduction parameters provides insights not only on the feeding event, but on its aftereffects. Together, fertility and mortality are the two main components used to describe mosquito's fitness (Marrelli *et al.*, 2006; Emami *et al.*, 2013). By also considering the fitness, this study aims to further the understanding on the long-term effects that feeding events might have on the life cycle of *An. coluzzii* mosquitoes. To the author's best knowledge, this is the first comprehensive report investigating several elements of *An. coluzzii* response when offered blood at different temperatures. In particular, the following three aspects were examined: a) behavioural responses, b) fitness after ingestion, and c) quantification of ingested blood. The following hypotheses were set out:

- H_0: The time between the beginning of the experiment and the first landing event is equal for all treatments.
  H_1: The time between the beginning of the experiment and the first landing event is shorter in treatments where the feeder is set at higher temperatures (i.e. mosquitoes employ less time to find a hotter feeder).

- H_0: Mosquitoes show similar behavioural responses when exposed to blood at different temperatures.
  H_1: Mosquitoes exhibit different behavioural responses when exposed to blood at different temperatures.

- H_0: The feeding propensity (i.e. number of mosquitoes that engage in blood-feeding) is the same regardless of the temperature of the offered blood.
  H_1: Mosquitoes show a higher propensity to feed on blood set at a host-equivalent temperature (36 °C) compared with the feeding propensity presented when exposed to blood set at temperatures outside the normal host range.

- H_0: Mosquitoes ingest equal quantities of blood irrespective of the temperature at which the blood is presented.
  H_1: Mosquitoes ingest more blood set at host temperature (36 °C) compared to

the quantity of blood ingested when the blood temperature is higher or lower than that of host temperature.

- H_0: The mortality rates of mosquitoes fed on blood at different temperatures are equal.

  H_1: The mortality rate of mosquitoes fed on blood set at 48 °C is higher compared to the mortality rates of those fed on blood at lower temperatures.

- H_0: The number of eggs oviposited by fed females is equal irrespective of blood meal temperature.

  H_1: The number of eggs oviposited by females that fed on blood at 36 °C is higher than the number of eggs oviposited by females that fed on blood at other temperatures.

Improving our understanding of the many factors involved in blood-feeding events will help to direct future work aimed to develop new ways to control the mosquito-host interaction, which might contribute to reducing the spread of mosquito-borne diseases (Benoit and Denlinger, 2017; Hol *et al.*, 2020).

6.2. Materials and methods

6.2.1. Mosquitoes

Insects were reared as described in section 3.1, with the mosquitoes being reared on defibrinated horse blood. Female mosquitoes used for the behavioural and physiological assay were between five and ten days old and were prepared and maintained as described in section 4.2.1. All assays were performed within the second and third hour of the scotophase, i.e. when *An. coluzzii* mosquitoes are most active (Hawkes *et al.*, 2012). Sugar feeders were removed four hours prior to the experiment. At the beginning of the dark phase of the light cycle, mosquitoes were selected and then individually transferred into 25 mL plastic vials, where they were kept in darkness for one hour to allow eye adaptation to dark conditions (Sato, 1957; Moon *et al.*, 2014).

6.2.2. Odour source

To activate host-seeking behaviour, mosquitoes were presented with a combination of whole human odour and pulsed carbon dioxide. Whole human odour was delivered by the experimenter, who sat in front of the experimental cage (see the Behavioural assay section) with her mouth being positioned approx. 10 cm away from the cage and at a height of 10 cm from its base. To limit changes in body odour, only one experimenter was used throughout all assays, and the person abstained from eating food with spices, drinking alcohol, using perfumes and strong perfumed soaps and clothes detergents (Pates *et al.*, 2001; Shirai *et al.*, 2002; Lefèvre *et al.*, 2010; Verhulst *et al.*, 2011b). The carbon dioxide was regulated and delivered as described in section 5.2.3., with the exception that the tube was positioned on one side of the cage, opposite from where the feeder was placed.

6.2.3. Blood-feeding system

Mosquitoes were presented with defibrinated horse blood that had a constant packed cell volume of between 40-50% (Thermo Fisher Scientific, USA). This was considered to be an adequate source of nutrition as a previous study demonstrated that the absence of fibrinogens did not cause a reduction in egg production in *Ae. aegypti* females (Harrison *et al.*, 2021). Furthermore, the colony used in this assay had been successfully reared for over 60 generations on this blood.

Blood was offered via a Hemotek membrane feeding system (Hemotek, UK). To prepare the feeding system the following protocol was followed: at the beginning of each replicate, a new batch of 4.5 mL of defibrinated horse blood and a sealed 3 mL standard Hemotek reservoir were heated to the desired temperature in a water bath (JB Aqua 12 Plus, Thermo Fisher Scientific, USA). The reservoir was sealed using a layer of swine intestine membrane (i.e. sausage casing) as described in Phasomkusolsil *et al.* (2013). A new piece of swine intestine membrane was used for each replicate, and the reservoirs were thoroughly washed with warm water in between replicates. Blood temperature was monitored using an immersion thermometer (Fisherbrand Red Spirit Filled thermometer, Thermo Fisher Scientific, USA) and once the desired temperature was reached, the blood was mounted in the reservoir, which was then placed on a preheated FU1 Hemotek feeder that was set to

the target temperature. This process ensured that the blood was heated in a gentle and homogenous way, which prevented direct heat damage to the cellular blood components. Furthermore, it ensured that the superficial temperature of the feeder was homogeneous.

When not in use, the horse blood and the swine intestine membrane, which was kept in a 10% saline solution, were refrigerated at 4 °C. A new batch of blood and swine intestine was used each week.

Four temperatures were tested: 30 °C, 36 °C, 42 °C, and 48 °C. The optimal feeding temperature for *An. coluzzii* mosquitoes was expected to be 36 °C, as this is the temperature that closely resembles skin temperature of a healthy person and this species is highly anthropophilic. The highest temperature (48 °C) was included as a reference point, as this temperature is considered to be substantially higher than the temperature of any possible hosts, while 30 °C and 42 °C were chosen as possible limit temperatures of superficial human skin, i.e. resembling the superficial temperature of a human hand when the environmental temperature is approx. 19 °C (Wang *et al.*, 2007), and the superficial temperature of a person with high fever (Odongo-Aginya *et al.*, 2005), respectively.

6.2.4. Behavioural assay

The experimental cage consisted of a 15 cm x 15 cm x 15 cm polyester mesh cage (NHBS, UK), which was kept in a climate-control room at 25 ± 2 °C and 60 ± 10% RH. During the experiments, the lights of the room were switched off and the only illumination was provided by a single dim red light bulb (STAR LED, Osram, Germany) which was further dimmed by placing it under a double layer of black mesh and further filtered using a light filter (number 787, LEE, UK) which only allowed transmission of approx. $\lambda > 650$ nm. At the beginning of the experiment, one individual mosquito was introduced in the cage and left to habituate for two minutes, with the experimenter sitting in front of the cage as described in the section "Odour source". After the habituation period, the carbon dioxide stimulus was initiated, the feeder was placed on the top left corner of the cage, ensuring that the membrane was in contact with the cage mesh, and the experiment commenced (Fig. 50).
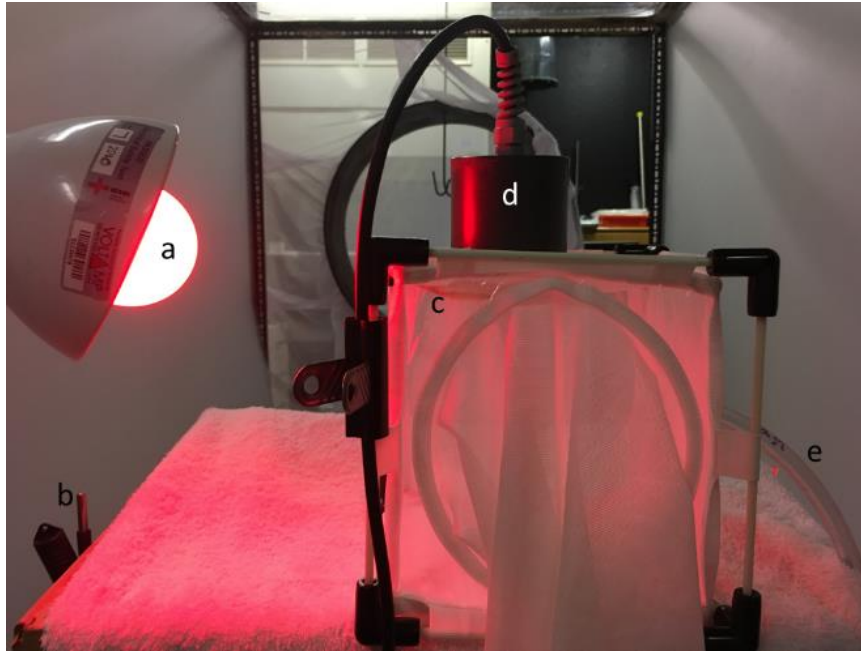
Figure 50: Photo of the set-up for the behavioural assay, where (a) indicates the light source, (b) the hygro-thermometer sensors, (c) the experimental cage, (d) the Hemotek feeder positioned with the feeding membrane in contact with the cage mesh, (e) tube delivering pulsed carbon dioxide. *The photo is taken from the position where the experimenter would be positioned. Note that room lights were turned off during the experiment and the red light source here is displayed without the dimming layers.*

For the entire duration of the experiment, which lasted ten minutes, the experimenter continuously observed the mosquito's behaviour and recorded the behavioural categories defined in Table 15 using a custom-made Phyton programme (version 3.9, Python Software Foundation, 2020) (Mandelli and Carnaghi, 2022a). The programme enabled the experimenter to take note of each behavioural event by pressing specific keys on the keyboard of a laptop that had its screen turned off to avoid adding light sources in the room. The programme automatically recorded the time at which the event took place, the duration of the event, and kept a separate count for each behavioural category.

At the end of the experiment, the feeder was removed from the top of the cage and the feeding status of the mosquito was assessed. As such, the mosquito was assigned to one of the following categories: a) fully fed, when the abdomen was fully extended and all red, indicating blood presence, b) partially fed, if blood could be seen in the abdomen but the abdominal segments were not extended, and c) not fed, if no blood could be seen in the abdomen. Mosquitoes that did not respond to the feeder in the first five minutes were deemed "unresponsive" and were not considered in the

analysis, whilst all mosquitoes that landed on the feeder, irrespectively of their feeding status at the end of the experiment, were deemed "responsive".

At the beginning of each replicate, the experimenter recorded the age of the mosquito and the light cycle that the mosquito was maintained at, while the surface temperature of the feeder was recorded both at the beginning and the end of each replicate. General notes from direct observation of the main behavioural traits displayed during the experiment were recorded for each replicate and were later used to generate a general qualitative description of mosquito's behaviour for each treatment.

For each treatment, replicates continued until a total of 25 mosquitoes responded to the feeder. The treatments were tested in a quasi-randomised order, between and within days, to exclude any effect of testing sequence (see section 3.3).

Table 15: Categories of behaviours recorded during the behavioural experiment. *The type of information refers to either the numerical count of the event (i.e. how many times the behaviour was displayed during the assay) or the duration of the event. Note that probing and sensing behaviours were often displayed in rapid succession and commonly alternated each other. Thus, it was not possible to record the duration of these events as separate entities and they were therefore combined under the category "foraging".*

Behavioural category	Definition	Type of information collected
Landing	Started when the mosquito alighted on the feeder and lasted until the mosquito moved away from the feeder, either by walking or flying away.	Numerical count; event duration
Foraging	Sequence of behaviours that encompass probing and sensing (see below). This started after the mosquito had alighted on the feeder and it began either probing or sensing. The event terminated when the mosquito moved away from the feeder or when it was deemed to be feeding.	Numerical count; event duration
Sensing	Repeated contact in short succession of the labium to the membrane feeder's surface. This behaviour generally occurred prior to probing.	Numerical count

Behavioural category	Definition	Type of information collected
Probing	Piercing the net or membrane with the stylet. This is accompanied by the characteristic vibrations of the head and palpi, which is the result of the repeated contractions of the powerful muscles that drive fascicle penetration (MacGregor, 1930). The event ended either when mosquitoes withdrew the stylet or when the palpi stopped vibrating, which indicated that imbibition had begun. This was confirmed shortly after by the appearance of blood in the abdomen.	Numerical count
Feeding	Started following a probing event when blood appeared in the abdomen and terminated when the proboscis was retracted from the membrane.	Numerical count; event duration
Grooming	Sequences of behaviours that encompassed the rapid rubbing or stroking-like movements of the legs' extremities against the proboscis, antennae, and other legs, as described in previous studies (Goldman *et al.*, 1972; Walker and Archer, 1988).	Numerical count
Resting	When mosquitoes were observed to remain on the walls of the cage without any notable displacement (i.e. without walking or flying). Note that grooming behaviour could occur when mosquitoes were deemed resting, as only the legs and head of the mosquitoes moved during a grooming event	Numerical count; event duration

6.2.5. Fitness assay

At the end of the behavioural assay, mosquitoes were transferred in individual 25 mL clear plastic vials that were sealed with a layer of netting secured with a rubber band (Fig. 51). The mosquitoes were then returned to their original light cycle and were kept under laboratory conditions as described in 3.1. A 10% sugar solution (prepared as described in 3.1) was provided by placing a soaked cotton wool pad on top of the netting. These individual sugar feeders were prepared at the end of each experimental day and were changed daily.
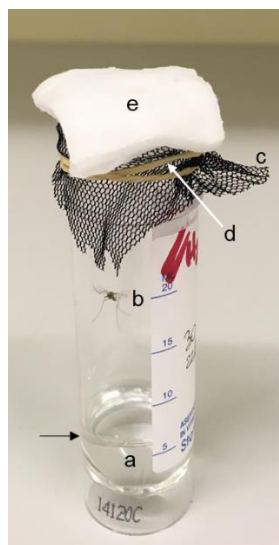
Figure 51: Photo of the plastic vials in which mosquitoes were kept after the behavioural assays. *In this image, the vial (~ 9 cm in height and 2.5 cm of diameter) contained 5 mL of deionised water at the bottom part (a), and the water level is indicated by the black arrow. The mosquito can be seen flying on the left side (b). The vial was sealed using a layer of mesh (c) and a rubber band (white arrow, d). On top of the vial, a 2 cm x 2 cm piece of cotton wool (e) soaked in sugar solutions was provided as energy and water source.*

Seventy-two hours after the behavioural assay, mosquitoes were transferred to a clean vial that contained 5 mL of deionised water (approx. 1 cm of height) to allow oviposition (Fig. 51). Mosquitoes were kept for a total of 14 consecutive days after exposure to the blood and were checked daily to record fitness indicators: survival (recorded in days, see below) and fertility (total number of eggs oviposited). Vials were examined in search for eggs and where eggs were found mosquitoes were transferred to a clean vial containing 5 mL of deionised water. Eggs were counted at least twice under a magnifying glass. Where the difference between the two egg counts was lower than 5% of the lower count, the mean of the two counts was taken as the final number

of eggs, whereas if the discrepancy exceeded 5% a new set of independent counts was carried out. For mosquitoes that oviposited on several days, the number of eggs found each day was summed to give the final number of eggs. For the survival component, mosquitoes were checked daily and if occurred, their death was recorded. Thus, the analysis was carried out on the mortality rates, which is inversely related to survival rates. The vials in which mosquitoes were kept for the first 72 h were stored at -20 °C and were later used to measure the quantity of blood ingested by mosquitoes, as described in section 6.2.6.

Due to technical issues, environmental conditions in the laboratory failed to be maintained, which caused abnormal mortality in mosquitoes collected during the behavioural assay. Consequently, it was decided to repeat the feeding event for the fitness assay using mosquitoes fed in groups of nine or ten at a time. This number of mosquitoes feeding at one time was deemed appropriate as the feeder was not overcrowded (a factor that can reduce feeding propensity (Rutledge *et al.*, 1964)) and each mosquito still had access to an adequate surface space on the feeder. Aside from the number of mosquitoes simultaneously exposed to the feeder in each replicate, all feeding conditions were the same as the conditions described for the behavioural assay. For each treatment, four groups (i.e. replicates) of mosquitoes were used, for a total of approx. 40 individuals per treatment. Once again, treatments were tested in a quasi-randomised order, between and within days, to exclude the effect of testing sequence. The conditions in which mosquitoes were kept and the daily checks were conducted as described above.

To summarise, two rounds of feeding were conducted: the first round was with mosquitoes being individually observed for the behavioural assay (25 mosquitoes per treatment), while the second round was conducted in groups, mosquitoes were not observed during the exposure to the feeder and were kept for the fitness assay (approx. 40 mosquitoes per treatment).

6.2.6. Quantification of blood meal size

The quantity of blood ingested by mosquitoes exposed to blood at different temperatures was estimated via a colourimetric assay in which the quantity of hematin, a by-product of blood digestion present in faeces, was assessed (Briegel, 1980;

Emami *et al.*, 2013). It has been previously demonstrated that hematin excretion follows an exact stoichiometric relationship with haemoglobin intake, with four moles of hematin being excreted for each mole of haemoglobin ingested, thus making hematin an ideal indicator of blood consumption (Briegel, 1986). To determine the hematin quantity, 1 mL of a 1% (w/v) lithium carbonate solution made with deionised water was added to the vials that contained the mosquitoes in the first 72 h post blood exposure. All vials were included in the assay, independently of whether they contained mosquitoes that had fed or had not feed. Where present, faeces were dissolved in the solution via gently pipetting the liquid directly on the spots until no stains were visible on the vial (Fig. 52).
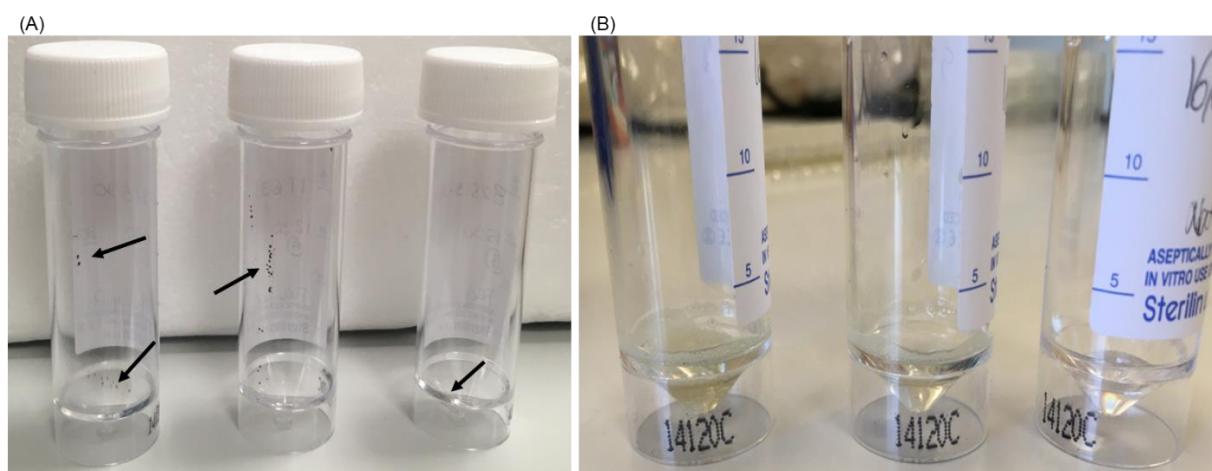
Figure 52: Photo of the plastic vials in which mosquitoes were kept. Arrows in panel (A) show mosquitoes' faeces, which contained hematin, while (B) displays three vials containing the lithium carbonate solution with dissolved hematin. In (B) the vials are arranged in order of decreasing concentration from left to right.

Hematin concentration was then estimated by reading the absorbance of the solution at 405 nm in an ELISA plate reader (Multiskan FC microplate reader, Thermo Fisher Scientific, USA). Three wells, each containing 200 µL of the solution were read per vial, and the final absorbance was taken as the mean of the three readings. The concentration of hematin was calculated by plotting the mean absorbance in the regression equation obtained from a calibration curve, which was prepared for each plate. This was done to ensure reliability on the readings between different plates. The calibration curves were checked to guarantee consistent values throughout the experiment. To prepare the calibration curve, ten solutions with hematin concentrations ranging from 1.25 to 40 µg/mL were read each time, and each solution

was read for three replicates to calculate the mean absorbance. The solutions used to create the calibration curve were kept in a refrigerator at 4 °C when not in use, while vials were stored at -20 °C at all times and were only briefly defrosted immediately prior to the assay. Vials collected from both feeding rounds (i.e. from the behavioural assay and the fitness assay) were analysed, which resulted in a total of over 64 vials per treatment. All results from vials that contained mosquitoes that were deemed "not fed" confirmed that those mosquitoes had not imbibed blood, as no hematin was detected with the colourimetric assay. Therefore, results from these vials were discarded and the quantification analysis was carried out only for vials that contained mosquitoes that imbibed blood.

6.2.7. Blood quality analysis

Anopheles mosquitoes strictly depend on the ingestion of blood to develop eggs, and in specific, egg formation is highly conditioned on the red blood cells component (intended as erythrocyte density) and plasma component of the blood (Harrison *et al.*, 2021), as erythrocytes and plasma account for the vast majority of the proteins found in blood. At the same time, haematological factors, such as viscosity and erythrocytes density influence blood-feeding success and feeding speed (Daniel and Kingsolver, 1983; Nacher, 2005; Emami *et al.*, 2013; Kim *et al.*, 2013). Furthermore, haematological parameters might also have consequences on mosquitoes reproductive fitness, as variations of these parameters might alter the nutritional quality of the blood (Lyimo and Ferguson, 2009; Harrison *et al.*, 2021). Therefore, a preliminary study was carried out to determine whether the main properties of defibrinated horse blood changed after exposure to various temperatures, and thus to establish whether any observed change in mosquito behaviour or physiology might be linked to blood quality.

Some studies indicated that blood viscosity is maintained approximately stable within temperatures ranging from 20 °C to 40 °C (Burton-Opitz, 1904; Eckmann *et al.*, 2000). In contrast, other studies suggested that increases in blood temperatures, from 25 °C up to 39.5 °C resulted in a decrease in blood viscosity (Çinar *et al.*, 2001; Pinho *et al.*, 2016). Although these studies report opposite results, it is commonly agreed that temperature-dependent changes in blood viscosity are attributed to either haemoconcentration or changes to erythrocytes, such as their flexibility, shape, and

degree of aggregation (Barbee, 1973; Lim *et al.*, 2010; Rostomily *et al.*, 2020). Therefore, this exploratory study focussed on erythrocyte properties, and specifically, on their morphology, size, number, and viability. Plasma proteins were not examined, as they are not thought to undergo significant changes due to heat unless exposed to extremely high temperatures. Vazquez and Larson (2013) showed that mouse plasma proteins endured a 2 h exposure to temperatures up to 45 °C without showing any significant form of denaturation, and only at 50 °C a significant protein degradation was observed. This supports a previous study that found that serum albumin, the most abundant protein in mammalian plasma, maintained its structural stability even when exposed to temperatures up to 60 °C (Wang *et al.*, 2005).

In addition to the four experimental temperatures, two extra temperatures were tested: 4 °C as a positive control, given that this is the ideal storage temperature for blood samples, and 60 °C, as the negative control, as at this point blood components should show degradation features (Wang *et al.*, 2005; Vazquez and Larson, 2013). Blood samples, which had been stored at 4 °C, were prepared for the study as follows: 3 mL of blood were gently warmed in a water bath (JB Aqua 12 Plus, Thermo Fisher Scientific, USA) and once the desired temperature was reached, samples were incubated at that temperature for ten minutes. This was done to match the duration at which the blood was left in membrane feeders when mosquitoes were exposed to them. Blood temperature was monitored at all times using an immersion thermometer (Fisherbrand Red Spirit Filled thermometer, Thermo Fisher Scientific, USA). After incubation, the samples were subjected to microscopy to examine erythrocyte size and morphology, and a trypan blue assay to determine erythrocyte viability. Samples were diluted with sterile phosphate-buffered saline solution with dilution factors of 1:2 for the microscopy observation and 1:1000 for the trypan blue assay. A new sample with fresh blood was prepared for each temperature treatment, and a new batch of samples was prepared for each test (microscopy and trypan blue assay).

To examine erythrocyte size and morphology, blood samples were mounted on a microscopy glass slide, covered with a cover slide and directly observed under a confocal microscope (Axio Observer, Zeiss LMS880, Germany) using a brightfield channel. After a first observation (with no magnification) which served to determine the general characteristics of the sample and the presence of agglomerations, images of different sections of the smear were recorded with a 20x magnification (Zen black

Software, ZEISS, Germany). These images were later used to determine the erythrocyte diameter (in µm), the shape following classification from eClinPath (Cornell University, 2021), and the number of spikes per erythrocytes. All parameters were taken for 15 different cells randomly selected in different images. Multiple images were taken for each smear, and attention was put into observing different areas of the smear to obtain a good representation of its entirety.

Erythrocytes' viability was inferred using the trypan blue exclusion test, which assesses cellular membrane integrity (Strober, 1997). For this, 10 µL of each diluted blood sample was mixed with 10 µL of trypan blue (Sigma-Aldrich, Merck, Germany). The sample was then placed on a haemocytometer and the number of viable cells (unstained), nonviable cells (stained blue), and the total number of cells per unit was annotated. Calculations for these parameters were carried out following Crowley *et al.* (2016) protocol. Four haemocytometer units were counted per treatment. Erythrocyte density was then calculated following Crowley *et al.* (2016) formula:

Cell density = (Mean number of cells counted in four units) x (volume of the unit) x (dilution factor)

6.2.8. Analysis and statistics

Behavioural assay

An initial screening of the data set revealed that for certain behavioural variables mosquitoes did not display the indicated behaviour (i.e. some mosquitoes did not feed or groom). Thus, data were screened separately for each variable, and in cases where mosquitoes did not display a particular behaviour, the data point was excluded (i.e. for that particular variable the replicate was deleted). This created a non-zero inflated *ad hoc* data set for each behavioural variable and resulted in some variables having a replicate number lower than 25. To compare the difference in the number of events between treatments of each behavioural variable, a GLM with negative binomial distribution and log link was used. Residual deviance levels were deemed satisfactory for all behavioural variables except for feeding events, and therefore a quasi-Poisson distribution was used for this variable. Multiple comparisons between treatments were done using a Tukey's *post hoc* test. The data on the times spent by mosquitoes doing each behavioural variable were fit on a linear model. Comparisons between treatments

of these data were carried out using an ANOVA, followed by a Tukey's *post hoc* test. Proportional data sets (i.e. proportion of mosquitoes that fed, proportion of mosquitoes that groomed, and proportion of mosquitoes that rested on the walls) were analysed for significant differences between treatments with a chi-square test. Expected values in each contingency table were checked to ensure they were larger than five. After the chi-square test was run, residuals were used to determine the temperature's effect on the deviation from predicted values.

To determine whether the number of mosquitoes that fed on blood at different temperatures was different between the two feeding rounds (i.e. round one: behavioural assay, and round two: fitness assay), results from each treatment were compared between the two rounds using a chi-square test. As there was no difference between these two groups, data were pooled together and the analysis on the feeding status was carried out on the pooled data.

An unconstrained principal component analysis (PCA) was carried out on untransformed data to determine the main variables that contributed to the variance of the data set. A selection process was carried out to retain meaningful behavioural variables and eliminate those that duplicated information, thus reducing multicollinearity. The analysis was carried out on a correlation matrix and the effect of the variables was standardised (i.e. standard deviation of all variables was set to be equal to one) so that all variables had an equal effect on the ordination process. A redundancy analysis (RDA) was then run on the results obtained from the PCA incorporating the temperature of the blood sources as an explanatory variable. To determine whether the addition of the explanatory variable significantly improved the fit of the model, the adjusted R^2 value was evaluated as a reference. Permutation tests were run to assess which constrained axes contributed the most to the explanation of the variation. Biplots and triplots were produced using axes that contributed to explaining most of the variation and the graphical representation was scaled to accurately represent correlation between variables.

Fitness assay

For the fitness assessment, mortality rates were compared between different treatments using a two-sided Fisher's Exact test. Similarly, the proportions of fed mosquitoes that oviposited were compared between treatments using a two-sided

Fisher's Exact test. To compare the number of eggs oviposited by females exposed to different blood temperatures a GLM with a log link and a negative binomial distribution was used. The feeding status was introduced in the model as a covariate. The data were also tested for best fit using an inverted quadratic model which was later introduced in the GLM.

Quantification of blood meal size

Data from both feeding rounds (i.e. round one: behavioural assay, and round two: fitness assay) were compared using an ANOVA to assess whether there was a difference in the two data sets. As no significant difference was found between rounds, data were pooled together and all further analyses were carried out on the pooled data set. Differences in hematin concentration between different treatments were assessed using an ANOVA followed by a Tukey's *post hoc* test. Estimates of blood ingestion speeds were calculated for each mosquito by dividing the concentration of hematin (proxy of ingested blood volume) by the total time that the mosquito spent feeding. This was done for all mosquitoes that had fed. Feeding speeds were then tested between different treatments using an ANOVA on the log-transformed data set.

Blood quality analysis

The proportion of erythrocytes with different morphology was analysed for significant differences between treatments with pairwise comparisons using a two-sided Fisher's Exact test. Data on erythrocyte dimensions were compared using an ANOVA followed by a Tukey's *post hoc* test, while the number of spicules per erythrocytes and total cell count were analysed using a GLM with Poisson distribution, also followed by a Tukey's *post hoc* test.

6.3. Results

6.3.1. Behavioural assay

A total of 110 assays were carried out, thus using 110 mosquitoes. Ten replicates were excluded from the analysis as mosquitoes were deemed unresponsive, thus giving a rate of 9.1% unresponsiveness.

Throughout all experiments, the mean ± SEM temperatures of the feeders were as follow:

- at 30 °C: 29.68 ± 0.09 °C
- at 36 °C: 36.21 ± 0.13 °C
- at 42 °C: 41.57 ± 0.16 °C
- at 48 °C: 47.44 ± 0.16 °C

In all replicates, the minimum and maximum temperatures registered were within ± 2 °C from the target temperature of each treatment.

A general summary of how mosquitoes allocated their time in each behavioural activity for each treatment is given in Fig. 53. Below follows a short description of the salient behavioural traits that mosquitoes displayed when exposed to blood at different temperatures, based on direct observation, followed by the analysis of quantified behavioural traits in the next section.

Mosquitoes that approached the feeder at the lowest temperature (30 °C) spent a relatively long time on its surface and displayed extended periods of foraging behaviour (i.e. probing and sensing). After several attempts of probing the feeder's surface, mosquitoes habitually flew away and displayed grooming behaviour. Generally, mosquitoes did not feed on the first landing event, but rather flew away and returned to the feeder several times before they started feeding, although often mosquitoes did not feed on blood at 30 °C at all, despite displaying foraging behaviours.

Mosquitoes that approached feeders set at the positive control temperature (36 °C) displayed a short period of foraging behaviour and often fed shortly after alighting in the first landing event. The majority of mosquitoes fed when presented with blood at this temperature. A small proportion of mosquitoes however displayed foraging behaviour for considerably longer periods of time. In those cases, mosquitoes often did not feed (either entirely or did not feed to repletion), flew to and from the feeder repeatedly, probed and sensed continually when on the feeder's surface, and groomed palpi and antenna for a long period of time. However, mosquitoes with this behaviour constituted only a small part of the total number of mosquitoes exposed to the positive control temperature.

In the treatment at 42 °C, mosquitoes overall behaved similarly to those exposed to the treatment at 36 °C. The majority of the mosquitoes displayed short bouts of

foraging behaviour and fed promptly after having landed on the feeder the first time. In the same way as described in the treatment at 36 °C, in the treatment at 42 °C a small number of mosquitoes flew to and from the feeder frequently and although they displayed several foraging behaviour, mixed with sparse grooming behaviour, those mosquitoes did not feed. Once again, these mosquitoes formed only a minor part of the total number of mosquitoes exposed to this treatment.

Mosquitoes exposed to the feeder set at 48 °C behaved in a radically different way compared to the mosquitoes exposed to the control temperature. When exposed to the highest tested temperature, mosquitoes spent little time on the feeder. Furthermore, it was observed that generally, when mosquitoes approach the feeder they remained around its periphery, with their thorax and abdomen positioned outside of the feeder area so that only the head and anterior pair of legs were over the feeder's surface. The foraging behaviour consisted mainly of repeated and energetic probing of the feeder's surface, with almost no time gap between each probing event. After a few seconds of being on the feeder and probing its surface, mosquitoes flew away and initiated grooming behaviour, after which they then returned to the feeder's surface. This sequence of events was repeated several times throughout the assay. In some cases, after numerous landing events were made, mosquitoes flew on the side of the walls and remained still for the remaining time of the assay. Only two mosquitoes were observed to feed.

Independently of the blood temperature, mosquitoes that fed to repletion left the feeder, and after a short flight, alighted on the walls of the cage and remained still for the remainder of the assay.

Altogether, this qualitative description suggests that mosquitoes approach feeders in a very distinctive manner depending on the temperature at which the blood is kept. This can be clearly noted in Fig. 53 and is further supported by the results of the different behavioural parameters analysis considered in the section below.
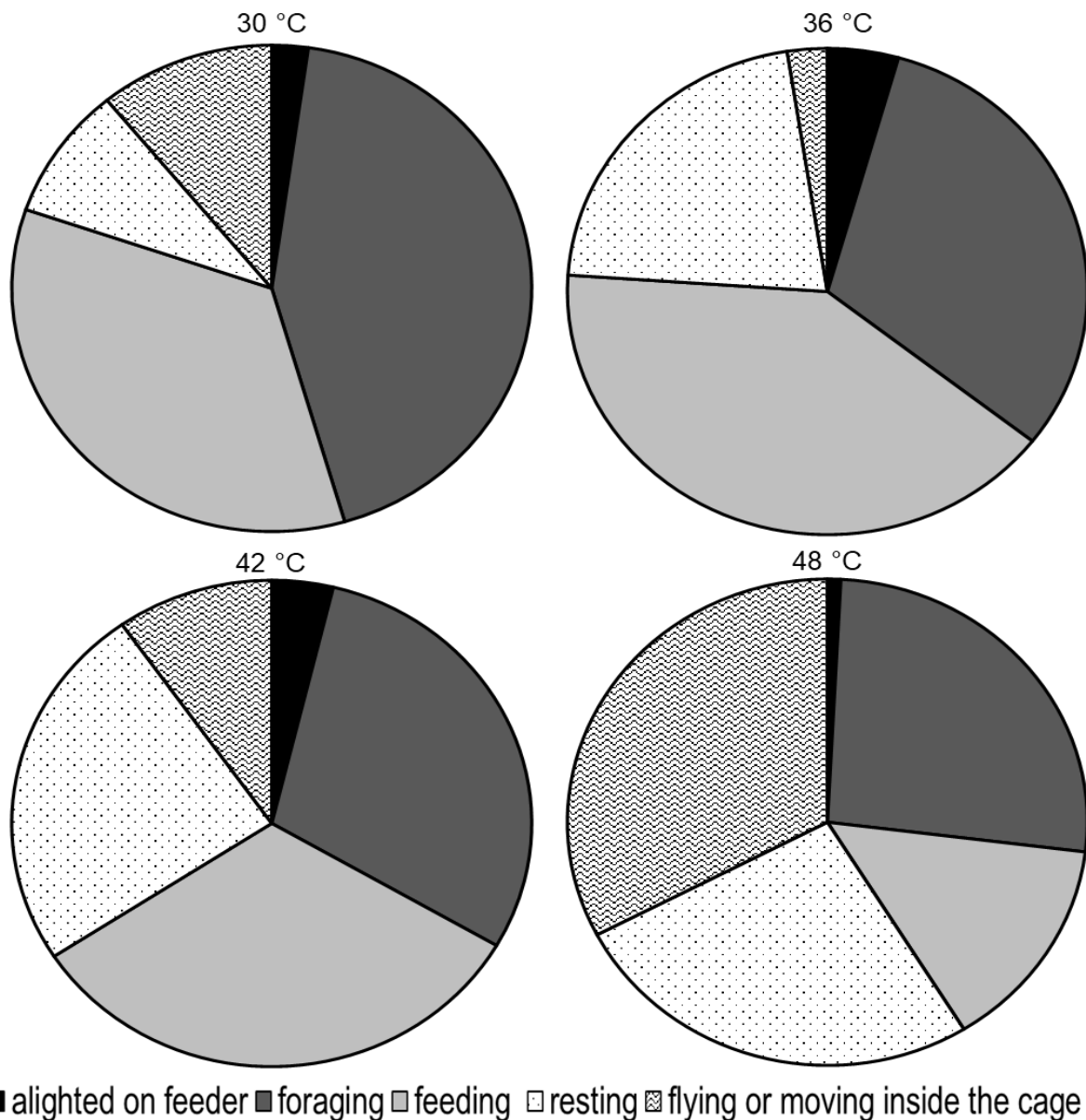
Figure 53: Summary of the proportion of time allocation for each behavioural activity in different treatments. Note that the category "alighted on feeder" refers to the time mosquitoes spent in contact with the feeder surface without displaying any foraging or feeding behaviour.

Feeding

The total number of mosquitoes that fed in both feeding rounds (i.e. round one: behavioural assay, and round two: feeding carried out for the fitness assay) is presented in Table 16. It is important to note that the analysis on the proportion of mosquitoes that fed was carried out on pooled data, whilst the analysis on the number of times mosquitoes fed in each replicate, and the time spent feeding was carried out on the data collected in the behavioural assay alone, as such information was not collected on round two. It can be noted that in round one, only two mosquitoes fed on

blood at 48 °C, thus the data set on the time spent feeding and the number of feeding events for this treatment is minimal and the analysis should be interpreted with care.

Table 16: Total number of mosquitoes that fed and did not feed in the two feeding rounds.

	Round one: behavioural assay		Round two: fitness assay		Pooled data from both feeding rounds		
Treatment	**Fed**	**Not fed**	**Fed**	**Not fed**	**Fed**	**Not fed**	**Total number of mosquitoes**
30 °C	12	13	28	13	40	26	66
36 °C	19	6	38	2	57	8	65
42 °C	21	4	37	5	58	9	67
48 °C	2	23	6	33	8	56	64

A significant difference was found in the proportion of mosquitoes that fed, either fully or partially, between different treatments (chi-square=112.92, d.f.=6, P<0.001). In particular, in the treatment where the feeder was set at 48 °C, fewer females fed to repletion (3.13%, Pearson residual=-5.41) and more females did not feed (87.50%, Pearson residual=6.47) compared to the expected numbers that would have resulted if the temperature was not affecting feeding behaviour (Fig. 54). Inversely, at 36 °C and 42 °C, more mosquitoes fed completely (81.54% and 74.63%; Pearson residuals=3.32 and 2.58, respectively) and fewer mosquitoes did not feed (12.31% and 13.43%; Pearson residuals=-3.34 and -3.24, respectively) compared with the predicted number of mosquitoes expected to feed if the temperature did not affect feeding (Fig. 54). Whilst no significant difference was found between the proportion of mosquitoes that fed at 36 °C (87.69%) and 42 °C (86.57%; chi-square=1.45, d.f.=2, P=0.48), significantly more mosquitoes fed at 36 °C compared with the corresponding proportion where the blood was at 30 °C (60.61%; chi-square=17.21, d.f.=2, P<0.001) and 48 °C (12.50%; chi-square=83.69, d.f.=2, P<0.001).
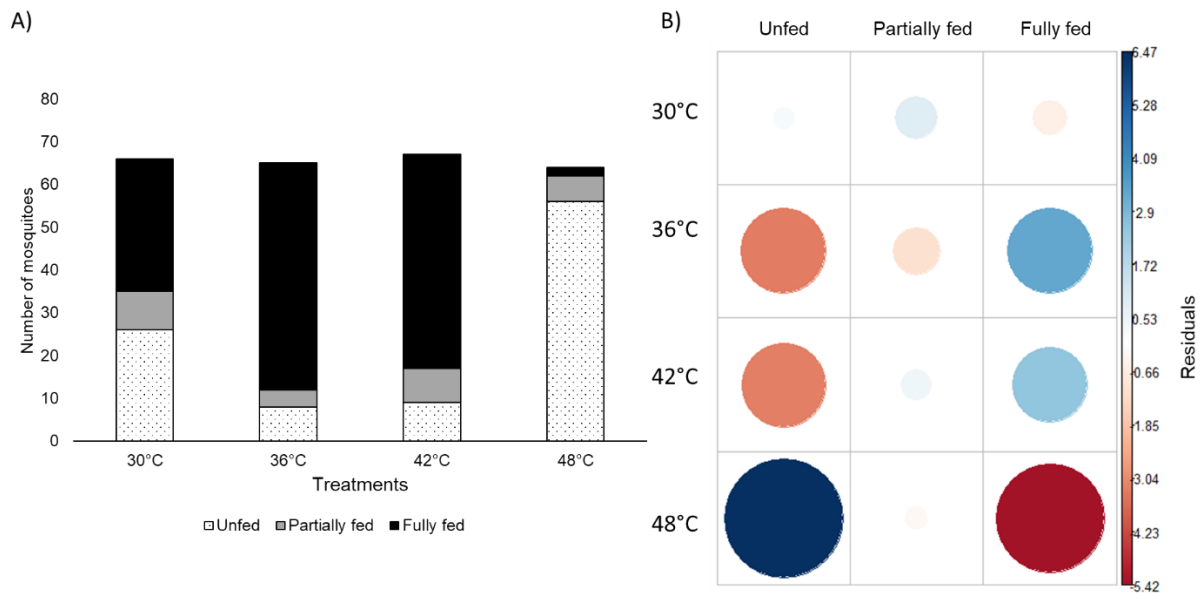
Figure 54: Mosquito feeding status according to blood meal temperature, using the data pooled from both feeding rounds. (A) Number of mosquitoes that were deemed fully fed, partially fed, or unfed. (B) Pearson residuals in each cell of the contingency table after the chi-square analysis. The intensity of the colour and the diameter of the circle indicate the magnitude of the residual, which denotes the contribution of the cell to the chi-square value. Negative residuals (red) imply a negative association between the treatment and the variable under examination and positive residuals (blue) imply a positive association between the treatments and the variable.

Excluding mosquitoes that did not feed, the analysis on fed mosquitoes (total n=54) revealed that although the mean ± SEM number of feeding events (i.e. the number of discrete times a mosquito fed in an assay) was highest at 36 °C and lowest at 30 °C, it was similar in all treatments (30 °C=1.58 ± 0.29, 36 °C=2.40 ± 0.27, 42 °C=2.28 ± 0.26, 48 °C=2.00 ± 0.79), with no statistical difference between different temperatures (GLM, chi-square=2.73, d.f.=3, P=0.24). Similarly, mosquitoes spent the longest feeding time at 36 °C and least time feeding at 48 °C, but no statistical difference was found in the mean total time (i.e. cumulative time) that individual mosquitoes spent feeding at different temperatures (mean ± SEM at 30 °C=208.00 ± 49.02 s, at 36 °C=243.22 ± 22.62 s, at 42 °C=194.52 ± 20.11 s, and at 48 °C=85.89 ± 5.74 s; ANOVA, F=1.29, d.f.=3, 51, P=0.29) (Fig. 55). When considering the average time spent by each mosquito in a singular feeding event, a progressive reduction in the mean times was seen with the increase in blood temperature (mean ± SEM at 30 °C=157.72 ± 30.90 s, at 36 °C=132.37 ± 23.94 s, at 42 °C=99.78 ± 23.36 s, and at 48 °C=42.95 ± 40.70 s) (Fig. 55). It is to be noted that such difference was accentuated in the means, whilst the median values remained similar in all treatments. This was

due to a high variance in the treatment at 30 °C, while at high temperatures mosquitoes behaved more uniformly.
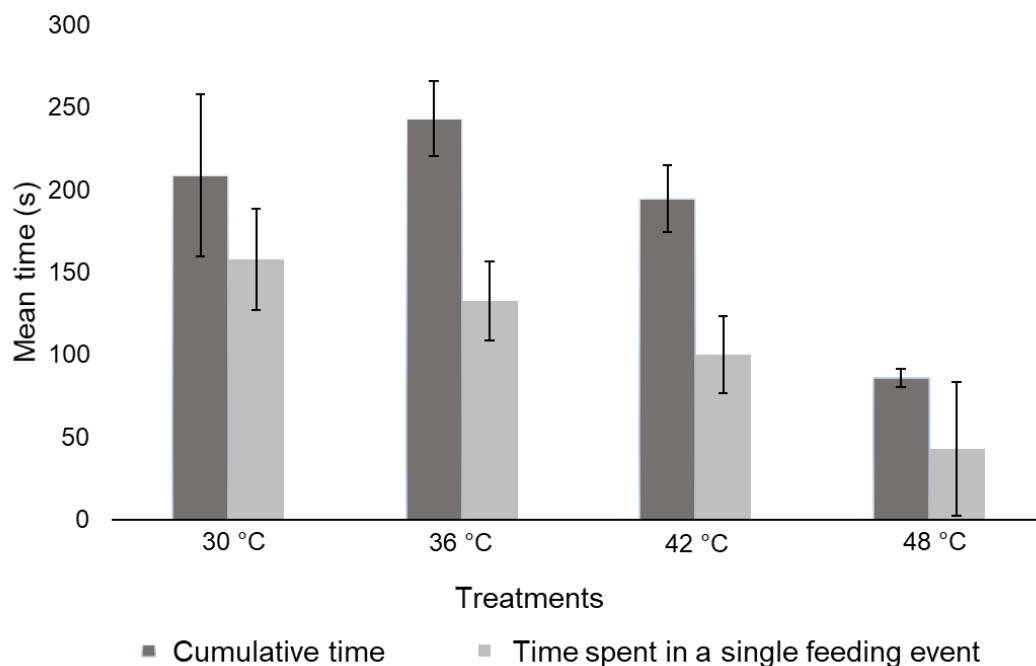
Figure 55: Mean time that mosquitoes spent feeding in different treatments. *The cumulative time was intended as the sum of all singular feeding events that a mosquito displayed in an assay (i.e. in a replicate), and this was averaged across all replicates for each treatment. The error bars indicate the SEM.*

Altogether these results indicate the strong propensity of *An. coluzzii* females to feed on blood at 36 °C and 42 °C. However, once the mosquito has engaged in feeding, no further difference is found in other behavioural parameters related to this activity, irrespectively of the temperature at which the blood is offered.

Landing

All mosquitoes considered in the analysis landed on the feeder, as those that did not land were considered unresponsive and excluded from the study (i.e. n=25 for all treatments). Combining all the replicates for each treatment, the results indicate that the total number of times mosquitoes visited the feeder was significantly different among treatments (GLM, chi-square=36.64, d.f.=3, P<0.001) (Fig. 56). In particular, mosquitoes landed on the feeder significantly more times in treatments where the blood was at 48 °C (mean ± SEM=13.72 ± 1.72) and 30 °C (mean ± SEM=9.60 ± 1.25) when compared to landings on feeders at 36 °C (mean ± SEM=4.84 ± 0.70; Tukey's test, t=5.44, P<0.001; t=3.51, P=0.002, respectively). Similarly, mosquitoes landed

significantly more times on the feeder at 48 °C compared with the mean number of landings per mosquito in the treatment at 42 °C (mean ± SEM=6.12 ± 0.85; Tukey's test, t=4.31, P<0.001), while no significant difference was detected in the number of landings per mosquitoes between treatments at 42 °C and 36 °C, and 42 °C and 30 °C (Tukey's test, t=1.17, P=0.65; t=2.36, P=0.08, respectively).

A significant difference was seen when comparing the mean total time spent by each mosquito on feeders at different temperatures (ANOVA, F=30.66, d.f.=3, 96, P<0.001). Specifically, as the blood temperature increased, the mean total time spent on the feeder decreased (mean ± SEM at 30 °C=462.20 ± 20.59 s, at 36 °C=434.53 ± 20.85 s, at 42 °C=371.10 ± 20.58 s, and at 48 °C=207.77 ± 20.58 s). Mosquitoes spent significantly less total time on the feeder at 48 °C than in any other treatment (Tukey's test, with 42 °C: t=5.61, P<0.001; with 36 °C: t=7.79, P<0.001; and with 30 °C: t=8.74, P<0.001) (Fig. 56). The mean total time spent by mosquitoes on the feeder at 42 °C was also significantly less compared to the corresponding time in the treatment at 30 °C (Tukey's test, t=3.13, P=0.01). Furthermore, in the treatment at 48 °C, mosquitoes remained on average for a shorter time in each singular landing event (mean ± SEM=20.69 ± 27.70 s) compared to other temperature treatments (mean ± SEM at 30 °C=126.83 ± 27.70 s, at 36 °C=192.43 ± 27.70 s, and at 42 °C=117.36 ± 27.70 s). In each visit, mosquitoes remained on average for a longer time on the feeder at 36 °C compared to any other temperature (Fig. 56). It is worth noting that similar to results reported in the feeding section, the variance was considerably smaller in the treatments at the highest temperature, while in the treatment at the lowest temperature the variance was greater, thus suggesting that overall at high temperatures mosquitoes behaved more uniformly.
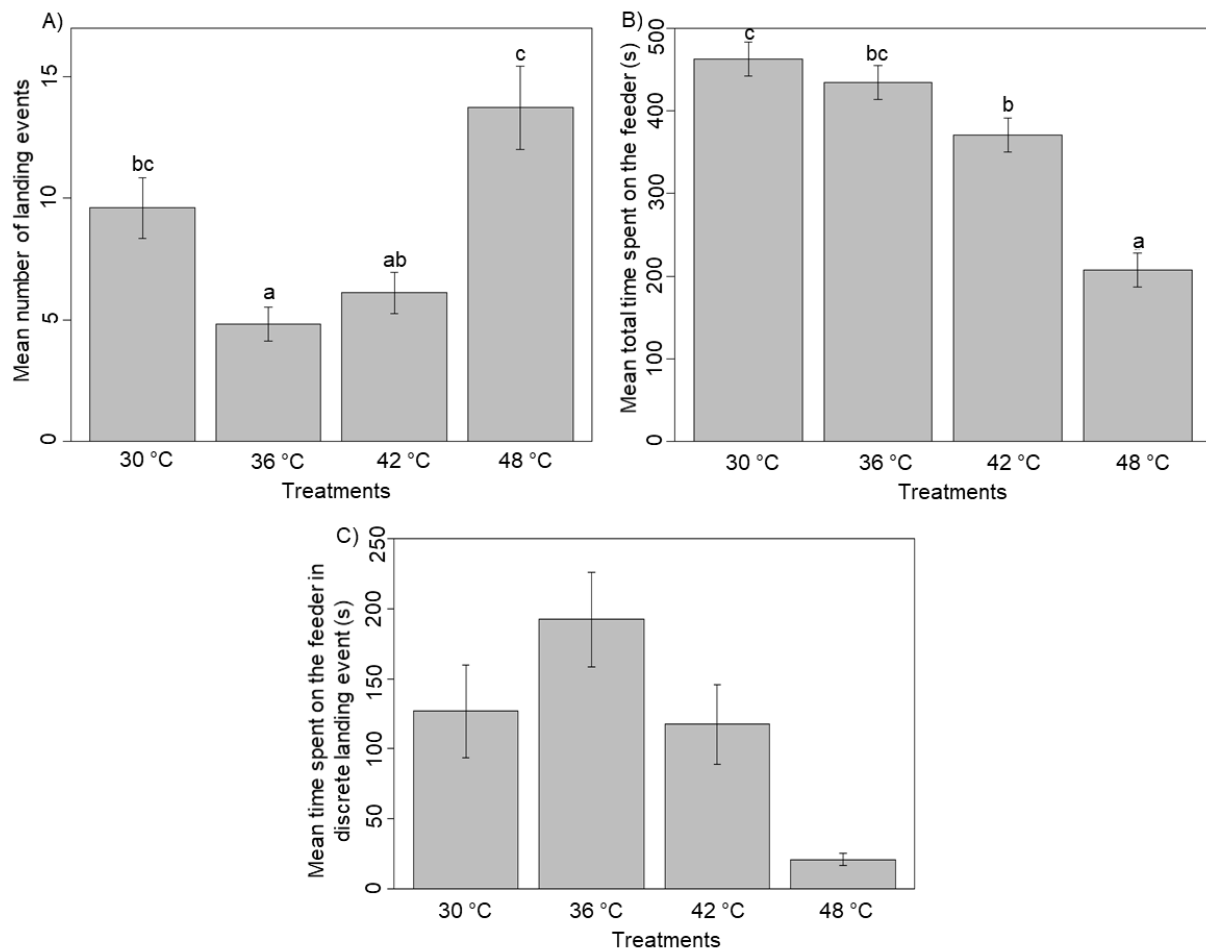
Figure 56: Landing responses on blood feeders at different temperatures. *(A) Mean number of landing events, (B) total amount of time mosquitoes spent on the feeder, (C) mean amount of time spent on the feeder in discrete landing events. All bar charts display means, the error bars indicate the SEM and, where present, different letters denote significant differences between treatments (Tukey's test, P<0.05).*

Taken together, the results reported above indicate that mosquitoes spend longer on feeders at lower temperatures. Interestingly, a greater number of visits did not correspond to a longer total time spent on the feeder, as in fact the more often the mosquito visited the feeder the shorter the length of time spent on the feeder on each visit. In contrast, mosquitoes visited the feeders at 36 °C and 42 °C less frequently, but on average, each visit lasted longer. This, combined with the results of the feeding parameter and the qualitative observation, suggests that when mosquitoes land on feeders at 36 °C and 42 °C they encounter a series of stimuli that drive them to promptly begin to feed and to do so at length, after which they leave the feeder, whereas feeders at lower (30 °C) and higher (48 °C) temperatures, though attracting mosquitoes to the surface do not induce full feeding behaviour, which suggests that in

these cases mosquitoes do not receive the decisive set of cues that induce the next step in the behavioural sequence.

No significant difference was found in the mean lag time that mosquitoes took to first land on the feeder when this was compared between treatments (ANOVA, F=0.54, d.f.=3, 96, P=0.65). Similarly, no significant difference was found between treatments when comparing the total time that elapsed from the moment of landing to the beginning of either foraging activities or feeding (ANOVA, F=1.34, d.f.=3, 96, P=0.28; F=1.74, d.f.=3, 51, P=0.17, respectively) (Table 17). This indicates that independently of the surface's temperature, mosquitoes initiated foraging activities promptly after landing.

Table 17: Mean length of time to start different behavioural traits according to feeder temperature.

Treatment	Mean ± SEM time to first landing event (s)	Mean ± SEM time elapsed from landing to the beginning of foraging (s)	Mean ± SEM time elapsed from landing to the beginning of feeding (s)
30 °C	48.61 ± 10.02	6.91 ± 0.98	197.21 ± 39.81
36 °C	40.56 ± 10.02	4.61 ± 0.98	112.20 ± 30.84
42 °C	46.19 ± 10.02	5.20 ± 0.98	178.94 ± 30.09
48 °C	58.89 ± 10.02	6.72 ± 0.98	33.97 ± 97.52

It is worth noting that the time mosquitoes took to first land on the surface, or to start probing after landing, or to start feeding after landing was consistently shorter for the control temperature (36 °C), thus suggesting that the optimal temperature might elicit a fractionally earlier response. However, no statistical difference was found in this data and therefore further investigation is recommended.

Foraging

All mosquitoes that landed on the feeder displayed a degree of foraging activities, thus all replicates were included in the foraging analysis (n=25 for all treatments). The total time spent in foraging activities (i.e. the time spent either probing or sensing) was significantly different in different treatments (ANOVA, F=5.68, d.f.=3, 96, P=0.001) (Fig. 57). In particular, mosquitoes spent significantly more time foraging on the feeder at 30 °C (mean ± SEM=259.29 ± 18.62 s) compared to any other treatment (mean ± SEM at 36 °C=186.79 ± 18.62 s, at 42 °C=177.42 ± 18.62 s, and at 48 °C=157.56 ± 18.62 s; Tukey's test with 36 °C: t=-2.75, P=0.04; with 42 °C: t=-3.11, P=0.01; and with 48 °C: t=-3.86, P=0.001). It is noteworthy that mosquitoes spent the majority (approx. 75%) of their time on the feeder at 48 °C in foraging activities, while this percentage was drastically lower (56%) when on the feeder at 30 °C, and even lower when at 36 °C and 42 °C (42% and 47%, respectively).

The number of foraging events (i.e. when a mosquito started probing or sensing *ex novo*) differed between treatments, with more foraging events recorded on 48 °C feeders, then 30 °C, followed by 42 °C, with fewest on 36 °C feeders. This difference between treatments was statistically significant (GLM, chi-square=103.81, d.f.=3, P<0.001) (Fig. 57). Specifically, mosquitoes initiated a foraging event significantly more times on feeders at 48 °C (mean ± SEM=12.23 ± 1.17) compared to the number of times a foraging activity was initiated at 36 °C (mean ± SEM=5.68 ± 0.65) and at 42 °C (mean ± SEM=6.68 ± 0.73) (Tukey's test with 36 °C: t=5.19, P<0.001, and with 42 °C: t=4.20, P<0.001). Similarly, foraging activities were initiated significantly more times by mosquitoes exposed to feeders at 30 °C (mean ± SEM=9.52 ± 0.96) compared to those exposed to feeders at 36 °C (Tukey's test, t=-3.41, P=0.004). In contrast, no difference was found in the number of times the foraging activity was initiated between treatments at 36 °C and 42 °C (Tukey's test, t=1.03, P=0.73), between 30 °C and 42 °C (Tukey's test, t=-2.39, P=0.08), and between 30 °C and 48 °C (Tukey's test, t=1.74, P=0.26).
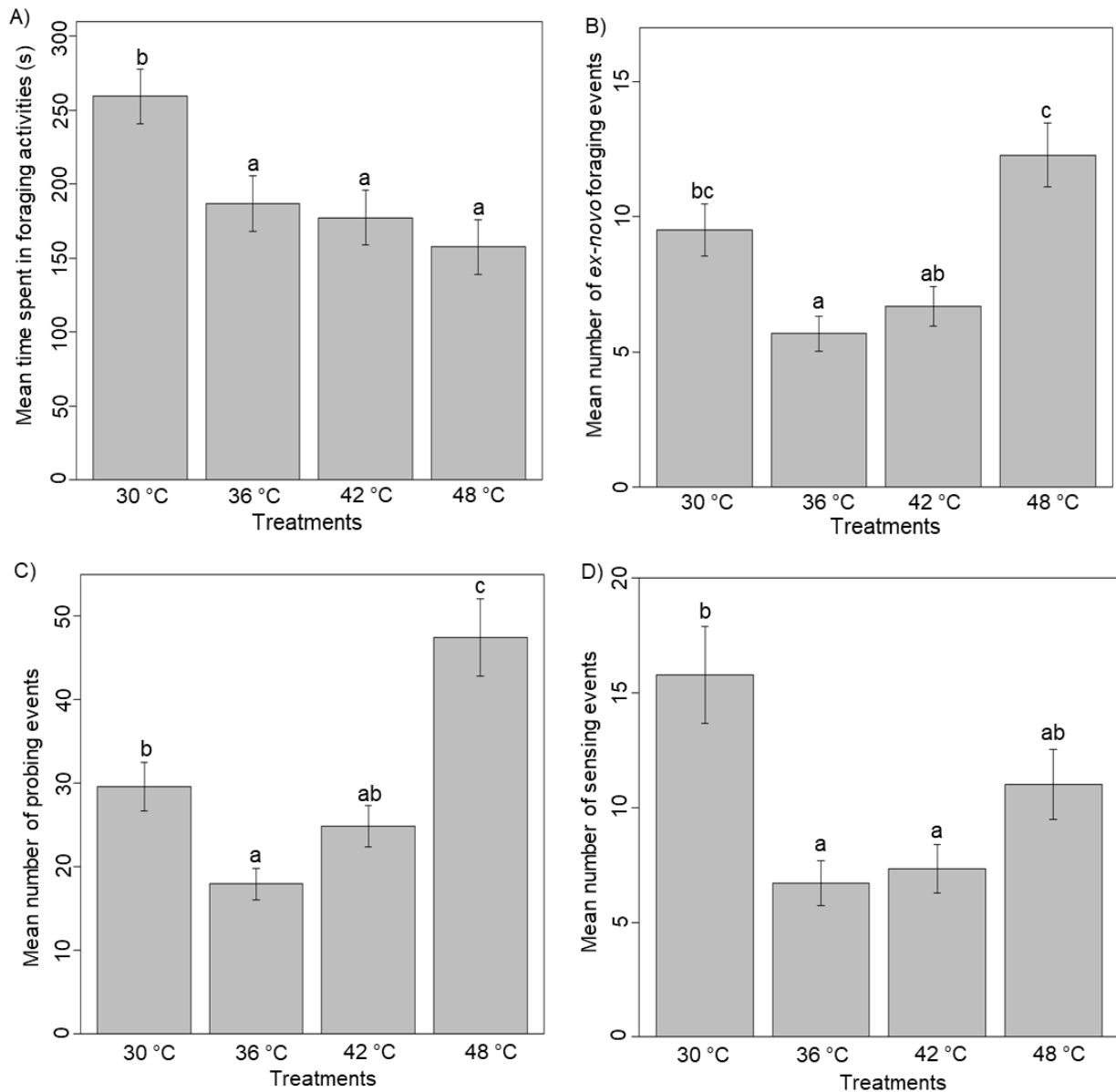
Figure 57: Summary of foraging behaviours at different feeder temperatures. Panel (A) displays the mean time that mosquitoes spent foraging, while (B) displays the mean number of times that mosquitoes initiated a foraging event ex novo. *(C) Mean number of probing events, and (D) mean number of sensing events. All bar charts display means, the error bars indicate the SEM and different letters denote significant differences between treatments (Tukey's test, P<0.05).*

When considering each of the foraging behaviours separately, a significant difference was found between treatments in the number of probing events (GLM, chi-square=105.39, d.f.=3, P<0.001) and the number of sensing events (GLM, chi-square=114.61, d.f.=3, P<0.001) (Fig. 57). Mosquitoes probed significantly more times when on the feeder at 48 °C (mean ± SEM=47.48 ± 4.60) compared to any other treatment (mean ± SEM at 30 °C=29.60 ± 2.95, at 36 °C=17.96 ± 1.86, at 42 °C=24.88

± 2.51; Tukey's test, with 30 °C: t=3.40, P=0.003; with 36 °C: t=6.84, P<0.001; with 42 °C: t=4.62, P<0.001). Similarly, mosquitoes probed significantly more times in treatments with blood at 30 °C compared with the number of times mosquitoes probed when they were exposed to blood at 36 °C (Tukey's test, t=-3.47, P=0.003). Furthermore, significantly more sensing events were recorded in the treatment at 30 °C (mean ± SEM=15.76 ± 2.10) compared to the number of sensing events recorded in treatments where the blood was set at 36 °C and 42 °C (mean ± SEM at 36 °C=6.72 ± 0.78, Tukey's test, t=-4.32, P<0.001; mean ± SEM at 42 °C=7.32 ± 1.05, Tukey's test, t=-3.91, P<0.001, respectively) (Fig. 57). Altogether, the results presented here indicate that when mosquitoes are exposed to blood at 36 °C and 42 °C, they tend to engage in fewer foraging activities. Once more, this supports the idea that when exposed to temperatures close to host body range, mosquitoes engage in feeding, while at the lowest and highest temperature mosquitoes undertake more foraging activities, which suggest that the stimuli received (low or high temperatures respectively) do not promptly trigger full feeding behaviour.

Grooming

A significant difference was found in the proportion of mosquitoes that performed grooming behaviour between treatments (chi-square=12.38, d.f.=3, P=0.006, n=25 for all treatments). In particular, mosquitoes groomed more in treatments where the blood was presented at the two extreme temperatures (at 30 °C 44.00% of the studied mosquitoes groomed, Pearson residual=1.27; at 48 °C 48.00% of the mosquitoes groomed, Pearson residual=1.64, respectively). In contrast, mosquitoes that were exposed to feeders at 36 °C and 42 °C groomed less (at 36 °C only 16.00% groomed, Pearson residual=-1.28; at 42 °C 8.00% groomed, Pearson residual=-1.64). Moreover, grooming behaviour was not equally distributed in mosquitoes with different feeding statuses (chi-square=29.6, d.f.=2, P<0.001, n for each treatment is indicated in Fig. 58), as more mosquitoes that did not feed displayed at least a grooming event compared to mosquitoes that fed either fully or partially (Fig. 58).
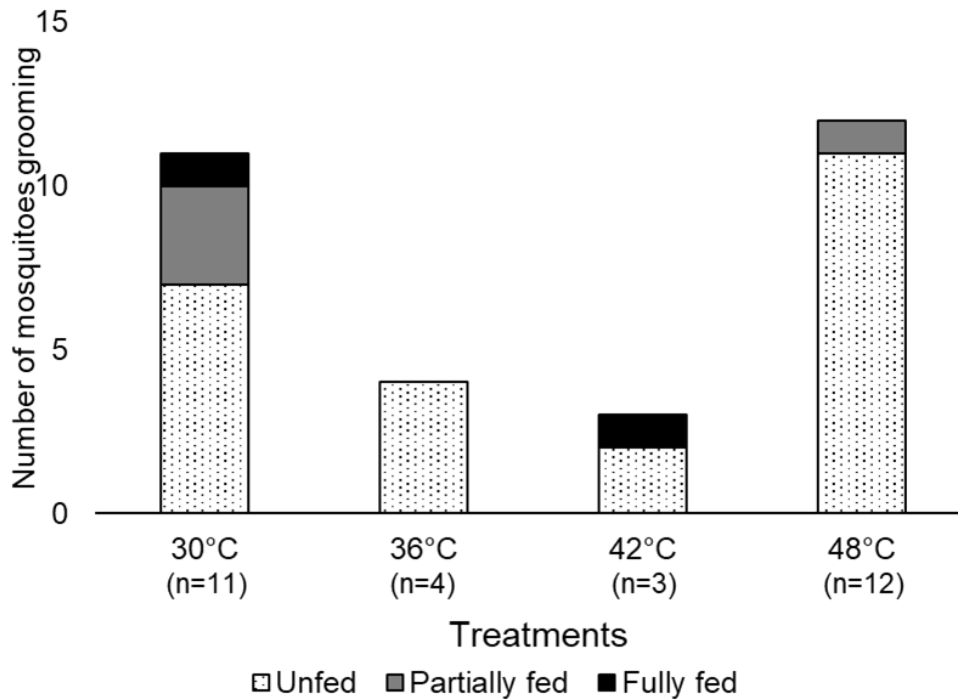
Figure 58: Number of mosquitoes that showed grooming behaviour in different treatments according to feeding status. *Note that feeding status was taken as the final feeding status of each mosquito at the end of the assay. Below each treatment, in brackets, is indicated the total number of mosquitoes that groomed.*

Although the mean number of grooming events per mosquito (i.e. discrete number of grooming events in each replicate) was more than double in treatments at the lowest and highest temperatures compared to the mean number recorded in treatments at the middle temperatures, no significant difference was found between different treatments (mean ± SEM for 30 °C=3.45 ± 0.62, for 36 °C=1.50 ± 0.64, for 42 °C=1.33 ± 0.70, for 48 °C=3.17 ± 0.57; GLM, chi-square=22.83, d.f.=3, P=0.08, n for each treatment indicated in Fig. 58). However, it is to be noted that only a small number of mosquitoes displayed grooming behaviour in the treatments with the feeder at 36 °C and 42 °C, thus the non-significant difference might be due to lack of data and further studies are recommended to verify this result.

Resting

In general, no significant difference was found in the time mosquitoes spent resting in different treatments (ANOVA, F=2.64, d.f.=3, 52, P=0.06). No statistical difference was found in the mean time mosquitoes spent resting at 30 °C (mean ± SEM=52.97 ± 31.38 s) compared to the time spent at 36 °C (mean ± SEM=128.64 ± 25.16 s) and at 42 °C (mean ± SEM=146.66 ± 22.83 s; Tukey's test, t=1.88, P=0.25; t=2.41, P=0.09,

respectively). Similarly, no difference was found in the time mosquitoes spent resting in the treatment at 48 °C (mean ±SEM=157.58 ± 23.54 s) compared to 36 °C (Tukey's test, t=0.84, P=0.83) and at 42 °C (Tukey's test, t=0.53, P=0.95). No difference was found in the resting time between the treatment at 36 °C and 42 °C (Tukey's test, t=0.53, P=0.95), while a statistical difference was found between the temperature extremes at 30 °C and 48 °C (Tukey's test, t=2.67, P=0.04) (Fig. 59).
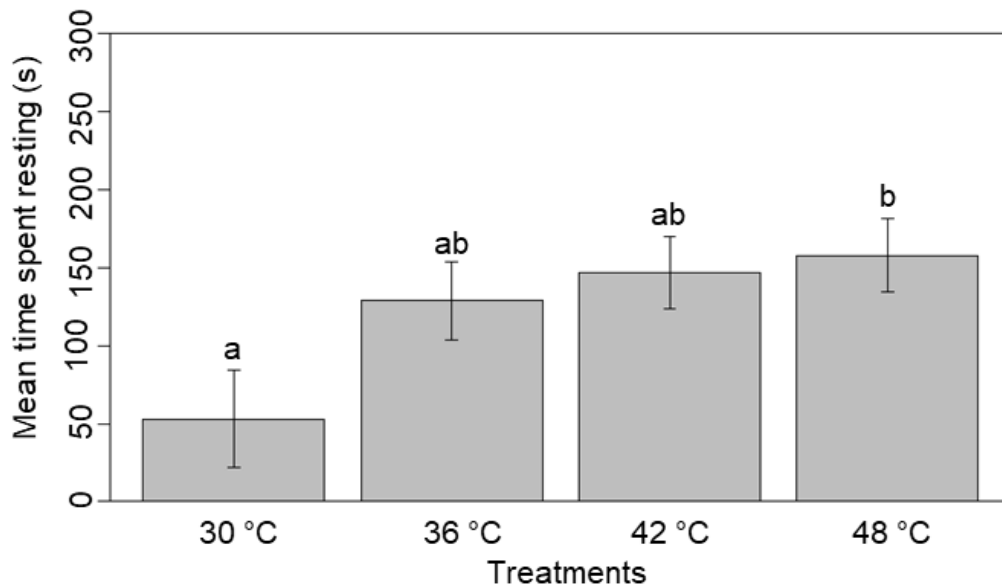
Figure 59: Mean time mosquitoes spent resting in different treatments. *Bars indicate the means, the error bars indicate the SEM and different letters denote significant differences between treatments (Tukey's test, P<0.05).*

No difference was found between treatments on the proportions of mosquitoes that rested on the walls (chi-square=6.17, d.f.=3, P=0.10). Independently of the treatments, the majority (60.71%) of mosquitoes that remained still were mosquitoes that had fed, and specifically, 65.79% of those mosquitoes rested on the walls after the feeding event. Furthermore, mosquitoes spent seven-times longer periods resting on the walls after they had ingested the blood meal, compared to the time they spent prior to feeding (total time spent resting before feeding=467.38 s, total time spent resting after feeding=3464.70 s).

Interaction between behavioural variables

To determine which behavioural variables contributed the most to the overall variance, and thus, which behavioural variables were the most different on mosquitoes exposed

to different temperatures, a PCA was carried out on the entire untransformed dataset collected in the behavioural assay. From the analysis, it resulted that the first two principal components (PC) represented 69.50% of the total variation of the data set (PC1=43.21% and PC2=26.29%), while a marked drop was seen in the percentage of the explained variance for the remaining PCs (Fig. 60). Furthermore, only the first two PC explained more than the variance that each variable would account for if all variables contributed equally (i.e. 100% variance divided by the nine variables=11.11%, which is the variance contribution of a single variable). This suggested that PC1 and PC2 contained more than one variable's worth of information. Altogether these results indicated that the first two PCs (PC1 and PC2) should be used as the main axes for the PCA.
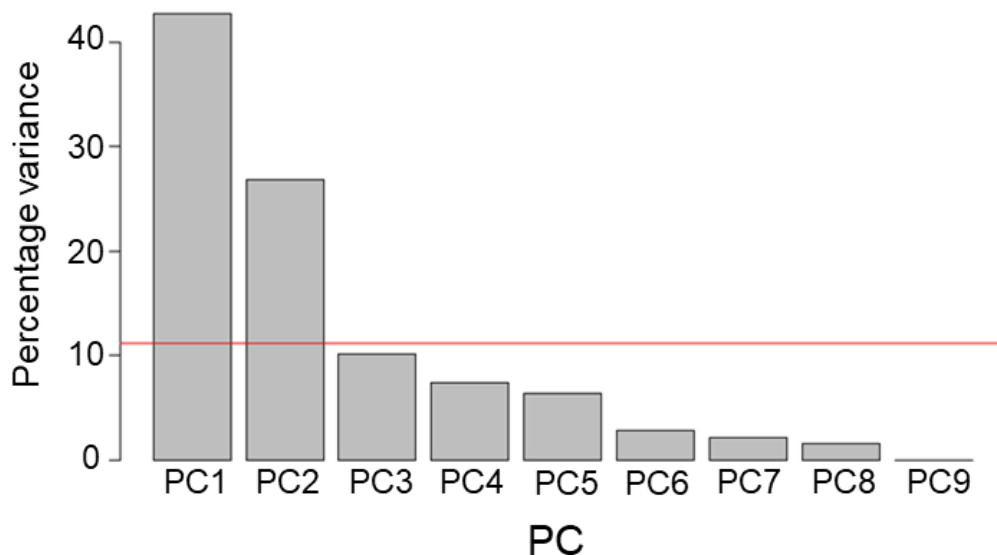
Figure 60: Plot indicating the percentage of the variance explained by each PC. The red line indicates the variance that each variable would have if all variables contributed equally (11.11%).

The first axis (PC1) was strongly positively influenced by the number of visits, probing, and sensing, and it was negatively correlated with the number of feeding events and the total time spent feeding (Table 18). The second axis was positively correlated with the total time spent on the feeder, along with the total time the mosquitoes spent foraging, and was negatively correlated with the time mosquitoes spent still (Table 18).

Table 18: Total loadings of each variable for the two main PCs. In each column, bold numbers highlight the variables that were major contributors to the respective PC. Note that the absolute value indicates the strength of the variable's effect on the PC, while the signs indicate whether there is a positive or negative correlation between the variable and the PC. The threshold value that indicated main contributors was taken to be the hypothetical loading of each variable if these contributed equally (0.33).

Variable	PC1	PC2
Number of visits	**0.44**	-0.03
Probing	**0.43**	0.03
Time spent on feeder	**-0.42**	0.24
Sensing	**0.38**	0.27
Number of feeding events	**-0.37**	0.25
Time spent on feeder	-0.13	**0.59**
Time spent still	-0.16	**-0.46**
Time spent foraging	0.31	**0.44**
Grooming	0.13	-0.23

The introduction of the explanatory variable significantly improved the fit of the model (permutation test: F=11.22, d.f.=3, P=0.001, adjusted R^2=0.24) although only approx. 25% of the total variation was explained by it. Only the first two RDA axes significantly contributed to the variation (permutation test for RDA1: F=23.98, d.f.=1, P=0.001; for RDA2: F=9.48, d.f.=1, P=0.001) and were therefore used for the triplot (Fig. 61).

By comparing the biplot with the triplot it can be seen that the addition of the explanatory variable (blood temperature) causes only minor changes in the position of the behavioural variables (Fig. 61). In particular, most variables maintained their positions, with the exception of grooming, which in the RDA analysis correlated more strongly with sensing behaviour and the number of visits, while in the PCA analysis it correlated more strongly with the time spent resting. These minor changes are expected given that only one explanatory variable was introduced in the model.

Furthermore, in both plots, dots of the same colour (each colour representing a treatment) cluster together, which suggests that mosquitoes exposed to the same treatment tended to behave more similar among themselves, compared to those exposed to other treatments. An exception to this can be seen for the yellow dots (mosquitoes exposed to blood at 30 °C), as in this case dots are less condensed in one area and can be found widely spread across the plots. This reflects the high variability found in mosquitoes' behaviour when they are exposed to blood at 30 °C and it corroborates the findings presented in the previous section where each behavioural variable was considered individually. Once more, this suggests a high variability in mosquitoes behaviour, which intensifies when mosquitoes are exposed to lower temperatures.

Clear relationships between variables can be seen from both plots. For example, results from the treatments at 36 °C and 42 °C were strongly correlated with the number of feeding events and the time mosquitoes spent feeding, while they were negatively correlated with the number of visits, sensing, and grooming behaviour. Vice-versa, the behaviour of mosquitoes exposed to blood at 48 °C was positively correlated with probing behaviour and negatively correlated with time spent on the feeder and time spent foraging. Thanks to the visual representation given in the biplot and triplot, it is possible to interpret all possible combinations of relationships between different behavioural traits and treatments. Aside from summarising the results found in the analysis when behavioural variables were examined individually, the plots provide, in an intuitive manner, a comprehensive overview of the interactions between the behaviours displayed, and clearly indicate the role that blood temperature played in clustering mosquitoes responses.
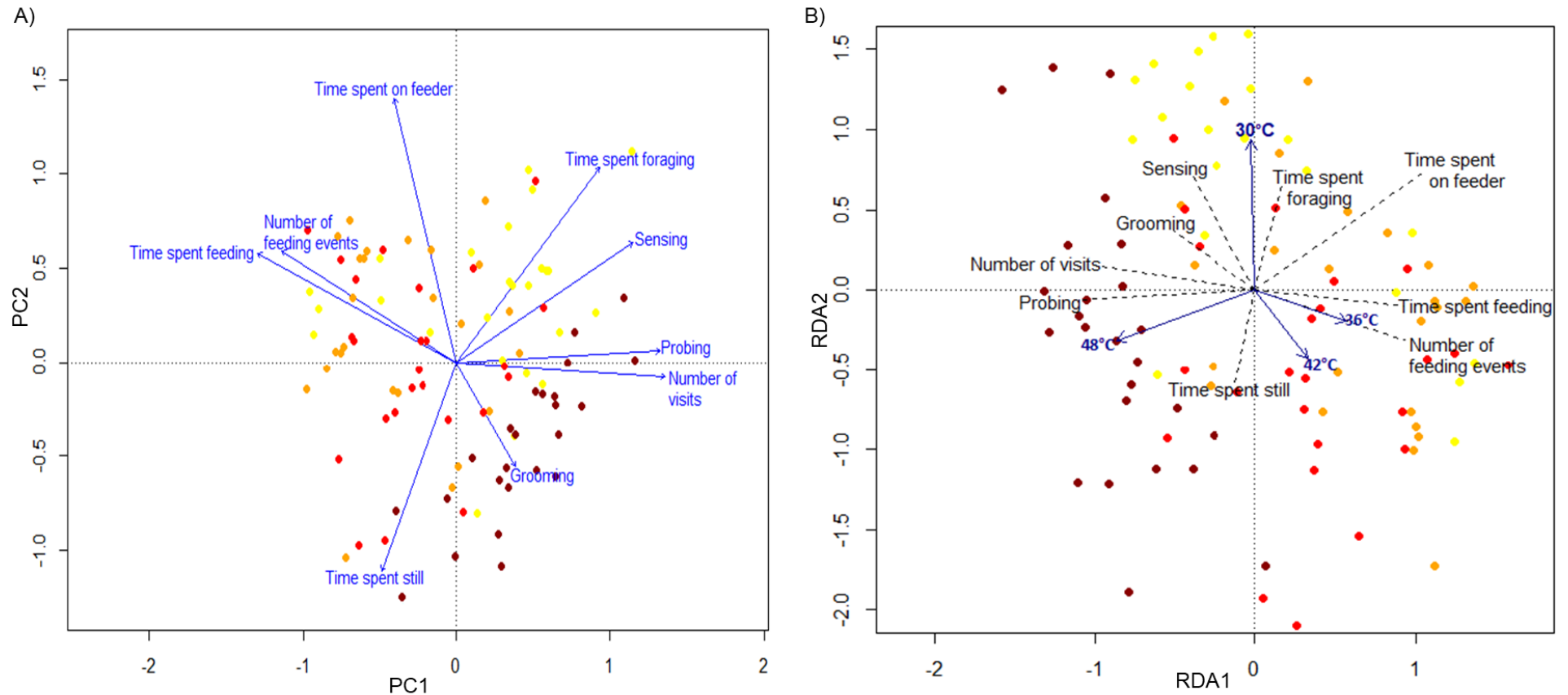
Figure 61: (A) Biplot and (B) triplot showing the correlation between (A) behavioural variables (blue arrows), and (B) behavioural variables (dashed lines) and the explanatory variable (i.e. treatments, solid lines). *The correlations are represented by the angles formed between the vectors of each variable, while the length of the lines indicate the total variance that each variable accounts for in the total data set. Variables positioned in quadrants opposite to each other are negatively correlated with each other. Each dot represents a replicate (i.e. a mosquito) and is colour-coded: yellow for the treatment at 30 °C, orange at 36 °C, red at 42 °C, and dark red for the treatment at 48 °C.*

6.3.2. Fitness assay

No significant difference was recorded in the mortality rates of mosquitoes exposed to different treatments (Fisher's Exact test, P=0.49) (Table 19). The overall mortality rate of all groups combined was approx. 6% (i.e. total mosquitoes found dead throughout the assay over the total number of mosquitoes used). The age of those recovered dead was between 13 to 15 days old. Both these measures align with typical life expectancy recorded under normal rearing conditions. The proportion of blood fed mosquitoes that were recovered dead (3.70%) was similar to the proportion of unfed mosquitoes that were recovered dead (2.50%). This similarity suggests that the ingestion of blood, independently of its temperature, did not cause a higher mortality. Further studies are recommended to substantiate the data presented here. It is interesting to note that all eight mosquitoes that ingested blood at 48 °C survived. Thus, it is not clear if imbibing blood at such high temperatures posed a thermal stress on mosquitoes physiology, but if it did, there is a suggestion that mosquitoes were able to cope with it.

Table 19: Results from the mortality assay.

Treatment	Dead mosquitoes that fed	Dead mosquitoes that did not feed	Mosquitoes alive at the end of the assay	Total number mosquitoes per treatment
30 °C	0	1	40	41
36 °C	3	0	37	40
42 °C	2	1	39	42
48 °C	0	3	36	39

To standardise oviposition rates between treatments, only mosquitoes that had fed were taken into account for the following analysis, as different proportions of mosquitoes fed in different treatments. A significant difference was found in the

proportion of fed mosquitoes that oviposited between different treatments (Fisher's Exact test, P=0.04) (Fig. 62). A higher proportion of the mosquitoes that fed on blood at 36 °C laid eggs (71.05%) compared with the corresponding proportion in the treatment at 48 °C (16.67%) (Fisher's Exact test, P=0.02). Only 51.35% of the mosquitoes that fed on blood at 42 °C laid eggs, while this percentage increased to 60.71% for the treatment at 30 °C. When introduced in the model as an independent factor, the blood temperature had a significant effect on the number of eggs oviposited by each female (GLM, chi-square=92.89, d.f.=3, P=0.008). Significantly more eggs were oviposited by mosquitoes that fed on blood at 36 °C compared with the number of eggs oviposited by mosquitoes that fed on blood at 48 °C (Tukey's test, t=-2.74, P=0.03). However, no significant difference was found in the number of eggs oviposited between the treatments at the three lowest temperatures (Fig. 62, Table 20). To summarise, when fed at 36 °C more females laid eggs, and those that oviposited laid more eggs compared to those fed on blood at 48 °C, but no statistical difference was found otherwise between the three lowest temperatures.
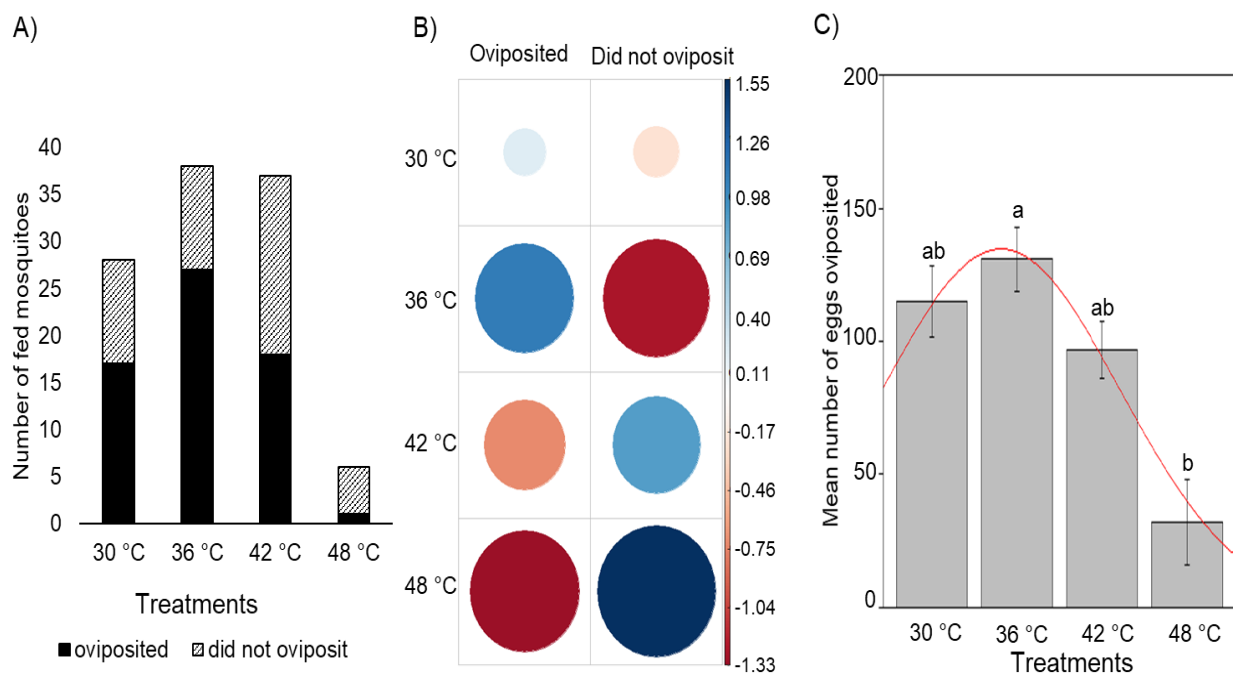
Figure 62: Oviposition data from mosquitoes that fed on blood at different temperatures. *(A) Number of fed mosquitoes that either did or did not oviposit. (B) Residuals in each cell of the contingency table. The intensity of the colour and the diameter of the circle indicate the magnitude of the residual. Negative residuals (red) imply a negative association between the treatment and the variable; positive residuals (blue) imply a positive association between the treatments and the variable. (C) Mean number of eggs laid in different treatments. The red line indicates the inverted quadratic curve.*

The feeding status (i.e. fully fed or partially fed) also affected the number of eggs laid (GLM, chi-square=78.06, d.f.=1, P<0.001). Significantly more eggs were laid by fully fed females that were exposed to blood at either 30 °C or 42 °C compared to the number of eggs laid by females that had only partially fed on blood at these temperatures (Tukey's test, t=-3.18, P=0.02; t=-3.45, P=0.008, respectively), while there was no difference when accounting for the feeding status in females that fed at the positive control temperature (Tukey's test, t=-0.78, P=0.98) (Table 20).

Table 20: Mean ± SEM numbers of eggs laid by individual female mosquitoes according to blood temperature and feeding status.

Treatment	Mean ± SEM number of eggs laid by fed females (comprising fully and partially fed)	Mean ± SEM number of eggs laid by fully fed females	Mean ± SEM number of eggs laid by partially fed females
30 °C	115.00 ± 14.71	131.69 ± 15.23	60.75 ± 12.99
36 °C	130.78 ± 12.56	132.88 ± 12.99	104.50± 30.99
42 °C	96.78 ± 10.59	104.24 ± 11.08	33.0 ± 10.49
48 °C	32.00 ± 14.23	N/A	32.00 ±14.23

Although no statistical differences were detected in the proportion of fed females that oviposited and in the number of eggs that females laid in the positive control compared to the treatments at 30 °C and 42 °C, there was some evidence of an optimal response curve with its maximal values at the control temperature (Fig. 62). Thus, the egg count data was tested with an inverted quadratic model to determine whether an optimal curve explained better the data distribution. Results corroborated the initial intuition, as data fitted significantly better in an inverted quadratic curve compared to a straight regression line (GLM, chi-square=71.81, d.f.=1, P=0.01). This suggests that there is a decrease in the number of eggs laid per female when the temperature of the imbibed

blood moves away from the optimum 36 °C. Thus, further studies with smaller temperature intervals and with a higher number of replicates are suggested to a) test whether the nondifference observed here is maintained in a more highly powered study, and b) determine the optimal temperature range beyond which the difference becomes significant.

6.3.3. Quantification of blood meal size

Hematin concentration, and therefore blood meal size, was not significantly influenced by the temperature of the blood. This was the case for the analysis carried out on the data set comprising results from fully fed mosquitoes and partially fed mosquitoes (ANOVA, F=1.43, d.f.=3, 167, P=0.24), and also when the data was separated with fully fed mosquitoes only (ANOVA, F=1.41, d.f.=3, 136, P=0.24) and partially fed mosquitoes only (ANOVA, F=0.49, d.f.=3, 27, P=0.70) (Table 21). Thus, temperature did not influence the volume of blood imbibed in any of the feeding statuses assessed.

Table 21: Hematin concentration recovered from mosquitoes that had fed on blood at different temperatures. Aside from the means ± SEM, the table also present the number of mosquitoes examined for each category.

Treatment	n	Pooled data from fully fed and partially fed mosquitoes	n	Fully fed mosquitoes	n	Partially fed mosquitoes
		Mean ± SEM concentration (µg/ml)		Mean ± SEM concentration (µg/ml)		Mean ± SEM concentration (µg/ml)
30 °C	40	12.83 ± 1.27	30	16.16 ± 1.40	10	2.86 ± 0.69
36 °C	57	12.67 ± 1.09	52	13.66 ± 1.08	5	3.42 ± 0.93
42 °C	54	11.55 ± 1.14	49	12.63 ± 1.16	5	4.13 ± 0.86
48 °C	8	7.16 ± 2.66	2	16.87 ± 4.65	6	3.00 ± 0.86

Feeding speed was extrapolated by dividing the quantity of hematin recovered on a vial by the total time that the mosquito from that vial spent feeding. This allowed a direct comparison between feeding speeds of mosquitoes in different treatments, without however producing indications on the exact volume of blood imbibed, as the direct parameter was based on hematin. Thus, feeding speed was defined as the quantity of blood (i.e. blood unit) ingested per second. A significant difference was found in the speed of blood ingestion between different treatments (ANOVA, F=3.54, d.f.=3, 49, P=0.02). Specifically, mosquitoes imbibed blood about four times faster when the blood was at 48 °C compared to the speed at which mosquitoes imbibed blood in the treatment at 36 °C and 30 °C (Tukey's test, t=3.06, P=0.02; t=2.64, P=0.048, respectively). No significant difference was found in the feeding speed in mosquitoes exposed to the three lowest temperatures (Fig. 63). It is to be noted that the number of replicates for which speed information was available is quite limited (n=19 for treatments at 36 °C and 42 °C; n=12 for the treatment at 30 °C; and n=2 for the treatment at 48 °C). Thus, care must be taken when considering the results here reported and further studies using a higher number of replicates and smaller temperature intervals are recommended.
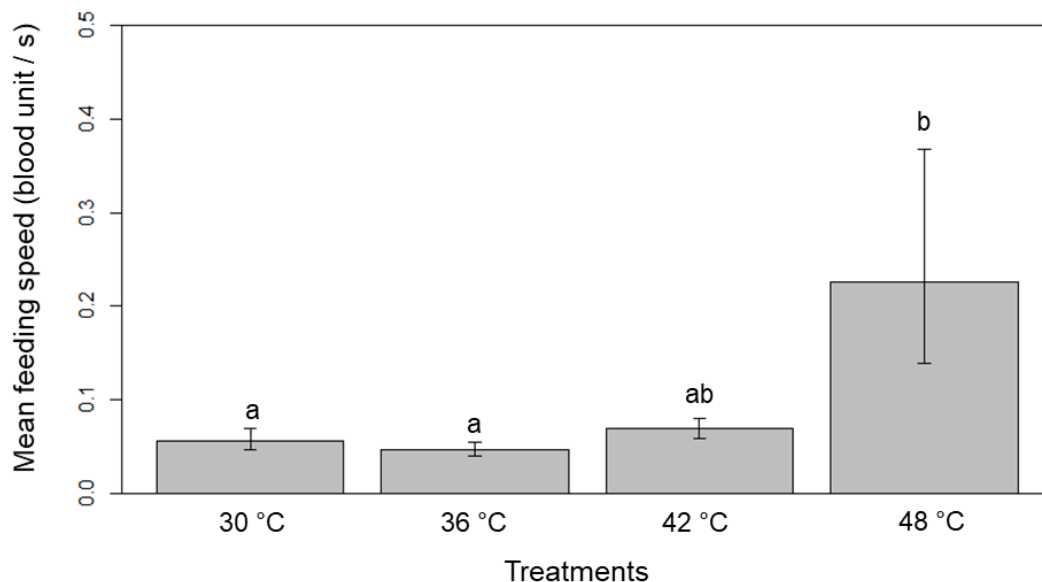
Figure 63: Mean feeding speed of mosquitoes exposed to blood at different temperatures. *Note that results presented here were anti-logged for ease of interpretation. A blood unit is intended as the parameter deriving from the hematin concentration recovered on the vial. Bars indicate means, the error bars indicate SEM and different letters denote significant differences between treatments (Tukey's test, P<0.05).*

6.3.4. Blood quality analysis

No immediate difference was seen in the overall quality, colour, and density of blood kept at the five lower temperatures. In contrast, blood that was kept at 60 °C was notably darker in colour and with clear signs of coagulation (Fig. 64).
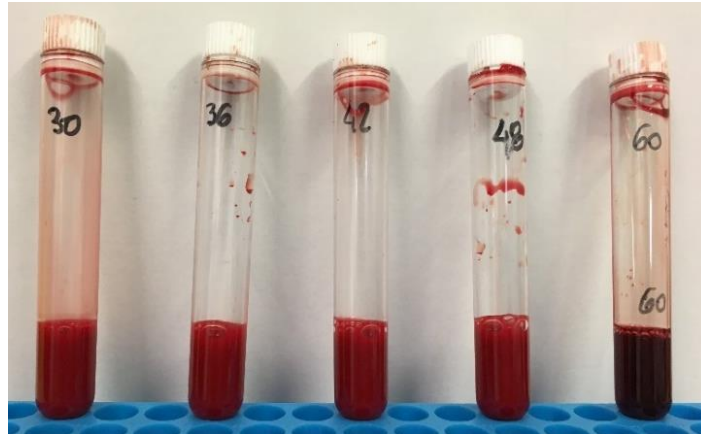
Figure 64: Photo of vials containing blood that was subjected to different temperatures for ten minutes. *From left to right, the temperature of each blood sample was 30 °C, 36 °C, 42 °C, 48 °C, and 60 °C. Note that the vial containing blood kept at 4 °C is not included in the photo.*

Microscopy images obtained from the control treatment (36 °C) were compared with stock images from veterinary sources ('Cells and Smears', 2021; Cornell University, 2021) and were deemed adequately similar to blood smears obtained from healthy horses in terms of morphology. Erythrocyte morphology appeared different when cells of different treatments were compared among each other. With an increase of temperature, erythrocyte morphology became more uniformly smooth, while at the control and lower temperatures, a higher number of acanthocytes (i.e. erythrocytes that present membrane protrusions) was recorded (Fig. 65).
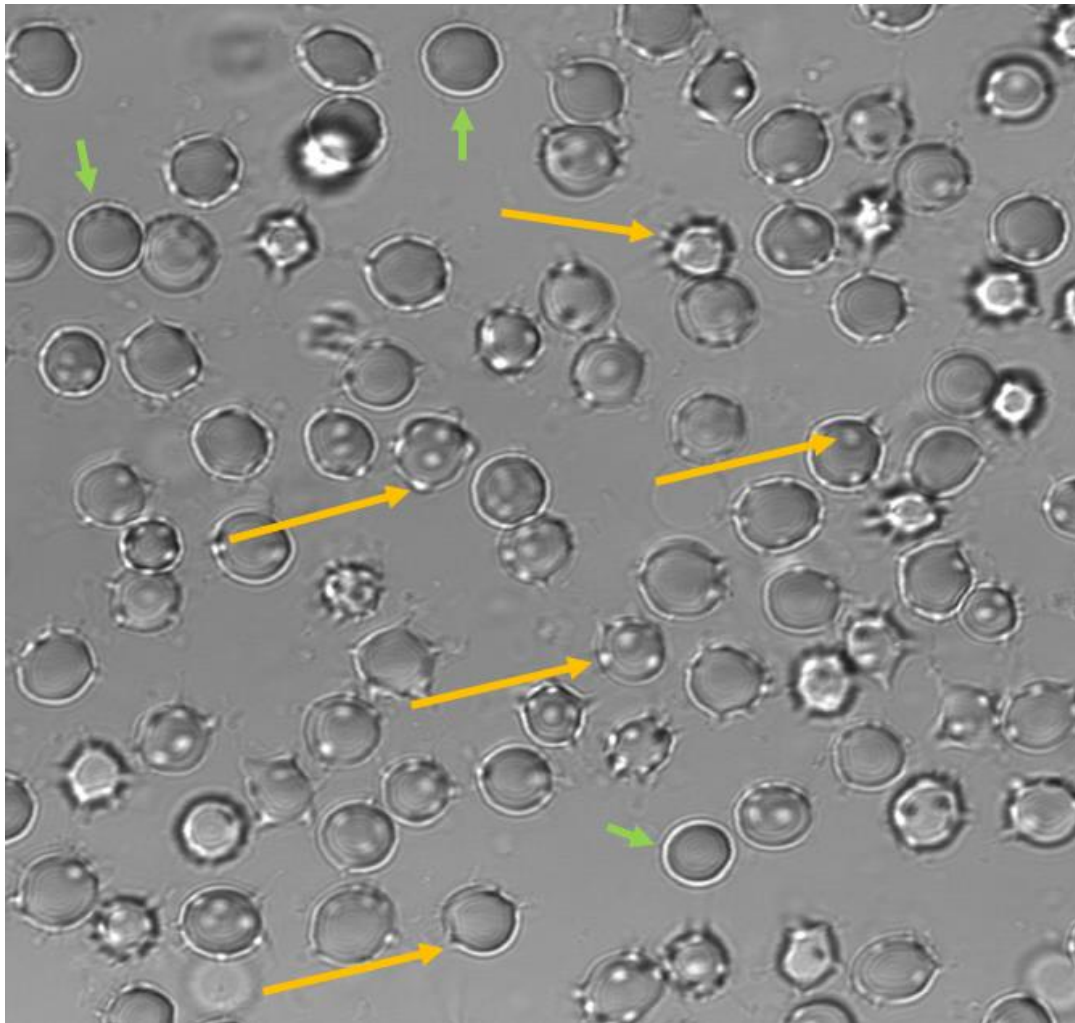
Figure 65: Erythrocytes with different morphology. *The short green arrows indicate uniformly smooth cells, while the long yellow arrows indicate acanthocytes and specifically point at the spicules, which can be seen as protrusions in the membrane if they are on focus, or as white points on the cell surface if the protrusion is out of focus.*

Specifically, the treatment at 36 °C had significantly more acanthocytes (46.67%) when compared with the treatment at 42 °C (6.67%; Fisher's Exact test, P=0.04) and at 48 °C (0%; Fisher's Exact test, P=0.006), while no difference was found in the number of acanthocytes between treatments at 36 °C (46.67%) and 30 °C (60.00%; Fisher's Exact test, P=0.72) (Fig. 66). Furthermore, the number of spicules (i.e. membrane protrusions) per erythrocyte also significantly decreased with the increase of temperature (GLM, chi-square=58.99, d.f.=4, P<0.001) (Fig. 66).
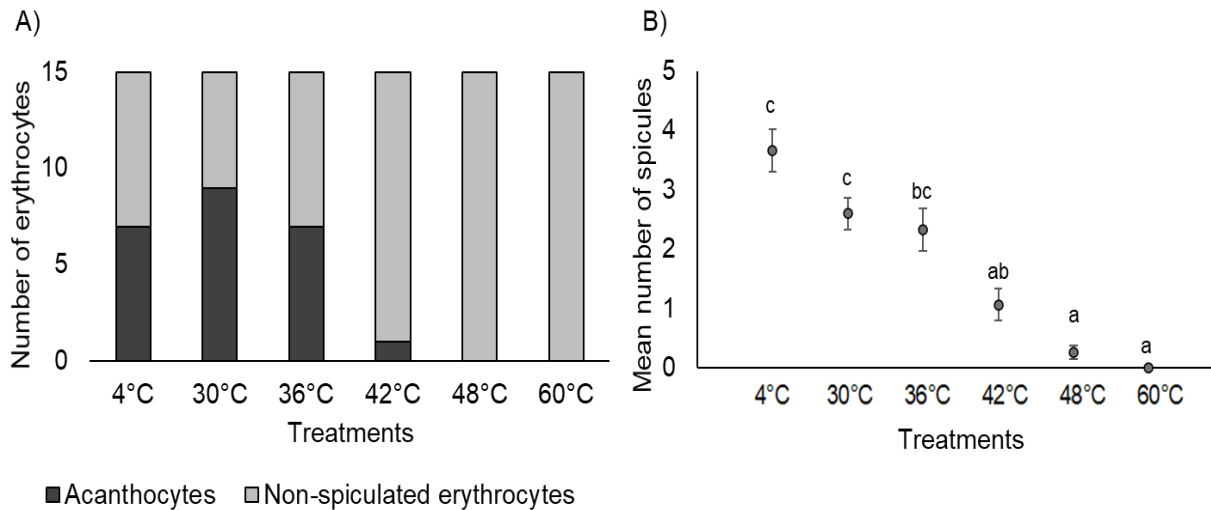
Figure 66: Changes in erythrocyte morphology following exposure to different temperatures. *(A) Shows the number of acanthocytes and non-spiculated erythrocytes counted in each different treatment. Note that a total of 15 erythrocytes were observed in different sections of the smear. Panel (B) shows the mean ± SEM number of spicules per erythrocyte in different treatments. It is worth noting that at 48 °C, the highest temperature tested in the behavioural study, all erythrocytes presented a uniform rounded membrane. In (B), the points indicate the mean while the error bars indicate the SEM. Different letters denote significant differences between treatments (Tukey's test, P<0.05).*

The diameter of cells that were exposed to the treatment at 36 °C fell within optimal range (optimal dimension between 5-6 μm (Grondin and Dewitt, 2010; Adili *et al.*, 2016), mean ± SEM diameter recorded in erythrocytes exposed to 36 °C=5.20 ± 0.09 μm). A significant difference was found in the diameters of cells exposed to different treatments (ANOVA, F=3.27, d.f.=5, 84, P=0.01). No significant difference was found in the diameter of erythrocytes exposed to the four temperatures tested in the behavioural study (i.e. 30 °C, 36 °C, 42 °C, and 48 °C) (Table 22, Fig. 67). However, a significant difference was found between the diameter of erythrocytes exposed to 36 °C and the two extreme temperatures (4 °C and 60 °C) (Table 22). For the full list of results from Tukey comparisons, see Table S2 in Appendix C.

Table 22: Results of the comparisons between erythrocytes exposed to host temperature and all other treatments. *The asterisk denotes significant difference between the treatments compared at a level of at least P<0.05 (Tukey's test).*

Host temperature	Mean ± SEM diameter (µm)	Compared treatment	Mean ± SEM diameter (µm)	t-value	P-value
36 °C	5.20 ± 0.09	4 °C	4.82 ± 0.09	3.05	0.04*
36 °C	5.20 ± 0.09	30 °C	4.86 ± 0.09	2.69	0.09
36 °C	5.20 ± 0.09	42 °C	4.91± 0.09	-2.25	0.23
36 °C	5.20 ± 0.09	48 °C	4.83 ± 0.09	-2.92	0.05
36 °C	5.20 ± 0.09	60 °C	4.73 ± 0.09	-3.67	0.005*
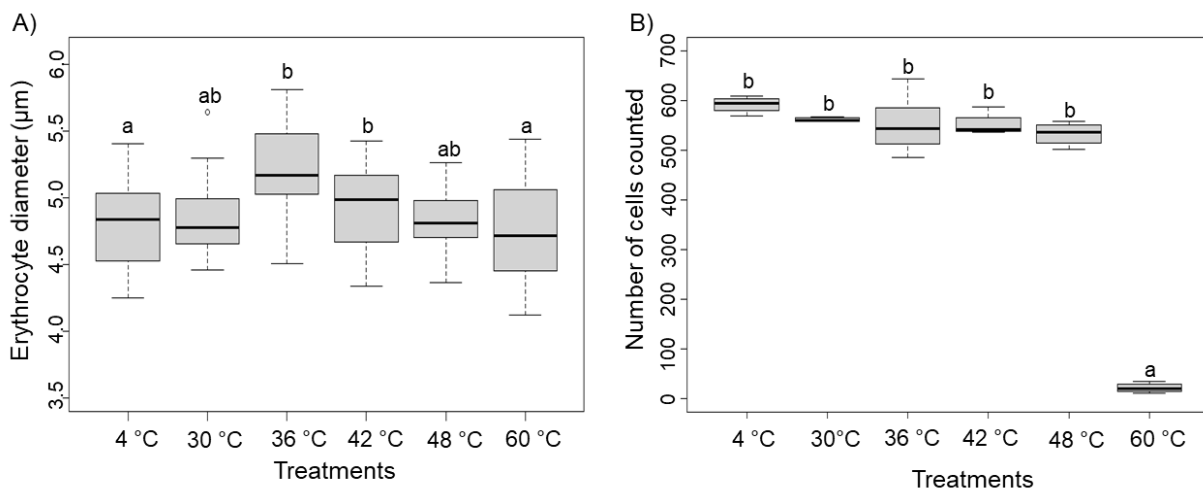
Figure 67: Cell attributes in defibrinated horse blood kept at different temperatures. *(A) Erythrocyte diameter (analysed with an ANOVA), note that CDC letters display the statistical results from all comparisons (Table S2 in Appendix C). (B) Number of cells counted per haemocytometer unit (analysed with a GLM). Four haemocytometer units were counted for each treatment. Both panels display box plots where the black bars indicate the medians, the upper and lower limits of the boxes indicate the interquartile range, and the whiskers indicate the maximum and minimum data points excluding outliers which are represented as small circles. Different letters denote significant differences between treatments (Tukey's test, P<0.05).*

Although the number of erythrocytes decreased with the increase of temperature, no significant difference was detected between treatments ranging from 4 °C to 48 °C

(Fig. 67). In fact, cell count fell within the range of standard erythrocyte count for the five lower temperatures tested (standard erythrocyte count for horse blood ranges from 6.0 – 12.5 million erythrocytes per µL (Tyler *et al.*, 1987), recorded means in million erythrocytes per µL for 4 °C=11.82, for 30 °C=11.20, for 36 °C=11.00, for 42 °C=10.98, for 48 °C=10.7). The cell count recorded in the blood kept at 60 °C was significantly lower compared with all the other treatments (Fig. 67) and was considerably below the standard erythrocyte count for horse blood (0.4 million erythrocytes per µL). For the full list of results from Tukey comparisons on the counted cell in the haemocytometer unit, see Table S3 in Appendix D.

Non-viable cells (i.e. dead cells or cells with ruptured plasma membranes) were observed only in the treatment at 48 °C and 60 °C, and in both cases in very low numbers. This indicates that although cells lost their 3D morphology after exposure to 42 °C, as evidenced by the significant decrease of spikes in the cell membrane, the cytoplasmatic membrane mostly remained intact and withstood temperatures up to 48 °C as in this treatment the cell count was still within the standard range and only a few non-viable cells were detected. However, the cell count drastically dropped for the treatment at 60 °C. From this information, it can be deduced that high temperatures caused erythrocytes to burst, rather than to slowly induce cell death, as only a small number of non-viable cells were recorded at 60 °C (i.e. dying cells would appear non-viable), and microscopy imaging showed deflated and empty erythrocytes bodies on samples exposed to 60 °C (Fig. 68).
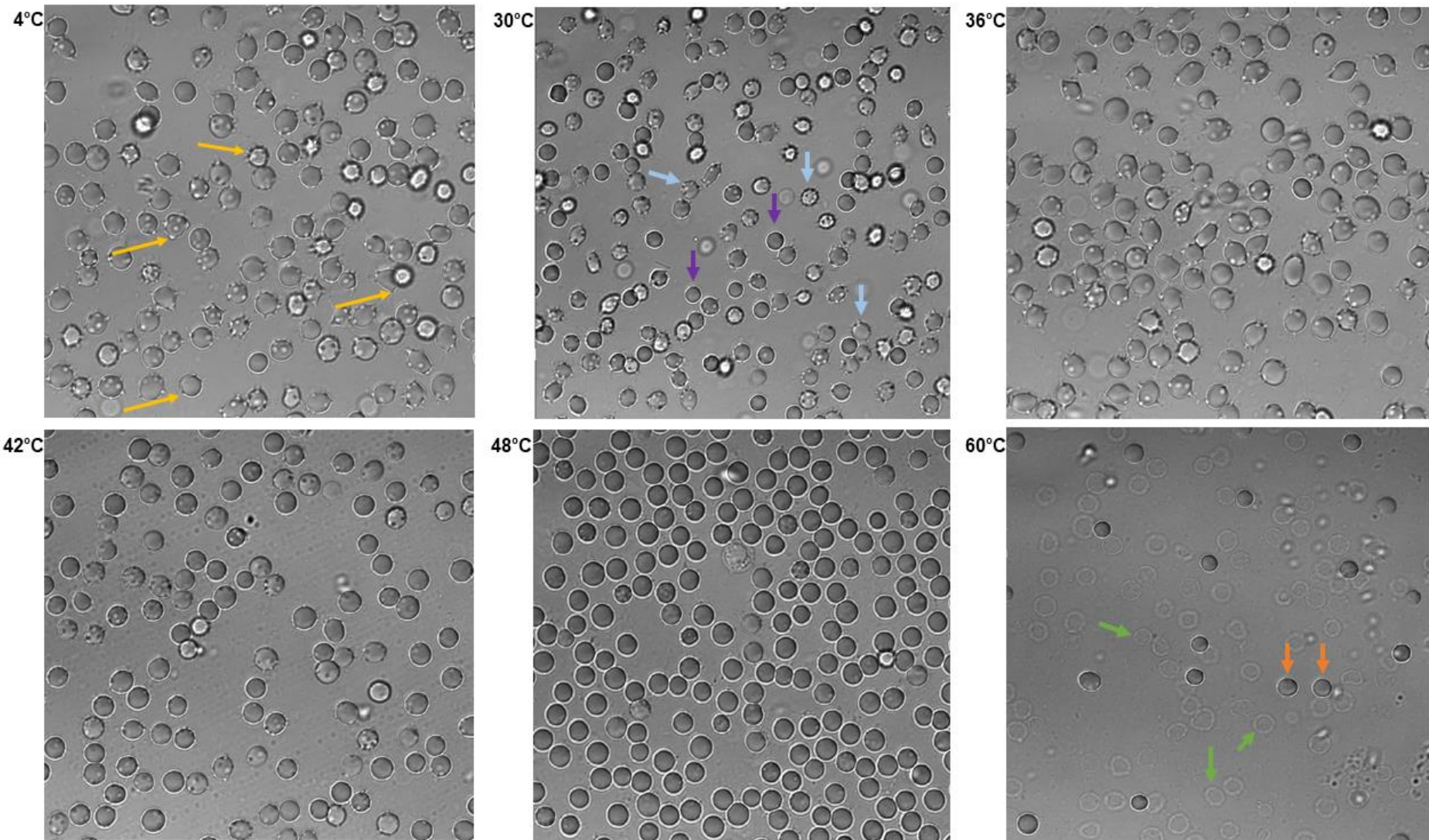
Figure 68: Erythrocytes imaging after treatment at different temperatures. *Yellow arrows indicate membrane protrusions, purple arrows indicate rounded cells, light blue arrows indicate acanthocytes, orange arrows indicate cells that maintained their integrity at 60 °C, while deflated cells (i.e. empty cells after rupture) are indicated by green arrows.*

6.4. Discussion

To the author's best knowledge, the results presented here constitute the first report of multiple pre-feeding, feeding, and fitness traits of *An. coluzzii* mosquitoes when presented with blood at different temperatures. Here, it was demonstrated that *An. coluzzii* females display different behaviours and propensities to feed when exposed to blood at different temperatures, thus indicating that temperature alone plays a major role in influencing short-range and feeding behaviour. In specific, mosquitoes displayed few foraging events and fed promptly on blood at 36 °C, whilst behaved radically different when presented with blood at 30 °C and 48 °C. Mosquitoes were equally successful in feeding on blood at temperatures that ranged from human physiological to febrile conditions. Results also demonstrated that mosquitoes produce similar number of eggs when they imbibe blood kept at temperatures between 30 °C and 42 °C, although a suggestion for an optimal oviposition response when imbibing blood at host temperatures was found. Further studies are needed to corroborate these findings. Furthermore, no major effects were seen in the mortality rates of fed mosquitoes, indicating that once the mosquitoes ingest the blood, independently of its temperature, they can endure the possible stresses associated with it, thus highlighting their remarkably flexible physiology.

The present study demonstrated that mosquito behaviour and feeding propensity for blood at 36 °C and 42 °C is substantially similar. Altogether, these results indicate a great elasticity in both their behaviour and physiology. From an evolutionary point of view, this suggests an adaptation to feed on human hosts at a range of temperatures encompassing the normal healthy human temperature of 36 °C up to temperatures of about 42 °C (i.e. severe fever temperature) (Odongo-Aginya *et al.*, 2005). Although *An. coluzzii* is considered to be strongly anthropophilic (Athrey *et al.*, 2017; Nguyen *et al.*, 2017), a previous study reported a population with a strong opportunistic trait (Lefèvre *et al.*, 2009), where blood meal analysis showed that approx. half of the fed mosquitoes collected in a rural area in Burkina Faso had ingested cattle blood. Cattle body temperature can vary depending on several environmental and physiological

conditions (Du Preez *et al.*, 1990), with physiological ranges of the core temperature fluctuating between 38 °C and 40 °C (Du Preez *et al.*, 1990; Beatty *et al.*, 2008), whilst the temperature of the peripheral body regions fluctuate more dramatically as it closely follows environmental temperatures (Du Preez *et al.*, 1990). Our results support the view of *An. coluzzii* as opportunistic, in that host blood temperatures up to 42 °C do not appear to inhibit or adversely modify feeding behaviour or success, identifying another trait that enables behavioural plasticity in terms of increasing diversity of host choice (Perugini *et al.*, 2020).

From an epidemiological perspective, it is interesting to note that *An. coluzzii* females presented a high propensity to feed on blood at fever temperatures, as about 86% of the mosquitoes presented with blood at 42 °C fed, either partially or fully. This propensity was not different compared with the one displayed for the control blood, where over 87% of the mosquitoes fed on the blood kept at 36 °C. Thus, there is no behavioural evidence of a preference for feeding on blood that has a thermal cue that could be associated with symptomatic malaria infection. As fever, the most common symptom of clinical malaria, is associated with the erythrocytic stage of the parasite's lifecycle, which is strongly linked with the production of its mosquito-infectious stage (i.e. gametocytes) (Crutcher and Hoffman, 1996), it is important to study all the factors that could influence host-vector interactions at this critical stage.

A study conducted on *Anopheles darlingi* (Root, 1926) measured the attractiveness of malaria patients in relation to different physiological conditions, e.g. presence of fever, and presence of malaria gametocytes (Batista *et al.*, 2014). This was done by counting the number of mosquitoes that at the end of the experiment were recovered in the olfactometer chamber where the foot of the patient was presented. Here the authors reported an increased attractiveness toward infected hosts which was accentuated by the presence of fever (temperature > 37.5 °C, with one case of fever > 40 °C). Notably, they found no correlation between host attractiveness and body temperature alone. However, when the body temperature was grouped with the presence or absence of *Plasmodium vivax* (Grassi and Feletti, 1890) gametocytes, they

found an increased mosquito attraction toward febrile patients if they carried gametocytes, while no difference in attraction was reported on patients, irrespectively of their gametocytes status, if they did not have fever. This suggests that is not the body temperature alone that increased host attraction, but rather an interaction between the increased temperature and the change in the host odour profile, which is caused by the parasite. In fact, it has been widely demonstrated that *Plasmodium* infections cause a change in host odour, which in turn increases mosquito attraction toward infected hosts, thus leading to a possible higher transmission rate. This has been reported in humans (Emami *et al.*, 2017; Robinson *et al.*, 2018) and birds (Cornet *et al.*, 2013; Díez-Fernández *et al.*, 2020).

In the study reported here, the only factor that was manipulated was the temperature of the blood, while the components of the odour profile were not actively altered. This could explain why there was no increased attraction towards blood at fever temperature, which supports the notion that increased attraction towards infected hosts is mediated by olfactory, rather than thermal cues. A recent paper reported that *An. coluzzii* rate of infection with *P. falciparum* gametocytes was negatively associated with fever, meaning that when mosquitoes were given infected blood via an artificial feeder from an infectious febrile patient, they were less likely to become infected with gametocytes (Ahmad *et al.*, 2021). This was attributed to the fact that gametocytes in febrile patients were less infectious, probably because of inactivation caused by the host immune response. Thus, greater overall host attractiveness might offset the low gametocytes' infectiousness when mosquitoes feed on humans with fever by increasing the overall rate of exposure to gametocytes. To date, many of the complex vector-host-parasite interactions are still unknown and the majority of the studies report only parts of interactions (i.e. focus only on vector-host, or vector-parasite, or host-parasite interactions). The results presented here help to clarify the role that blood temperature, and therefore body temperature, might have on mosquito behaviour. However, this study did not take into account possible changes that might occur when the parasite is introduced in the equation. Thus, further studies that encompass all levels of the

vector-host-parasite cycle are recommended to better understand the changes that take place when the three organisms interact with one another.

Perhaps the most surprising result reported here is the fact that although the feeding rate in the hottest treatment was significantly smaller compared to any other blood temperature, eight mosquitoes successfully fed on blood at 48 °C. To the author's best knowledge, this is the first report of mosquitoes feeding on blood at such high temperatures. However, it is important to emphasise that given the small number of mosquitoes that fed at 48 °C, the analyses carried out for this treatment on parameters related to feeding behaviour should be considered with care and interpreted as a preliminary overview, rather than definitive results, as the low number of replicates rendered difficult to draw firm conclusions. This also applies to considerations based on the oviposition and the survival of mosquitoes that fed at 48 °C. It has to be taken into consideration that for high and low blood temperatures (i.e. blood temperatures outside the normal range of host temperatures) the feeding acceptance rate is expected to be minimal. This, unfortunately, hinders data collection. Therefore, it is suggested for future studies to use smaller temperature intervals, as this will increase the number of data points and will limit the low response rate to only the extreme points. Further studies should also include more replicates. These precautions should render the analysis more powerful.

Previous studies have reported that even by just alighting on a warmer surface (e.g. a feeder or a host) haematophagous insects experience a rise in their body temperatures (Benoit and Denlinger, 2017; Reinhold *et al.*, 2021). This temperature increase can be explained by simple conduction, where the heat is transferred from the feeder surface to the mosquito. Given the physical properties of conduction, it follows that the mosquito's body temperature will rise accordingly to the surface's temperature, meaning that the hotter the surface the more the body temperature will increase. Thus, even by just resting for prolonged periods of time on a hot surface, mosquitoes might experience thermal stress. Host-seeking *Ae. aegypti* females have been reported to avoid surfaces at 50 °C and 55 °C (Corfas and Vosshall, 2015). This partially aligns with the results

reported in the present study, which showed that mosquitoes spent considerably less time on feeders at 48 °C compared to other treatments, although there was no indication of a complete avoidance behaviour (i.e. there was no suppression of landing behaviour). Furthermore, it is interesting to note that generally, when landing on a feeder at 48 °C, mosquitoes remained on the periphery of the feeder, typically with their thorax and abdomen positioned outside of the circumference of the feeder. A similar response was found in *Cx. quinquefasciatus*, which preferred the outer rim of blood feeders (set at 36 °C) as there the blood was slightly cooler (Reinhold *et al.*, 2021). Altogether, these results indicate that although mosquitoes were attracted to the hottest feeder, both the time spent on the feeder and the area of contact were minimal, suggesting a certain degree of avoidance behaviour, which could thereby reduce thermal stress associated with excess heat exposure.

Nevertheless, eight mosquitoes ultimately fed on blood at 48 °C, and surprisingly, all eight mosquitoes survived the event. However, only two mosquitoes went on to form eggs. In contrast, 27 out of 38 mosquitoes that fed in the fitness assay on blood at host temperature oviposited, giving an oviposition rate of about 71%. Blood quality was similar irrespective of heat treatment, which suggests that it was the effect of heat exposure that compromised the development of the eggs. This could have several explanations. It is possible that blood digestion, and therefore nutrient availability for vitellogenesis, was limited due to a) enzyme inactivation (Benoit *et al.*, 2011), or b) inactivation of symbiotic gut bacteria (Lahondère and Lazzari, 2012), both of which are potentially caused by heat exposure. Another possibility is that the blood was properly digested, but the nutrients and energy derived from it were used in the processes required to repair the damaged caused by the thermal stress. This field remains unexplored and further studies are needed to clarify this.

Imbibing blood at extreme temperatures might pose a serious thermal risk to the mosquito physiologically, and it would be interesting to investigate what mechanisms *An. coluzzii* mosquitoes use to withstand such a stressful event. A recent study showed that another haematophagous species, the kissing bug

Rhodnius prolixus (Stål, 1859), thermoregulates its body temperature when blood-feeding via a counter current heat exchange mechanism. This cools down the temperature of the ingested blood before it reaches the abdomen, thus protecting the insect from thermal stress (Lahondère *et al.*, 2017). A different thermo-regulatory mechanism was found in the mosquitoes *An. stephensi* and *Cx. quinquefasciatus*. In this case, the body temperature of the insect is decreased as droplets of the fluid excreted during the prediuresis are kept attached to the abdomen, which creates an evaporative cooling effect (Lahondère and Lazzari, 2012; Reinhold *et al.*, 2021). Prediuresis, i.e. the excretion of rectal fluid during blood-feeding, is common in haematophagous insect species (Lahondère and Lazzari, 2012), and prior to the discovery of its thermoregulatory role, it was thought to primarily serve as a way to concentrate host erythrocytes during feeding whilst eliminating the excess of liquid (Briegel and Rezzonico, 1985; Lahondère and Lazzari, 2013). Evaporative cooling mechanisms have also been recently reported in the soft tick *Ornithodoros rostratus* (Aragão, 1911) (Lazzari *et al.*, 2021), whilst more dated studies had reported evaporative cooling in moths (Adams and Heath, 1964), honeybees and bumblebees (Heinrich, 1976, 1979), and aphids (Mittler, 1958). Thus, it seems that this mechanism is common to several groups of arthropods as a response to events associated with high temperatures.

Another mechanism that protects against the thermal damage caused by blood-feeding is the synthesis of HSPs, a group of chaperon proteins that preserve the enzymatic function and the structural integrity of proteins, and can aid to repair the damage caused by heat exposure (Benoit *et al.*, 2011; Pereira *et al.*, 2017). The expression of HSPs has been reported to be upregulated in relation to blood-feeding events in soft ticks, hard ticks, bed bugs, kissing bugs, sand flies, and mosquitoes (Pereira *et al.*, 2017). Although this physiological response seems to be highly conserved throughout the taxa, the level of upregulation greatly varies across species, and it depends on the strategy (i.e. toleration or regulation) used to bypass thermal stress. For instance, *Ae. aegypti* is classified as a conformer species (i.e. thermotolerant), while *Anopheles* are thought to be regulators. As such, *Ae. aegypti* significantly upregulate HSPs as

the principal response to heat stress, whereas *Anopheles* mosquitoes thermoregulate primarily via evaporative cooling, and therefore the levels of HSPs are only slightly upregulated (Benoit *et al.*, 2011; Lahondère and Lazzari, 2015). It is interesting to note that these mechanisms have been studied only in vectors that were fed blood at host temperatures, thus it is not known how these different responses may be expressed when mosquitoes feed on blood at higher temperatures. It would be interesting to carry out a thermographic analysis on *An. coluzzii* mosquitoes feeding at different temperatures to determine whether their body temperature increases when feeding on hotter blood or if evaporative cooling is sufficient to maintain the temperature within a suitable physiological range, independently of the temperature of the blood. It is hypothesised that evaporative cooling might be an efficacious method only when dealing with blood within a limited temperature range. Thus, if results from the thermographic analysis indicate an increase in body temperature when imbibing hot blood, further studies (e.g. using transcriptomic analysis) might help to further elucidate whether *An. coluzzii* mosquitoes also employ other mechanisms (e.g. enhanced production of HSPs) to cope with extreme temperatures.

It is interesting to note that mosquitoes presented with blood at 30 °C showed a pronounced variability in their behaviour. This was found in both the analyses of individual behavioural parameters as well as in the PCA and RDA analyses. Within treatments from 36 °C to 48 °C, mosquitoes behaved similarly to individuals of the same treatment, thus indicating that each heat cue elicited a rather uniform response. On the other hand, in the treatment at 30 °C mosquitoes behaved radically different among themselves, with some mosquitoes displaying a behaviour closer to those exposed to 36 °C and 42 °C, and some others displaying a behaviour more similar to mosquitoes exposed to the highest temperature. It has been previously reported that *An. coluzzii* females rely on multiple cues to successfully find a host, and host odour was reported to play a crucial role in eliciting short-range attraction, with the heat cue further increasing this response (Carnaghi *et al.*, 2021). Similarly, in the study presented here, which used odour-activated host-seeking females, the temperature of the feeder was a decisive factor that triggered the next steps of

the feeding behavioural sequence. In the treatment at 30 °C, the absence of a sufficient thermal cue left the activated mosquitoes to continue exploring the feeder and other parts of the cage, and mosquitoes initiated different behaviours in a more randomised manner (hence the recorded high variability), as the cue that drove the advancement in the feeding behavioural sequence was missing.

Interestingly, mosquitoes exposed to the two extreme temperatures (30 °C and 48 °C) reported behaviours somewhat similar to one another, being the treatments where mosquitoes displayed more foraging activities and grooming behaviour, visited the feeder more frequently, and had lower feeding rates. The main differences between these treatments were that at the lowest temperature mosquitoes spent more time on the feeder and displayed a pronounced variability in their behaviour (as suggested above, due to the lack of the signal that drove the progression in the behavioural sequence), whilst at the highest temperature the behavioural responses recorded were uniform. This could be explained by the fact that high temperatures could pose possible detrimental consequences, and therefore responses to such cues might be driven by a more strict selective pressure, which would then translate into a more uniform behavioural outcome. In both cases however, the absence of a suitable heat cue led to the continued expression of appetitive behaviours associated with searching for the blood meal. This translated into more foraging behaviour, where the type of behaviour depended on the temperature cue. Whilst at the lowest temperature mosquitoes frequently explored the surface by repeatedly tapping their labella (the tip of the labium) against the membrane (i.e. sensing behaviour), at the highest temperature the most common foraging behaviour was probing. In both treatments, mosquitoes displayed significantly more foraging behaviours (both probing and sensing) compared to the positive control, which suggests that at host temperatures the suitable set of cues were detected quicker, and those cues led mosquitoes to move onto the next steps of the feeding sequence.

Sensing behaviour has been previously reported in studies that used a variety of mosquito species (Gordon and Lumsden, 1939; Griffiths and Gordon, 1952;

Grossman and Pappas, 1991; Choumet *et al.*, 2012; Sparks *et al.*, 2013; Hol *et al.*, 2020). Given that the labium hosts a large number of sensory sensilla, and specifically, of gustatory receptors (Goldman *et al.*, 1972; Sparks *et al.*, 2013), important information is acquired when tapping the labium against the surface. This process allows them to further determine the suitability of the surface and influences their response to initiate the series of events that lead to engorgement (Sparks and Dickens, 2017). Therefore sensing is thought to be the first sign of the intention to feed (Gordon and Lumsden, 1939). Although the low heat cue emanating from the feeder at 30 °C was not always sufficiently strong to trigger a uniform response in mosquitoes (i.e. progression in the sequence of behaviours that takes mosquitoes to feed), the presence of host odour and the feeble heat cue continued signalling the presence of a possible host. Thus, mosquitoes were driven to continue repeating the first step of the foraging behaviour (i.e. sensing). In contrast, as a result of the avoidance behaviour toward high temperatures, the contact with the feeder at 48 °C was minimised by skipping this foraging stage and directly starting the probing behaviour. By probing, the chemoreceptors on the labrum, one of the fascicles that mosquitoes insert into the host tissue, are directly exposed to the phagostimulants and therefore the presence of blood can be detected (Werner-Reiss *et al.*, 1999). A study conducted on *Ae. aegypti* reported that this foraging behaviour was persistently repeated until a blood supply was encountered (Griffiths and Gordon, 1952). This is in accordance with the results reported here, where for example, on feeders at 48 °C mosquitoes insistently probed many times before desisting.

Altogether, the findings of this study suggest that the sequence of behaviours that mosquitoes employ from landing to feeding are not fixed, and they can be flexibly used in a context-dependent manner to distinguish potential hosts from non-hosts or objects. It is interesting to note that even when offered the feeder at the control temperature, mosquitoes often probed several times before starting to imbibe the blood. This is in accordance with results reported by Choumet *et al.* (2012), who described *An. gambiae* probing the skin of a mouse multiple times before moving to another behaviour. It is expected for this

behaviour to be strongly negatively selected in the wild, as this would disturb and alert the host (Gillett, 1967; Choumet *et al.*, 2012). However, two factors need to be taken into consideration when comparing probing behaviour with wild mosquitoes. First, here the feeder was prepared using a membrane, which could have altered the probing response (Friend and Smith, 1977). Secondly, as the mosquitoes used in this study derived from a colony that had been kept in a laboratory for several generations, some of the behaviour selected in the wild might have reverted under these new conditions. This would be in accordance with a previous study where it was reported that the difference in feeding rate between wild *Aedes africanus (Theobald, 1901)* females (which fed faster) and colonised *Ae. aegypti* females (which fed slower) could be attributed to the different selective pressure applied to the populations (Gillett, 1967). Thus, it is possible that the probing behaviour observed here is not a direct reflection of the behaviour displayed in wild mosquitoes and further studies using different populations of the same mosquito species might help to clarify this point.

It is noted that significantly more mosquitoes displayed grooming behaviour in the treatments at 30 °C and 48 °C compared to the two middle treatments. This could be explained by the fact that when exposed to the lowest and highest temperature mosquitoes might have received either a lack of stimuli or opposing stimuli (i.e. feeders possessing both attractive and repellent properties). Grooming behaviour frees obstructive matter from the sensilla on the antenna or the proboscis that might otherwise interfere with the functioning of the receptors hosted in them (Goldman *et al.*, 1972; Walker and Archer, 1988). Thus, it can be expected that mosquitoes employ such behaviour when receiving confounding stimuli as a manner of detecting if the stimuli they were receiving persist after cleaning the sensilla, which would indicate that the stimuli are currently emitted. Therefore, it is not surprising that mosquitoes groomed more when exposed to the highest and lowest blood temperatures.

In this study, the time that elapsed from the beginning of the experiment until the first landing event was similar across all heat treatments, thus suggesting that feeders that present more extreme thermal cues do not reduce the time needed

for the mosquito to find the surface. This indicates that, at close range, a person with a fever might not be more easily detected, solely because of the increased temperature, compared to a person without a fever. However, two factors of the experimental set-up have to be considered, as they may have influenced the time it took mosquitoes to first reach the feeder. First, the cage used was relatively small, meaning that test mosquitoes may have been able to detect the heat from any position in the cage, irrespectively of the absolute temperature of the feeder. Previous studies have reported different distances at which the heat dissipating from a warm object elicited a behavioural response in mosquitoes. These distances ranged from only few cm from the heat source (Van Breugel *et al.*, 2015), to several tens of cm from the source (Wright, 1968; Zermoglio *et al.*, 2017), to even one to two metres from the heat source (Bowen, 1991; Davis and Bowen, 1994). The discrepancies in the reports might be due to the fact that several factors, such as the orientation of the object, the presence of water vapor, and the angle between the object and the mosquito could have influenced heat detection and heat dissipation. It is therefore difficult to make a generalisation of the distance range at which heat is detected by mosquitoes. Unfortunately, in the study presented here, it was not possible to record the distance range at which the heat plume affected the surrounding air, thus, it is not possible to determine if the cage was sufficiently large to allow for the different heat cues to be detected from different distances. Therefore, this remains an unclear factor that could have influenced mosquitoes response time.

Secondly, the feeder in this experimental set-up was positioned on the top of the cage in an upside-down position (i.e. with the feeding surface facing the bottom of the cage). This might have prevented the formation of convection currents that normally help carry stimuli (odour, heat, vapour) over longer distances (Khan *et al.*, 1968). Thus, further studies that take into account these factors are recommended to clarify whether mosquitoes might respond faster to objects set at high temperatures depending on their relative position. Nonetheless, although mosquitoes might not benefit from a hotter host temperature to better find a meal, finding and feeding on a febrile host might still come with some advantages. For example, hosts with fever might be more tired and drowsy, which might result in

lower vigilant anti-vector defensive behaviour. Given that blood-feeding is very risky for mosquitoes due to the host's defensive behaviour, feeding on hosts with a lower level of defensive behaviour might be an advantageous strategy for mosquitoes (Burkot, 1988). On the other hand, imbibing warmer blood might increase the thermal stress that a mosquito has to endure, and the quality of the blood of a diseased person might be poorer compared to that of a healthy individual (Kelly, 2001; Logan, 2008). Thus, many factors may offer advantages or disadvantages to the fitness of mosquitoes that imbibe blood from febrile patients.

Outcomes from the exploratory study confirmed that no major alterations were noted in the quality of blood kept at temperatures ranging from 4 °C to 48 °C, thus suggesting that the observed differences in mosquito behaviour when exposed to the different treatments were the result of the physical thermal cue, rather than blood properties. Furthermore, the fact that blood properties remained mostly unaltered might also explain why mosquitoes imbibed similar quantities of blood, irrespectively of the temperature at which this was presented. These results are in accordance with Grossman and Pappas (1991), who reported that the meal size of *Ae. aegypti* females exposed to blood ranging from 29 °C to 36.2 °C remained constant (approx. 3 µL) in all treatments. Thus, it can be concluded that the likelihood of accepting a blood meal depends on the temperature of the blood, but once the mosquito commences feeding, the positive feedback received from the blood drives it to continue imbibing, normally to repletion, irrespectively of the temperature of the blood. Certain characteristics of the blood (e.g. its tonicity, the presence of sodium ions, and the presence of phagostimulants) are sufficient cues to keep haematophagous insects engaged in feeding until fully engorged (Galun *et al.*, 1985; DeVries *et al.*, 2016). This is due to the fact that chemoreceptors in the cibarial determine the acceptability of the meal while this is being imbibed, and positive signals resulting from this receptors reinforce the imbibing process (Friend, 1978). The conclusion drawn from this study is in line with previous descriptions of *Culiseta inornata* (Williston, 1893) behaviour, where it was reported that the heat signal and the presence of a membrane on the feeder affected the number of mosquitoes that accepted to

feed. Specifically, the heat and the membrane triggered a feed-forward effect that biased the nervous system of the insect, directing the response into full "blood-feeding mode" (Friend, 1978). The authors also concluded that once the insect has started feeding in a particular mode (e.g. blood-feeding, sugar-feeding), abnormal signals, e.g. an unexpected heat cue or different phagostimulants, evoke much smaller responses than what they would normally if the insect had not started feeding. Nonetheless, with increments of the blood temperature is logical to think that the consequential thermal stress on the insect will also increase. Thus, it is possible that when the stress reaches a certain threshold level, mosquitoes might interrupt the feeding. This idea needs testing, but it could explain why out of the eight mosquitoes that fed at 48 °C, six did so only partially, which suggests that this temperature approached the maximum thermal tolerance of *An. coluzzii*.

One of the blood parameters that might have differed between treatments is viscosity, which generally decreases with an increase in fluid temperature. Studies that reported stable viscosity at different temperatures only took into account blood warmed up to physiologically relevant temperatures for humans (up to 42 °C) (Eckmann *et al.*, 2000; Pinho *et al.*, 2016), thus it is possible that viscosity in blood at 48 °C might have been lower compared with the viscosity of blood at host temperatures. As mosquitoes feed faster on blood with lower viscosity (Daniel and Kingsolver, 1983; Grossman and Pappas, 1991; Kim *et al.*, 2013), it is hypothesised that the notable faster feeding speed found in the present study for the treatment at 48 °C may be due to the decrease in blood viscosity. Grossman and Pappas (1991) reported that the feeding speed in *Ae. aegypti* increased from 1.2 µL/min when feeding on blood at 29 °C to 2.2 µL/min when the blood was at 36.2 °C. Thus, they concluded that even a change of blood temperature of only 7.2 °C could increase the feeding efficiency. The authors attributed this increase in efficiency to a lower viscosity in blood at the higher temperatures, however this is in contradiction with results reported by Eckmann *et al.* (2000) and Pinho *et al.* (2016), so other factors might have influenced the feeding rate. Similarly, another study conducted on tsetse flies showed how these females fed to repletion in a quicker period on an artificial

feeder when this was set to 42 °C (fed in about 30 s) compared to one set at 37 °C (approx. 50 s of feeding time) (Lahondère and Lazzari, 2015). In the study presented here no difference was found in the feeding speed of mosquitoes imbibing blood ranging from 30 °C to 42 °C. The discrepancies between studies could be due to species-specific differences or differences in the methodology used to quantify blood ingestion. Further studies are recommended to clarify this.

Studies that analyse sequences of behaviour can be challenging given that not every studied individual performs all the behaviours, which then translates into a data set with large numbers of zeros (Slater, 1973; Walker and Archer, 1988). This makes the analysis difficult, as it creates problems with sample size and with classical statistical assumptions needed for most tests (Slater, 1973; Walker and Archer, 1988). In the study reported here, these problems arose for some of the parameters analysed (for example when looking at feeding or grooming behaviour). To bypass these issues, a conservative analysis method was chosen, where indications on the frequency of the event were evaluated using proportion analysis, whilst more in-depth testing was carried out in data sets where the individuals that had not completed the behaviour were excluded. Nonetheless, sample size affected some of the analyses, principally those related to the feeding behaviour, and the corresponding conclusions. Thus, further studies are suggested to confirm the preliminary results obtained on said analyses.

6.5. Conclusions

The results described here constitute the first short-range and feeding characterisation of *An. coluzzii* mosquitoes when presented with blood at different temperatures. Although a general indication of optimal feeding response was obtained for mosquitoes exposed to blood at 36 °C, females behaved comparatively similar when exposed to blood at 36 °C and 42 °C, and to a lesser extent, when exposed to 30 °C. Therefore, the study presented here describes a great plasticity in feeding acceptance and good ability to utilise blood's nutrients, as mosquitoes not only imbibed blood at a wide range of temperatures but also used the digested nutrients to oviposit similar number of

eggs irrespective of the blood's temperature. From an evolutionary point of view, the ability to imbibe and digest blood at a wide range of temperatures might result in an advantageous trait, as it might allow mosquitoes to feed on a wide range of hosts. Surprisingly, mosquitoes repeatedly attempted to feed on blood at 48 °C, and no difference was found in the mortality rates of mosquitoes that fed at such high temperatures and mosquitoes that did not feed. It would be interesting to determine if this great protection against high temperatures is the result of evaporative cooling alone (Lahondère and Lazzari, 2012) or if other mechanisms play a role in such exceptional response. Understanding the behaviour and physiology of *An. coluzzii* mosquitoes related to blood-feeding opens new avenues for vector control applications as, for example, disrupting the mechanisms that mosquitoes use to cope with the stresses associated with blood-feeding could translate into blocking feeding altogether.

7. GENERAL DISCUSSION

For centuries, mosquitoes have been the source of extensive nuisance for humans (Becker *et al.*, 2010). Thus, it is not surprising that much research has focussed on the study of these insects. In particular, as mosquitoes are vectors of several diseases, the research carried out aimed to elucidate traits in their biology and behaviour that could be exploited in control methods designed against them. As such, a vast amount of effort had been put into the investigation of host-seeking behaviour and on the cues used by mosquitoes to successfully detect and feed on a host (see section 2.4). However, the majority of these studies focussed on the response to odourants (Zwiebel and Takken, 2004; Zermoglio *et al.*, 2017; Lazzari, 2020), which drive the activation and long-range orientation phase (Cardé, 2015). As a result of this large number of studies, there are now several promising odour blends that can be used in traps to increase mosquito catches (Okumu *et al.*, 2010b; Verhulst *et al.*, 2011a; Mukabana *et al.*, 2012; Mweresa *et al.*, 2016).

However, relatively little research had concentrated on studying the last phases of host-seeking behaviour, particularly in *Anopheles* mosquitoes. Consequently, prior to the commencement of this project, little was known about the sequence of cues used by landing mosquitoes (Zhou *et al.*, 2018). Thus, this project aimed to evaluate the effect of several cues used by *An. coluzzii* host-seeking females during the landing and post-landing phases. To address this, a series of behavioural assays were designed to systematically test different cues, either presented alone or in combination with other cues. Altogether, the result obtained in Chapters 4-6 corroborated previous notions of mosquito host-seeking behaviour and provided significant new insights into the factors that guide the close-range orientation, landing, and post-landing phase in *An. coluzzii* mosquitoes. An interpretation of the results obtained in this project, when considered in their entirety, is presented below.

It has been well established that different cues guide different stages of the host-seeking behaviour, yet some aspects of how these cues affect mosquito

behaviour are still unclear (Cardé, 2015). For example, the types of interactions that host cues have when presented in combination and the distance over which these cues influence mosquito behaviour are still debated (Cardé, 2015). The general consensus on the role of the different host cues and the approximate range at which they direct mosquito behaviour is well illustrated in Cardé (2015) and Cardé and Gibson (2010). To summarise this: carbon dioxide and host body odour are considered to be important cues in the activation and long-range orientation phases, the visibility of the target or the host is thought to direct host-seeking behaviour at a mid to short-range distance, depending on the mosquito species, the angular resolution of the eye, and the lighting conditions (Bidlingmayer and Hem, 1980; Land *et al.*, 1997), while the thermal cue is thought to play a crucial role only in proximity of the host, although some studies have reported that mosquitoes responded by orienting towards a thermal cue even when this was presented metres away (Bowen, 1991; Davis and Bowen, 1994). Host emits all these cues simultaneously (Zhou *et al.*, 2018), and mosquitoes therefore might use multiple cues at the same time to detect (Lazzari, 2020), and ultimately land on a host.

In the present research, by quantifying the landing response of mosquitoes presented with different combinations of cues it was possible to determine that these cues interact in a synergic manner, rather than in a simple additive manner, and that, if considering only the landing response, mosquitoes were capable of bypassing the absence of either the thermal or the visual cue provided that one of these cues was presented together with host odour (Chapter 4). This indicates that sensory information is integrated in a way that allows for flexible and accurate responses that vary depending on the set of cues received, thus attaining a context-relevant behavioural outcome (Carnaghi *et al.*, 2021). A previous study described how the information deriving from visual and olfactory stimuli was integrated to facilitate host tracking in *Ae. aegypti* (Vinauger *et al.*, 2019), however, similar integration mechanisms are yet to be described for the thermal and olfactory cue. Nonetheless, results from Chapter 4 clearly indicated that the thermal cue synergically interacts with the host odour, as demonstrated by the fact that the combination of host odour and thermal cue elicited

significantly more mosquitoes to land (~53%), compared to the proportion expected if these two cues interacted in a mere additive manner (~21%). Thus, considering these remarkable results, the interaction between cues was further explored in Chapters 5 and 6. Given that host odour proved to be essential in recording any significant landing response, i.e. is a prerequisite for this behaviour (Zhou *et al.*, 2018; Reinhold *et al.*, 2022), presumably because without this cue mosquitoes flew less and were therefore less likely to encounter further host cues (Van Breugel *et al.*, 2015; Carnaghi *et al.*, 2021), all experiments carried out in Chapters 5 and 6 were done so in the presence of host odour. The carbon dioxide stimulus was adjusted and dispensed in a pulsed manner, as this proved to be more attractive to mosquitoes compared to a continuous carbon dioxide flow (Gillies, 1980; Geier *et al.*, 1999; Dekker *et al.*, 2001; Dekker and Carde, 2011).

Having demonstrated the relative importance of the olfactory, visual, and thermal cues when presented individually and in combination, the effect that variations of these cues had in driving landing was further tested (Chapter 5). Here, it was shown that the thermal cue induced significantly more mosquitoes to land on the target only when covering an area at least half the size of a consistent visual stimulus. Aside from corroborating results obtained in Chapter 4, this result highlights the complexity of the elaboration of sensory information, as different facets of cue presentations elicit different behaviours in a mosquito. It could be that the sensory system conveys not only a dichotomous presence/absence but also specific details on the presentation of the stimulus, as for example, information on whether the stimulus is presented adjacent to another one might be important. It is interesting to note that contrary to the results obtained for the heated area, no size-effect was detected in the landing response for the unheated area (i.e. increments in the unheated area size were not matched with increments in landing response). However, results obtained with the 3D tracking system indicated that, irrespective of the size of the heated area, the number of visits to the target, the time spent flying in their proximity, and the total distance flown around them were similar for all treatments, thus suggesting that all targets, even the ones where the entire surface was unheated, elicited similar

short-range attraction, but only sufficient thermal cues triggered landing specifically.

Results from the 3D tracking system also indicated that independently of the final response (i.e. landing or steering away) and of the size of the heated area, mosquitoes approached the black targets within centimetres from the surface. This reinforced the idea that the visual cue plays an important role as an attractant (Van Breugel *et al.*, 2015; Hawkes and Gibson, 2016; Zhou *et al.*, 2018). This type of information is vital when considering the efficacy of surveillance or control devices (Cribellier *et al.*, 2018, 2020). Tools with different modes of action need to elicit specific responses in mosquitoes. For example, sticky traps or insecticide-treated surfaces require mosquitoes to land, while suction traps only require mosquitoes to closely approach the inlet airflow. The results described above suggest that suction traps which incorporate specific visual characteristics in their design may significantly reduce the final distance between flying mosquitoes and the trap, thereby potentially increasing the number of attracted mosquitoes that fly within range of the suctioning air flow and thus improving their overall capture rate. Comparably, if the trapping or killing mechanism requires physical contact (i.e. landing), then the incorporation of a thermal signature of a specific size could improve the overall capture or killing rate. Knowing the precise behavioural steps elicited by a set of cues can therefore be crucial for designing and improving control tools. Here the implementation of a 3D tracking system substantially augmented the information obtained in behavioural assays, as it provided information on the events that took place when mosquitoes approached a target. This methodology for behavioural studies is highly recommended (Cribellier *et al.*, 2020).

The effect of the thermal cue was further evaluated in Chapter 6, where different post-landing behavioural traits were examined in relation to different feeder temperatures. Results from this study indicated that depending on the magnitude of the thermal cue, mosquitoes responded with different arrays of behaviours, and the optimal "landing-probing-feeding" sequence was carried out when mosquitoes were exposed to physiologically normal host temperatures and fever

host temperatures. Altogether, results found in Chapters 4-6 suggest that the thermal cue is a crucial factor in eliciting landing, however, the response elicited depends on specific aspects of this cue, such as the area size in which it is presented and the magnitude of the stimulus. As described by Zermoglio *et al.* (2017) and Lazzari (2019), the thermal energy emanating from a host that reaches a mosquito depends on three factors: the size of the area emitting the energy, the temperature differential, and the distance between the host and the insect. *Aedes aegypti* females were reported to be capable of differentiating between two heat sources set at different temperatures if these were placed at equal distances from the mosquito, or if they were placed at different distances provided that their temperature was identical (Zermoglio *et al.*, 2017). However, they were not capable of distinguishing the size of the heat source (i.e. they did not show a preference for flying toward large or small objects kept at the same temperature and equal distances). Results from Chapter 5 suggest that the area size of the thermal cue is an important factor that determines landing in *An. coluzzii* mosquitoes. This finding opens a new set of questions with regards to heat perception in mosquitoes, as it might be that the size of the thermal source plays a role only when considering landing, whilst it does not influence the attraction response, hence the lack of preference shown for objects of different sizes reported by Zermoglio *et al.* (2017). It is important to note that the experimental design used in Chapter 5 was substantially different from that used by Zermoglio *et al.* (2017); e.g. Zermoglio *et al.* (2017) examined the attraction response in a two-choice assay using *Aedes* mosquitoes, while here the variable examined was landing and the experiment was conducted on *An. coluzzii* in a no-choice assay. Therefore, a direct comparison of the two studies would not be appropriate, and more investigation aimed to examine the capability of *Anopheles* mosquitoes to distinguish the size, the distance, and the temperature of heat sources should be carried out to further our understanding on this matter.

Another point to consider when examining results from Chapters 5 and 6 is that in Chapter 5, the thermal signature only incremented the landing response when at least half of the visual target was heated, whilst in Chapter 6 mosquitoes consistently landed on the small artificial feeder, which had a heated area ~ 30

times smaller compared to the heated area used in Chapter 5 when half of the target was heated. This could be due to the fact that in Chapter 5 the experimental set-up consisted of targets being presented in a large wind tunnel that allowed free movement and did not limit flight activity close to the targets, whilst in Chapter 6 mosquitoes were kept in a small (15 cm x 15 cm x 15 cm) cage, thus mosquitoes were more likely to encounter the surface of the feeder. Moreover, the blood in the feeder might have released additional odours, which might have increased mosquito attraction toward the small feeder. Additional studies should be carried out to elucidate the reason of these contrasting results.

It is interesting to note that while in Chapter 4 no avoidance response was detected even when mosquitoes were exposed to the target heated at 45 °C (63% of the mosquitoes landed on target at 45 °C, 66% of the mosquitoes landed on target at 35 °C), signs of avoidance behaviour were recorded in mosquitoes exposed to feeders set at 48 °C (Chapter 6). This discrepancy might be in part due to the 3 °C difference in the temperature and in part due to the fact that the two studies examined different variables. In Chapter 4 the information collected only indicated whether the mosquito landed or not whilst Chapter 6 aside from providing information on the landing rates, also considered detailed aspects of the behaviour and provided a more comprehensive overview of the overall behavioural response elicited by targets at high temperatures. In particular, results from Chapter 6 indicated that although *An. coluzzii* mosquitoes landed on the feeder set at 48 °C, they remained on the surface only for a few seconds in each visit and reduced to a minimum the portion of the body that was in direct contact with the hot surface. Thus, by using a methodology that permitted the analysis of multiple behavioural variables, more accurate information was acquired than the information that could be inferred solely from the landing rate, which suggested no avoidance behaviour even for high temperatures. It has been common practice to measure the effect of a cue by counting the number of insects that arrive at the source or that are caught in the trap (Dekker *et al.*, 2002, 2005). However, as demonstrated here, inferring a full behavioural response exclusively from results obtained from one variable could generate misleading results. Additionally, the measurement of a landing response does not

necessarily correspond to a measurement of an attraction response (Zhou *et al.*, 2018), as these two responses are not always triggered by the same set of cues. Thus, to obtain a full picture of the effect that a cue might exert on a mosquito, behavioural assays should take into account different behavioural variables, and the methodology should be accurately designed to manipulate the right set of variables and detect their specific effects.

Taken together, results from this project provide useful insights on how host-seeking *An. coluzzii* females respond to different cues during the landing and post-landing phase. From an application perspective, elements of these findings could be used to improve the efficacy or optimise the cost-efficiency of surveillance or control tools employed against this vector. For example, results from this project suggest that the use of a thermal cue in traps is highly recommended (Cribellier *et al.*, 2020, Chapter 4), and the area of the thermal cue can likely be optimised without undermining the catching rate (Chapter 5). The overall size of the target is also an element that could be optimised (Lindh *et al.*, 2009), and as demonstrated here, it would be important to consider possible sources of cue that might compete with the target, as this could alter the number of catches (Chapter 5). Furthermore, the different post-landing responses could be exploited to improve the efficacy of feed-kill traps or insecticide-treated targets, by for example, improving the amount of contact time with the toxic substance or adjusting the position of suction devices to collect mosquitoes at specific distances from attractive features (Chapter 6).

Altogether, the findings presented in this thesis enhance the current understanding of the factors that direct the complex sequence of behaviours that drive mosquitoes in the close-range, landing, and post-landing phase. This new information could be used to modify surveillance and control tools against these important vectors, thus contributing to the reduction of mosquito-spread diseases.

7.1. Limitations

The findings of this project have to be seen in light of the following limitations:

- The research for this study was conducted using mosquitoes that have been colonised and maintained in the laboratory for several years. Although maximum care was taken to maintain the conditions in the laboratory in a way that reflected natural conditions of tropical regions (e.g. environment kept at 26 ± 2 ºC and 60 ± 10% RH, with a photoperiod of 12:12 h LD cycle), it is normal to assume that colonised mosquitoes experience different conditions than those that live in the wild (Leftwich *et al.*, 2016), and therefore biological traits of mosquitoes used in the experiments differed from those of wild mosquitoes (Ng'habi *et al.*, 2015). For example, when reared in the laboratory, mosquitoes had constant access to sugar feeders, while this would not be the case in the wild. Thus, the lack of selective pressure imposed by the wild environment might have allowed for certain traits to spread among mosquitoes populations, while other traits that are advantageous in the wild might have been negatively selected in laboratory conditions (Leftwich *et al.*, 2016; Ross *et al.*, 2019). Therefore, as for all experiments conducted on living organisms, it is important to consider that some variability might be found when conducting experiments with different populations (Ross *et al.*, 2019). Care must be taken when comparing results of experiments that used different populations (i.e. laboratory-reared colonies and wild populations), or when results obtained from a specific population are used to generalise the overall characteristics of the species.

- All the research undertaken in this project was conducted under laboratory conditions, in either a wind tunnel or cages. These methods offered control over the conditions in which the experiments were carried out, but constrained mosquitoes to a limited physical area and offered a rather simplistic environment, while animals in the wild are constantly presented with a multitude of stimuli and information (Dukas, 1998). For example, mosquitoes in the wild must navigate a complex environment that presents several barriers (e.g. bushes, huts, nets). In order to reach the host and feed

on its blood, mosquitoes must bypass these barriers. Furthermore, the wild environment also offers a series of objects and animals that emanate a wide range of cues (e.g. warm rocks could emanate thermal and visual cues, animals emit carbon dioxide). As these cues are presented simultaneously, multiple sensory stimuli might compete with each other and might signal confounding information (i.e. mosquitoes might receive two cues coming from opposite directions) (Dukas, 1998). Adding to this, it has to be considered that mosquito behaviour is also regulated by internal factors (i.e. their physiological state) (Bowen, 1991; Barrozo *et al.*, 2004; Hawkes *et al.*, 2012). Altogether, these considerations indicate that in the wild, mosquito responses take into account a larger range of factors than the ones presented under laboratory conditions, and therefore their behavioural response might deviate from predictions obtained in laboratory settings.

- The low number of replicates obtained for some of the variables in Chapter 6 (i.e. only few mosquitoes imbibed blood at 48 °C) decreased the power of the analysis carried out for these variables, and interpretation of these analyses should therefore be done cautiously. Further studies, using a higher number of replicates and smaller temperature increments between treatments are recommended.

7.2. Future work

The work described in this thesis has advanced the knowledge of the scientific community with regards to the landing and post-landing behaviour of *An. coluzzii* mosquitoes, a main vector of malaria. Building from the findings obtained in this project, additional studies should be carried out to further explore mosquito behavioural and physiological responses during the last phases of host-seeking. Additional research on these topics may provide insights on components of mosquito biology that could be targeted as a way of reducing vector-host interaction and therefore, as factors that could be used in vector control methods. Possible new areas of study include:

- Exploring the effect that other host-associated physical stimuli might have in driving landing and feeding behaviour in order to achieve a fuller understanding of all the components that might play a role in directing these complex behaviours. For example, it would be interesting to quantify the effect that water vapour has in eliciting landing, given that it was previously reported that the addition of this cue incremented the distance at which warm targets were detected by mosquitoes (Van Breugel *et al.*, 2015; Zhou *et al.*, 2018), and several studies reported an augmented landing response when water vapour was added in the equation (Wright and Kellogg, 1962; Khan and Maibach, 1966; Eiras and Jepson, 1994). However, a recent study indicated that water vapour did not influence the landing response in wild mosquitoes (Zhou *et al.*, 2018). Thus, much remains unclear on the effect that this cue might have on eliciting landing. Results from this thesis demonstrate that even when host odour was presented together with visual and thermal cues, approx. 33% of the mosquitoes did not land on a target. Thus, it would be interesting to determine if the addition of other cues reduces the proportion of non-responding mosquitoes, or if independently of the cues presented, the percentage of non-responding mosquitoes remains unaffected. This could direct future effort in determining what cues should be included in trap devices and may indicate sensory neurology relationships between water vapour and other stimuli.

- Similarly to the point above, it would also be of interest exploring the effect that other host-associated physical stimuli might have in driving the post-landing behaviour. This should not be restricted solely to the post-landing phase of host-seeking mosquitoes, but should also examine post-landing behaviour in resting or sugar-seeking mosquitoes, as this could provide useful information that could be used to improve feed-kill traps (Traore *et al.*, 2020; Stromsky, V. E., Hajkazemian *et al.*, 2021) or resting-traps (Panella *et al.*, 2011; Brown *et al.*, 2018). The addition of chemo stimuli on the target could augment the time that mosquitoes spend

alighted on the surface, or the acceptability of the meal, both factors that could help improve the delivery of insecticides, toxins, or biocontrol agents.

- Further exploring the effect that variations of physical characteristics of a target may have on mosquito catches (e.g. exploring the landing response on horizontal targets set at different heights, or the effect of targets with different shapes). This field might provide information on further modifications that could be implemented in traps for their optimisation. As demonstrated by the success story of controlling tsetse flies using improved baits (Lindh *et al.*, 2009; Torr and Vale, 2015), by methodically examining the effect of different target components, it is possible to drastically optimise their effectiveness and reduce their cost.

- Reproducing the landing behavioural studies under semi-field and field conditions to validate whether the results obtained in the laboratory studies presented here remain unvaried or if the intricacy of a natural environment leads to a deviation from the results of this thesis. Results of field studies might also provide more realistic insights on target effectiveness when presented with several different sources of competition (i.e. the effectiveness of traps when presented in an environment where hosts might be present). Given that the majority of the behavioural experiments conducted in the past tested the responses of lab-reared mosquitoes in experiments conducted in enclosed tunnels, there is a call for better understanding the behaviours of wild mosquitoes when exposed to complex natural environments (Zhou *et al.*, 2018).

- Determining whether the body temperature of *Anopheles* mosquitoes feeding on hot blood differs from the body temperature of mosquitoes feeding on blood kept at physiological host temperature. If no difference is found in the body temperature irrespectively of the temperature of the blood at which mosquitoes are feeding on, it would be interesting to assess what mechanisms contribute to the maintenance of the stable temperatures (i.e. if evaporative cooling is sufficient to control temperature

changes or if other methods come into action). On the other hand, if the body temperature of mosquitoes feeding on hot blood results higher compared to those feeding on host temperature blood, then research should focus on determining what mechanisms are used to overcome the possible thermal stress associated with hot temperatures (Benoit *et al.*, 2019).

- Determining whether mosquitoes exposed to blood at different temperatures express different chemosensory genes. This could help elucidating the role of specific genes in determining avoidance or landing behaviour (Greppi *et al.*, 2020). Similarly, the expression of thermoregulatory genes should also be studied to better understand the physiological mechanisms involved in thermoregulation of mosquitoes exposed to blood at different temperatures.

7.3. Conclusions

This project aimed to evaluate the effect that different cues had on the behaviour of *An. coluzzii* mosquitoes during the landing and post-landing phase of host-seeking females. The research presented here quantified for the first time the effect that three host-associated cues (host odour, thermal, and visual) had in eliciting a landing response (Objective 1, Chapter 4), and furthermore, demonstrated that when presented all together, these host-associated cues interact synergically to increase the number of mosquitoes landing on the target. A synergic interaction was also seen between host odour and the thermal cue (Objective 2, Chapter 4). Building from these findings, it was further explored the effect that physical characteristics of a heated target (presented with host odour) had on the landing response of host-seeking mosquitoes (Objective 3, Chapter 5). It was found that the size of the target and the size of the heated area of the target played a major role in driving landing, whilst no effect was found for either trap orientation. Specifically, more mosquitoes landed on large targets compared to those that landed on smaller targets, but small targets were more efficient in terms of number of mosquitoes caught per cm^2. Moreover, more mosquitoes landed on the target when at least half of the area was heated. Using a 3D

tracking system it was possible to further extract information on the flight parameters of mosquitoes approaching the targets with different characteristics; this complemented the interpretation of landing catches obtained on the different targets (Objective 4, Chapter 5). For example, it showed that when both target sizes were presented simultaneously, mosquitoes not only landed more on large targets, but they also visited them more times compared with the number of visits recorded for small targets. Finally, the landing and post-landing behaviours in relation to blood offered in artificial feeders kept at different temperatures were examined (Objective 5, Chapter 6). This was accompanied by a physiological evaluation of the mosquitoes' status after exposure to blood. Here it was found that mosquitoes fed with equal propensity and equal success on host temperature blood (36 °C) and fever temperature blood (42 °C), whilst blood at low temperature (30 °C) only elicited a minor feeding response. Surprisingly, mosquitoes abundantly displayed foraging behaviour even when presented with hot blood (48 °C). Altogether, the findings presented in this thesis enhance the current understanding of the factors that direct the complex sequence of behaviours that drive mosquitoes in the landing and post-landing phases. This new information could be used to modify surveillance and control tools against these important vectors, thus contributing to the reduction of mosquito-spread diseases.

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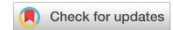
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Multimodal synergisms in host stimuli drive landing response in malaria mosquitoes

Manuela Carnaghi, Steven R. Belmain, Richard J. Hopkins & Frances M. Hawkes

Anopheles mosquitoes transmit malaria, which affects one-fifth of the world population. A comprehensive understanding of mosquito behaviour is essential for the development of novel tools for vector control and surveillance. Despite abundant research on mosquito behaviour, little is known on the stimuli that drive malaria vectors during the landing phase of host-seeking. Using behavioural assays with a multimodal step approach we quantified both the individual and the combined effect of three host-associated stimuli in eliciting landing in *Anopheles coluzzii* females. We demonstrated that visual, olfactory and thermal sensory stimuli interact synergistically to increase the landing response. Furthermore, if considering only the final outcome (i.e. landing response), our insect model can bypass the absence of either a thermal or a visual stimulus, provided that at least one of these is presented simultaneously with the olfactory stimuli, suggesting that landing is the result of a flexible but accurate stimuli integration. These results have important implications for the development of mosquito control and surveillance tools.

Mosquitoes can transmit a range of pathogens that collectively cause the death of over 700,000 people each year[1]. Of these, malaria, which is transmitted by mosquitoes of the *Anopheles* genus, causes the highest annual mortality[1]. Insecticidal bed nets and indoor spraying of insecticides have significantly reduced malaria in endemic regions by targeting the mosquito vector; these two interventions account for nearly 80% of the estimated 663 million cases averted between 2000 and 2015[2]. However, there has been a worrying increase in the strength and distribution of physiological and behavioural resistance to insecticides within vector populations, compromising the long-term utility of these control measures[3–5]. Thus, there is an urgent need for new vector control and surveillance tools, the most effective of which must be designed taking into account the behaviour and ecology of the vector[6,7].

Although vector behaviour has been intensively studied, research has often focussed on responses to host odour[8,9], which is only one of the many host cues that shape host-seeking behaviour[10]. Initial activation and long-range orientation (over many metres) is mediated by volatile odours and carbon dioxide[10], and the detection and behavioural responses to these stimuli are well-characterised in several disease vector species[8,11–13]. Within the range of visual resolution, which depends on the species and the size of the object, but can be up to 5 m away, visual stimuli are also attractive[14]. In addition, visual feedback is crucial for mosquito orientation and upwind flight as they use the optomotor anemotaxis mechanism to adjust flight manoeuvres and their orientation[14]. When in a plume of host odour, *Anopheles coluzzii* rapidly decrease their mean ground speed and closely approach visually conspicuous targets to within a few centimetres, but do not make contact[15]. At the very close range, thermal and humidity gradients detected by thermoreceptors on the mosquito antennae[16,17], proboscis[18], and maxillary palps[19], and hygroreceptors on the antennae[20,21], influence the final stages of close-range orientation and landing[9,10,22,23]. Recent studies have characterised the function of heat receptors in both *Aedes aegypti* and *An. gambiae* sensu stricto mosquitoes, elucidating their function in heat avoidance and heat-seeking, respectively[24,25]. However, the response to thermal stimuli varies when there is simultaneous stimulation of olfactory receptors. In the presence of human odour, *An. gambiae s.s.* mosquitoes fly faster, with more convoluted flight trajectories and land significantly more often on a surface if it is heated to 34 °C, representative of human skin temperature[26].

Landing, the final stage of host-seeking behaviour, is the crucial moment of vector-host contact. However, research to systematically unravel the effect of different sensory modalities on this behaviour has been relatively neglected[9,27,28]. Published studies present only the response to one or two stimuli at a time[15,26,29,30], or focus on diurnal vectors of the genus *Aedes*, which display some different behaviours to the nocturnal *Anopheles*[31,32]. Thus, in this study we set out to determine the relative importance of thermal, visual and odour cues in mediating

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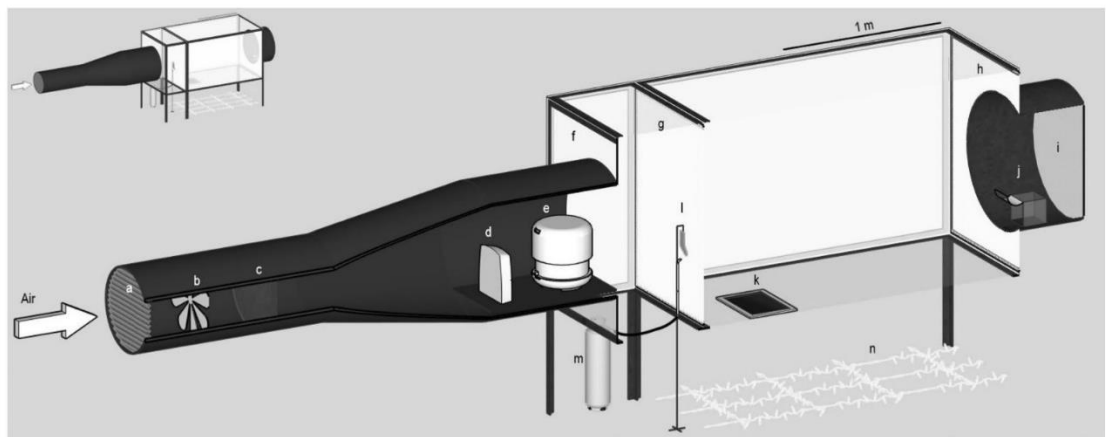
Figure 1. Wind tunnel and flight arena schematic. (a) shutter, (b) impelling fan, (c) charcoal filters, (d) fan heater, (e) atomising humidifier, (f) brushed cotton screen, (g) upwind white net screen, (h) downwind white net screen, (i) terminal downwind netting, (j) release cage, (k) landing target, (l) odour delivery platform, with worn sock and carbon dioxide release tube, (m) carbon dioxide tank, (n) lighting array.

landing behaviour of the primary vector of malaria in sub-Saharan Africa, *Anopheles coluzzii*. We used a multi-modal step approach, where stimuli were presented first alone, then in combination to facilitate a quantification of the effect of each stimulus and how the behavioural effect changed as a result of multimodal integration. We report here the strong synergistic effects between thermal and odour cues, and among all three host-associated cues, which amplified the landing response, and the crucial role of host odour in enabling landing behaviour. Understanding mosquito responses to specific host cues provides insights into behaviours that could be exploited to improve current surveillance and control tools, or provide the basis for new behaviourally-based interventions.

Results

The study was carried out in a large wind tunnel (Fig. 1)[15], where mosquitoes were released at the downwind end and a target was presented at the upwind end. Twelve different treatments were tested, representing all possible combinations of the following factors: visual variables (the target was either transparent or visually conspicuous), odour variables (presence or absence of volatile odours from a human foot plus constant carbon dioxide) and thermal variables (the target was set at either 25 °C, 35 °C, or 45 °C). The number of mosquitoes recovered from the surface of the target and in different parts of the wind tunnel flight arena were counted. A total of 124 assays were conducted, a minimum of ten replicates were carried out for each treatment, releasing a mean number of 25 ± 1 (SEM) mosquitoes per replicate, thus using a total of 3074 mosquitoes.

The presence or absence of the odour cue had no significant effect on the activation rate (number of mosquitoes which left the release cage during an assay) ($\chi^2 = 1.00$, d.f. = 1, $P = 0.53$). Similarly, no effect was found on the activation rate independently of the visibility of the landing target ($\chi^2 = 1.46$, d.f. = 1, $P = 0.45$) or the temperature of the target ($\chi^2 = 0.93$, d.f. = 2, $P = 0.83$). Thus, none of the host-associated stimuli presented had a significant effect on the activation behaviour of *An. coluzzii* (Fig. 2A).

Landing overview. Overall, mosquitoes were equally recovered in all quadrants of the landing target and no significant difference was found between landings on the downwind quadrants compared to the upwind quadrants ($\chi^2 = 0.86$, $P = 0.64$). This allowed verification that the flight arena conditions were symmetrical.

When considered as independent factors, all three variables tested had a significant effect on driving the landing response of *An. coluzzii* (analysis of deviance, for temperature: $\chi^2 = 40.92$, d.f. = 2, $P < 0.001$, for visibility: $\chi^2 = 26.25$, d.f. = 1, $P < 0.001$, for host odour: $\chi^2 = 503.97$, d.f. = 1, $P < 0.001$). Presence or absence of host odour had the strongest effect on landing behaviour which was enhanced by the addition of visual and thermal cues. Overall, the highest number of catches was obtained in assays where all the three cues were presented together (Fig. 2B).

It is interesting to note that independent of other variables, no difference was ever observed when comparing the number of landings between corresponding treatments where the surfaces were at 35 °C and 45 °C. This indicated that as long as the surface was warmer than the environment (at 25 °C), it elicited a similar landing response, and even the highest temperature did not produce a behavioural cut-off in the landing response.

To establish the role of each host-associated cue and its relative importance in driving landing behaviour, we undertook a series of comparisons between each permutation of possible treatment combinations (Table 1).

Significantly more mosquitoes landed on the target when host odour was the only cue presented compared with the number of landings when the cue was missing. On the other hand, the visibility of the target, when presented alone, did not significantly affect the number landings on the target. Although a small number of individuals landed on the black target, this was not significantly different from the mean number of mosquitoes that landed on a transparent target. Similarly, when the temperature of the target was the only cue offered, it did not
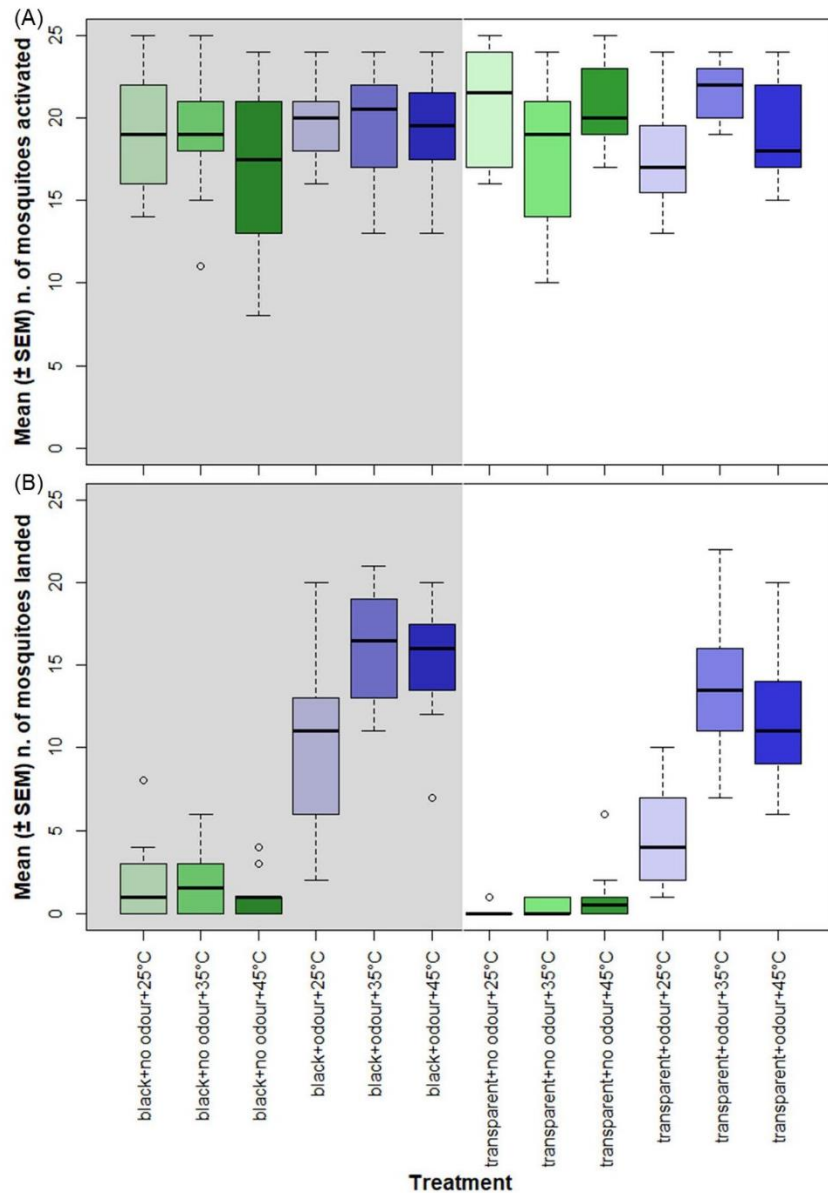
Figure 2. Mean number (±SEM) of mosquitoes activated (**A**) and mean number (±SEM) of mosquitoes found on the landing surface (**B**) for each treatment. Bold black bars indicate the median; upper and lower limits of a box indicate the interquartile range; whiskers indicate the maximum and minimum data points, excluding outliers, which are indicated by the circles. The y-axis shows the twelve different treatments, where "black" and "transparent" indicate the different visual cues offered, "no odour" and "odour" indicate the absence or presence of host odour, and the temperature indicates the thermal cue used. Blue bars indicate host odour was present during assays, green bars indicate it was absent. Paler colours denote lower temperatures and darker colours indicate higher temperatures. Background colour indicates the presence (grey) or absence (white) of the visual cue.

influence landing behaviour relative to a transparent target at 25 °C. Collectively, these results show that when no other stimuli are present, neither a visual nor a thermal stimulus alone are sufficient to induce landing (Fig. 3).

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Factor compared	Treatment 1	Mean (± SEM)	Treatment 2	Mean (± SEM)	z-value	P value
Odour cue	**O** + T + 25 °C	4.8 (±0.88)	**N** + T + 25 °C	0.2 (±0.13)	4.37	P < 0.001***
Visual cue	N + **B** + 25 °C	2.0 (±0.82)	N + **T** + 25 °C	0.2 (±0.13)	−3.08	P = 0.07
Thermal cue	N + T + **25 °C**	0.2 (±0.13)	N + T + **35 °C**	0.4 (±0.16)	0.80	P = 1
Thermal cue	N + T + **25 °C**	0.2 (±0.13)	N + T + **45 °C**	1.1 (±0.59)	2.20	P = 0.49
Thermal cue	N + T + **35 °C**	0.4 (±0.16)	N + T + **45 °C**	1.1 (±0.59)	1.71	P = 0.83
Odour cue in presence of thermal cue	**O** + T + 35 °C	13.3 (±1.41)	**N** + T + 35 °C	0.4 (±0.16)	6.79	P < 0.001***
Odour cue in presence of thermal cue	**O** + T + 45 °C	11.8 (±1.24)	**N** + T + 45 °C	1.1 (±0.59)	7.21	P < 0.001***
Odour cue in presence of visual cue	**O** + B + 25 °C	10.4 (±1.77)	**N** + B + 25 °C	2.0 (±0.82)	6.30	P < 0.001***
Odour cue in presence of visual and thermal cue	**O** + B + 35 °C	15.9 (±1.05)	**N** + B + 35 °C	1.7 (±0.62)	8.21	P < 0.001***
Odour cue in presence of visual and thermal cue	**O** + B + 45 °C	15.2 (±1.00)	**N** + B + 45 °C	1.2 (±0.42)	8.14	P < 0.001***
Visual cue in presence of odour cue	O + **B** + 25 °C	10.4 (±1.77)	O + **T** + 25 °C	4.8 (±0.88)	−4.17	P < 0.001***
Visual cue in presence of thermal cue	N + **B** + 35 °C	1.7 (±0.62)	N + **T** + 35 °C	0.4 (±0.16)	−2.57	P = 0.25
Visual cue in presence of thermal cue	N + **B** + 45 °C	1.2 (±0.42)	N + **T** + 45 °C	1.1 (±0.59)	−0.20	P = 1
Visual cue in presence of thermal cue and odour cue	O + **B** + 35 °C	15.9 (±1.05)	O + **T** + 35 °C	13.3 (±1.41)	−1.18	P = 0.99
Visual cue in presence of thermal cue and odour cue	O + **B** + 45 °C	15.2 (±1.00)	O + **T** + 45 °C	11.8 (±1.24)	−1.69	P = 0.84
Thermal cue in presence of visual cue	N + B + **25 °C**	2.0 (±0.82)	N + B + **35 °C**	1.7 (±0.62)	−0.47	P = 1
Thermal cue in presence of visual cue	N + B + **25 °C**	2.0 (±0.82)	N + B + **45 °C**	1.2 (±0.42)	−1.35	P = 0.96
Thermal cue in presence of visual cue	N + B + **35 °C**	1.7 (±0.62)	N + B + **45 °C**	1.2 (±0.42)	−0.90	P = 1
Thermal cue in presence of odour cue	O + T + **25 °C**	4.8 (±0.88)	O + T + **35 °C**	13.3 (±1.41)	5.64	P = 0.001***
Thermal cue in presence of odour cue	O + T + **25 °C**	4.8 (±0.88)	O + T + **45 °C**	11.8 (±1.24)	4.92	P = 0.001***
Thermal cue in presence of odour cue	O + T + **35 °C**	13.3 (±1.41)	O + T + **45 °C**	11.8 (±1.24)	−0.76	P = 1
Thermal cue in presence of visual cue and odour cue	O + B + **25 °C**	10.4 (±1.77)	O + B + **35 °C**	15.9 (±1.05)	2.69	P = 0.19
Thermal cue in presence of visual cue and odour cue	O + B + **25 °C**	10.4 (±1.77)	O + B + **45 °C**	15.2 (±1.00)	2.47	P = 0.30
Thermal cue in presence of visual cue and odour cue	O + B + **35 °C**	15.9 (±1.05)	O + B + **45 °C**	15.2 (±1.00)	−0.33	P = 1

Table 1. Tukey contrast comparisons between mean landing rates for each permutation of possible treatment combinations. *O* Odour, *N* No odour, *T* transparent, *B* Black. In bold is highlighted the changing variable in each comparison. ***Denotes significant difference between the treatments.

Interactions between host odour and other host-associated cues. The addition of host odour to either the thermal cue or to the visual cue had a significant effect on the landing behaviour, as it greatly increased the number of mosquitoes recovered on the target in the respective treatments. Furthermore, significantly more mosquitoes landed on the target when all three cues were presented together when compared to treatments where the odour cue was absent, or when compared to the treatment where only the odour cue was presented. However, the number of mosquitoes caught on the surface when all three cues were offered was not significantly higher than the number caught when either the visual or thermal cue were missing. Thus, odour always plays a major role in triggering the behaviours that take mosquitoes to land and its absence results in low landing proportions. Visual and thermal cues increase landings if introduced as the only other cue presented along with host odour, while their role becomes secondary when host odour and either one of the visual or thermal cue are already present, increasing the landing behaviour in a minor manner.

Interaction between visual cue and other host-associated cues. More mosquitoes landed on black target surfaces in the presence of host odour compared to the number that landed on transparent targets in the presence of host odour. However, no effect was recorded on the landing behaviour when, in the absence of host odour, the mosquitoes were presented with a black surface at 35 °C or 45 °C compared with landings respectively on a transparent surface at 35 °C or 45 °C. This indicates that, in absence of host odour, the visibility of a heated target surface does not influence the landing behaviour. Equally, in the presence of host odour, no significant difference was found in the number of landings between treatments where the visually conspicuous target surface was set at 35 °C or 45 °C when compared with a treatment which was identical apart from having a transparent surface. These results indicate that the visibility of the target did not increase the landing behaviour if the other two factors, i.e. host odour and thermal cue, were present. Thus, mosquitoes can bypass the absence of the visual cue if sufficient non-visual host-associated stimuli are present.

Interaction between thermal cue and other host-associated cues. In treatments where host odour was absent and the target surface was visually conspicuous, the temperature of the surface did not influence the landing behaviour. Specifically, no significant effect was found when comparing the number of mosquitoes

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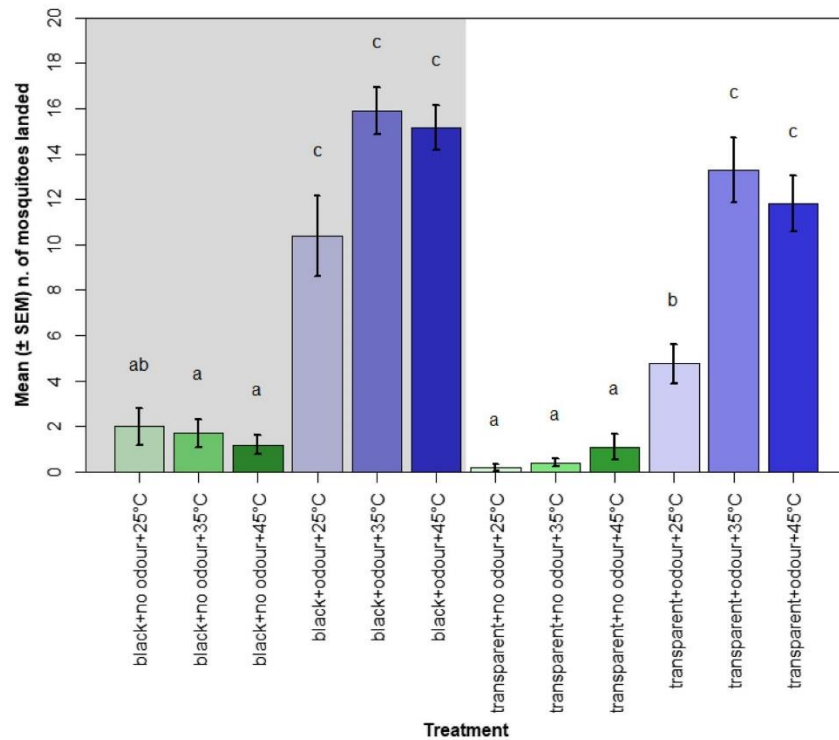
Figure 3. Mean number of mosquitoes (±SEM) found on the landing surface in each treatment. The y-axis shows the twelve different treatments, where "black" and "transparent" indicate the different visual cues offered, "no odour" and "odour" indicate the absence or presence of host odour, and the temperature indicates the thermal cue used. Blue bars indicate host odour was present during assays, green bars indicate it was absent. Paler colours denote lower temperatures and darker colours indicate higher temperatures. Background colour indicates the presence (grey) or absence (white) of the visual cue. Different letters denote significant differences between treatments (Tukey contrast comparisons test, $P < 0.01$).

caught on a black surface at 25 °C with the number caught on the same surface at 35 °C and 45 °C. On the other hand, when the thermal cue was provided simultaneously with host odour, a substantial effect on the landing response was recorded. A higher number of mosquitoes were caught in the treatment where the transparent target was set at 35 °C and 45 °C and was presented with host odour when compared to the same target at 25 °C. Furthermore, the number of landings were not different in treatments where the black surface was set at either 35 °C or 45 °C and was presented with host odour compared to the respective treatment where the temperature was 25 °C. These results indicate that a thermal cue does not increase landing if other host-associated stimuli, i.e. host odour and visual cue, are present. Thus, mosquitoes can compensate for the absence of the thermal cue if they receive other sufficient information to locate the host.

Quantification of the role of each stimulus on triggering landing behaviour and synergistic effect of combined stimuli. As human body temperature was the most behaviourally relevant thermal cue, and no significant difference was found between the number of mosquitoes recovered on target surfaces set at 35 °C and at 45 °C, the latter temperature was removed for this analysis. We calculated what proportion of landing response was attributable to each stimulus in isolation. The thermal stimulus alone, i.e. surface at 35 °C, elicited 1.57% of mosquitoes to land on the target, the visually conspicuous target 7.98%, and host odour 19.14%, while when all three stimuli where presented simultaneously, 66.47% of the mosquitoes landed on the target. Host odour and the visual cue when presented simultaneously, elicited 41.60% of the mosquitoes to land, while 52.77% landed when they were presented with host odour and the thermal cue. Ultimately, in absence of host odour, when the visual and thermal cue were combined, only 6.8% of the mosquitoes landed on the target surface.

To understand the role of the different host-associated stimuli when presented in combination with other stimuli, we compared the number of mosquitoes that landed when exposed to different treatments with the number of mosquitoes expected to land if the effect of each stimulus in that combination would act in an additive manner (Fig. 4).

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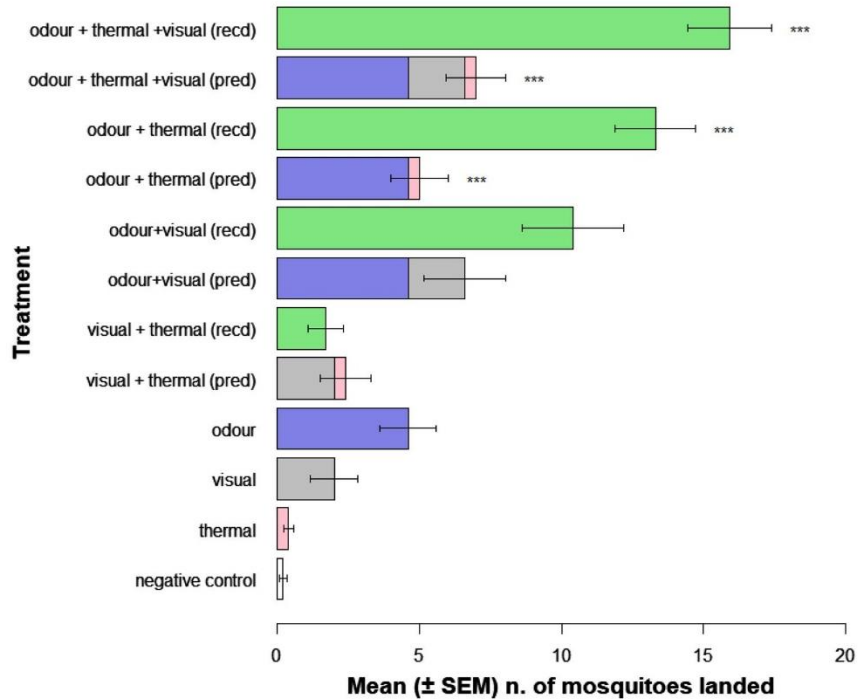
Figure 4. Predicted and recorded effects of host-associated stimuli on the mean landing response (±SEM) of host-seeking An. coluzzii, when stimuli were presented alone and in combination. Negative control is set based on the number of mosquitoes landing on a target when no thermal, visual or odour stimuli are presented. Recd, recorded mean from experiments; pred, additive predicted mean derived from simulated data set produced using recorded baseline values. ***Denotes significant difference at $P < 0.001$ between the recorded effect and the predicted effect for each respective treatment.

The most notable finding of our study is indicated in Fig. 4, where it can be seen that the recorded effect of the host-associated cues presented together exceeded considerably the effect that would have resulted by a simple sum of the singular effects of each stimulus. The recorded number of mosquitoes that landed when the three host-associated cues were presented simultaneously (Mean ± SEM = 15.9 ± 1.05) was significantly higher when compared to the number of mosquitoes that were expected to land if the effect was given by the addition of singular effects of each stimuli (Mean ± SEM = 7.0 ± 1.48) ($\chi^2 = 24.15$, d.f. = 1, $P < 0.001$). Similarly, the odour cue acted synergistically with the thermal cue in eliciting landing behaviour, as the recorded number (Mean ± SEM = 13.3 ± 1.41) significantly exceed the expected number given by the sum of the effect of the odour cue and the effect of the thermal cue (Mean ± SEM = 5.0 ± 1.01) ($\chi^2 = 26.19$, d.f. = 1, $P < 0.001$). However, we found no synergism between odour and visual cues (Mean ± SEM recorded landings = 10.4 ± 1.77, expected = 6.6 ± 1.43, $\chi^2 = 2.84$, d.f. = 1, $P = 0.09$) or thermal and visual cues (Mean ± SEM recorded landings recorded = 1.7 ± 0.62, expected = 2.4 ± 0.90, $\chi^2 = 0.42$, d.f. = 1, $P = 0.52$).

Discussion

The findings reported here demonstrate for the first time a synergistic effect between odour, visual and thermal cues in eliciting landing behaviour in host-seeking *An. coluzzii* females. When all the host-associated cues were provided, the total number of mosquitoes landing on the surface was more than twice the number that would have been theoretically expected to land if the response was merely additive, based on the landing response attributable to each cue individually. A synergistic effect was also recorded for the odour and thermal cue, which indicates the ability of mosquitoes to integrate different types of sensory information ahead of a behavioural response. There are many advantages in using different cues that signal the same resource to produce a behavioural response. Sensitivity to a stimulus may be increased by integrating information derived from other cues, thus enhancing the response to the first cue[33,34]. Relying on several cues may also allow the insect a parsimonious use of sensory organs[33], acquiring the most accurate information in the least costly way[35]. In environmental conditions where one cue may not be detectable, the presence of another cue, carried on a parallel sensory system, might still provide sufficient information to allow successful resource location. For instance, mutant mosquitoes that had a single sensory modality disrupted were still able to detect a host or target[25,29].

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Many host-seeking insect species rely on the integration of information which derives from different stimuli to locate their host. For example, some parasitic wasps use vibrational and visual cues when host-seeking[36]. Bark beetles flexibly integrate multimodal sensory information at a close-range (≤ 2 m) to regulate their landing behaviour[37]. Triatomines also integrate multimodal signals, where different contexts are defined by a specific combination of cues[33]. The observed behavioural integration described in our study suggests that host-seeking *An. coluzzii* adopt a similar resource-searching strategy. Recent studies suggest that for host-seeking mosquitoes, sensory integration occurs centrally, i.e. in the mosquitoes' brain, rather than peripherally[29]. This has been corroborated for the visual-olfaction interaction using calcium tracing imaging[38]. It remains to be seen if a similar mechanism is used for the heat-olfaction interaction.

Our experiments revealed a non-synergistic effect between odour and visual cues. Visual cues are thought to evoke a range of different behavioural responses in mosquitoes. The optomotor mechanism, which was first reported in diurnal/crepuscular mosquitoes[39] and later in nocturnal mosquitoes[40] enables mosquitoes to spatially orient themselves relative to visual cues in the environment. However, visual cues may also be associated with potential hosts, and so can be attractive depending on the insect's physiological condition, the presence of other host-associated stimuli and the precise nature of the visual cue. In the present study the target was considerably larger when compared to the black tiles that were provided on the wind tunnel floor to facilitate optomotor anemotaxis, and additionally, the target could also present other host cues. Thus, we expect that in accordance to findings of a previour study that used a similar experimental set up[15], the black tiles were not attractive to mosquitoes. When flying close to an object, visual cues may elicit an avoidance mechanism, i.e. flying either to the sides or upwards to avoid the object that is perceived as a barrier[14,15,41,42]. On the other hand, when additional host cues are presented, the visual avoidance mechanism is suppressed and visual cues act as attractants. This was corroborated in two laboratory studies[15,30]. It is worth noting that in these two studies, mosquitoes exposed to host odour were recorded hovering above a visually conspicuous surface without alighting on it, thus suggesting that other cues were missing. In a female mosquito located within centimetres of a visually conspicuous body, the frontal and lower field of view would be completely occupied by the view of the body. Thus, this stimulus would likely not provide any significant information that suggests that the body is in fact a host, and so would be unlikely to trigger the landing behaviour. Furthermore, nocturnal mosquitoes that feed inside human dwellings are expected to operate under very limiting light conditions, thus it is no surprise that the landing response is not governed by visual cues alone. We propose that in the context of host-seeking, visual cues serve as attractants and their role is important in the medium to close-range phase of location, while its effect in triggering landing behaviour is likely to be negligible, due to diminished resolution at very close range. A study conducted on *Ae. aegypti* also suggested that visibility is a characteristic that increments the facility by which the target is found by odour-induced searching mosquitoes, however, the "permission to land" is elicited by other stimuli, such as warm, wet convection currents[43]. Interestingly, a recent study reported how mosquitoes might use self-induced airflow patterns to avoid collision with surfaces[44], suggesting that other cues (such as airflow) might provide directional information during the landing phase. Further studies to explore this are recommended.

We found that the addition of the odour cue to a visually conspicuous target at host temperature produced a tenfold increase in landing if compared to the treatment without the odour. Previous research showed that *An. gambiae* females landed up to 25 times more on a surface heated to 34 °C when carbon dioxide pulses were introduced in the arena, while in the absence of the odour stimulus the warm surface was ignored[26,45,46], suggesting that odour may gate the landing response elicited by thermal cues[29,47]. Furthermore, visual attraction in host-seeking *Ae. aegypti* proved to also be odour-gated[30], wherein carbon dioxide modulates the response of the lobula neuropil, a region dense of synaptic connections in the mosquito's optic lobe, to discrete visual stimuli[38]. Our results support the view by which host odour is crucial in facilitating host-seeking and landing behaviour, as mosquitoes were more prone to land on a visually conspicuous and warm object when exposed to host odour. However, even in the absence of the odour cue, mosquitoes were recovered from the landing target, indicating that despite the absence of an olfactory cue, a small proportion of the tested mosquitoes responded to only thermal and visual cues. This is supported by another study with *Ae. aegypti*, which proposed that different cues might be able to interact thereby increasing a behavioural response, while remaining independent from each other[30]. Under this view, *An. coluzzii* mosquitoes might still approach and land on a warm object, even in the absence of host odour. Nonetheless, as thermal cues are hypothesised to only be detectable within a close range from the host[10,25,26,29], and as heat alone might not effectively signal to mosquitoes the presence of a host, where an inanimate object heated by the sun may reach host-like temperatures, it might be more advantageous for mosquitoes to only orient towards and land on warm objects that also present other host-associated cues. It has to be taken into account that objects with different visual characteristics (i.e. transparent versus black) might also have different thermal transfer properties. To limit this issue in this study, the black panels that provided the visual cue where positioned underneath the target where they were not heated, as the target only emitted heat from its upper surface, thus convection currents produced by the upper heated surface of the target were unlikely to be altered.

Although fewer mosquitoes were recovered from the target surface at 45 °C, no difference was found with the number recovered on the surface at 35 °C, suggesting that mosquitoes did not differentiate between temperatures. This disagrees with a previous study on *An. gambiae*, where mosquitoes significantly preferred landing on targets at host temperature (34 ± 2 °C) compared with targets at lower (27 °C) and higher (41 °C) temperatures[48]. Two other studies which were conducted in still air using *Ae. aegypti* recorded a heat avoidance behaviour for high temperatures (40 °C or above), while both studies recorded a preference (intended as either directional choice or time spent closer to the thermal cue) for temperatures closer to human body temperature[9,24]. As the study presented here was conducted in a wind tunnel with flowing air, it is possible that the moving air might have contributed to alter the thermal cue given by the heated target, not by altering the surface's temperature as this was maintained stable throughout the assay, but by cooling the temperature of the air surrounding the target.

This might in part explain the differences between our results and previous studies. On the other hand, our results are in accordance with behaviour recorded on other blood-sucking insects (e.g. triatomines), that exhibit host-seeking behaviours in response to objects at temperatures from 2 °C above ambient temperature and up to 47°C[33]. Further studies, which should be carried out taking into account air movement, are needed to define the upper and lower limit of temperatures that elicit a landing behaviour in this mosquito species.

In summary, the strong synergistic effect between odour, visual and thermal cues indicate the robust interaction of these factors, which incremented the landing response. This has important implications for practical vector control interventions. Understanding the role of different cues used in triggering attraction and landing may be essential for the development or improvement of tools for delivering insecticides on contact, as well as traps for surveillance and control[49]. By unravelling tsetse response to different cues and their specific characteristics, researchers were able to identy key components that were then incorporated into an improved vector control approach[6]. The results described here bring new information that could be used to modify surveillance and control tools against mosquitoes, particularly where landing or contact is required. As demonstrated in this study, the addition of a visual, but more importantly, of a thermal cue in an odour releasing trap could greatly increase the number of mosquitoes caught, improving the efficiency of control methods[42,50,51].

Taken together, the results reported here support the view that considers landing behaviour as a result of a complex series of stimuli integration, where the information deriving from different stimuli is integrated to permit a flexible, yet highly accurate and context-relevant behaviour.

Methods

Mosquitoes. A colony of *An. coluzzii* was established at NRI's laboratory in 2017 using eggs derived from a colony at Institut de Recherche en Sciences de la Santé, Burkina Faso, which originated from wild gravid females collected in Vallée du Kou, Burkina Faso, 11°23′14″N, 4°24′42″W. Mosquitoes were identified to species level by PCR. Rearing of the colonies was carried out following established protocols[15].

Wind tunnel. The experiment was carried out in a wind tunnel (1.2 m tall × 1.2 m wide × 2 m long)[15] (Fig. 1) which allowed mosquitoes to execute flight manoeuvres and reduced the constraining effects caused by the walls of small flight arenas used in other studies[26,52]. The arena was kept at 25 ± 2 °C and 65 ± 5% RH, and the air was drawn at the upwind end by an impelling fan from outside the building, to avoid using room air which accumulates human emanations[53]. Before entering the flight arena, the air was purified by passage through activated charcoal filters, heated, humidified and pushed through a screen of brushed cotton to create a laminar airflow. The air was pulled out of the laboratory room by an extractor fan at the downwind end. Thus a constant airflow of approximately 0.2 m s⁻¹ was maintained in the flight arena[15,26,28,54]. During the experiments, the only source of illumination consisted of a series of warm white LEDs which were placed in an even array on the laboratory floor, below the floor of the flight arena, and provided a homogenous light level of 0.001 W m⁻², similar to full moonlight illumination[55]. The wind tunnel wall and floor panels consisted of opal Perspex, which diffused the incoming light. To permit mosquitoes to orient themselves and navigate using the optomotor mechanism, nine small visually conspicuous squares (10 cm per side) and two large squares (20 cm per side) were placed randomly on the floor of the wind tunnel. Mosquitoes were released at the downwind end of the wind tunnel, from a release cage (15 × 15 × 15 cm) positioned at the centre of the crosswind axis (60 cm from the walls) and approximately 35 cm above the wind tunnel floor, and therefore centred with the odour stimulus releasing point.

Landing target. To test landing responses which could incorporate different combinations of thermal and visual stimuli, a landing target was designed based on a commercially available transparent heated glass unit (E-GLAS sample—Saint Gobain, UK). The unit (3 × 40 x 30 cm) consisted of two sheets of glass and a layer of electrically conductive material in the middle, which allowed the glass surface to be warmed to up to 50 °C via mains power supply. During experiments, the surface temperature of the landing target was controlled using a thermostat (ReptiZoo, China) which maintained the desired temperature (± 1 °C). To achieve a highly accurate temperature control, a layer of heat sink paste (RS Components, UK) was interposed between the thermostat probe and the glass surface. At the start and end of each assay, the temperature of the surface was thoroughly scanned with an infra-red laser gun thermometer to ensure that the temperature was within ± 2 °C from the desired experimental temperature. Visual properties of the target could also be controlled; the glass could be either left transparent to provide a very low contrast stimulus or made highly visually conspicuous with the addition of opaque black plastic secured underneath the glass.

To capture mosquitoes landing on the target, the surface was covered by a layer of thin (< 1 mm) adhesive film (FICSFIL Barrettine, UK), which consists of a transparent plastic sheet coated with a transparent layer of strong glue. The landing target was positioned horizontally (flat) on the floor of the wind tunnel, at the centre of the crosswind axis (40 cm from the lateral walls) and 30 cm from the upwind end of the flight arena.

Odour treatment. Human host odour, comprising a combination of human skin odour and carbon dioxide, was used as the olfactory stimuli. Human foot odour was sourced from 15 denier sheer knee-high 100% polyamide-nylon socks worn for 24 h by the experimenter (MC), who voluntarily consented to donate the worn socks. No ethical approval was required for this study as no data was collected about the experimenter. To limit changes in body odour, the socks were worn by the same volunteer[56]. One hour prior to wearing the socks, the volunteer washed her feet with water and fragrance-free soap. Throughout the duration of the experiment, the volunteer abstained from consuming food with spices and alcohol, did not use perfumes, perfumed soaps and clothes detergents, as these substances can affect the collected odour[57–59]. The worn socks were used in experiments for a maximum of one week, when not in use were kept in a sealed zip-lock bag at -20 °C to minimize

nature portfolio   8

Treatment	Visual cue	Thermal cue	Odour cue
1 (negative control)	Transparent	25 °C	Absent
2	Transparent	35 °C	Absent
3	Transparent	45 °C	Absent
4	Black	25 °C	Absent
5	Black	35 °C	Absent
6	Black	45 °C	Absent
7	Transparent	25 °C	Present
8	Transparent	35 °C	Present
9	Transparent	45 °C	Present
10	Black	25 °C	Present
11 (positive control)	Black	35 °C	Present
12	Black	45 °C	Present

Table 2. Summary of the twelve tested treatments which resulted from all possible combinations of the three studied host-associated cues.

variation of the odour components[54,60]. In addition to human body odour, carbon dioxide at approximately the concentration in human breath[56] (flow rate of 5 L min^{-1}, approx. 4.5% concentration) was also offered. The tube releasing carbon dioxide was positioned in the centre of the crosswind axis, 3 cm behind the netting and at 25 cm high above the wind tunnel floor, while the socks were presented in the same position but at 40 cm above the arena floor. Both the carbon dioxide releasing method and the wind tunnel conditions used in this study were comparable with conditions used in a previous study[15]. Translating this information into our study, it follows that the landing target was positioned in alignment with and directly below this plume, with the bottom edge of the plume modelled to touch the landing target.

Experimental procedure. A new group of non-blood fed, four to twelve days old female mosquitoes was used for each bioassay. Four to five hours before the commencement of assays, sugar feeders were removed from adult cages. The mosquitoes were collected between one and two hours before assays using a mouth-aspirator and were kept in small WHO tubes (4.5 cm diameter and 12 cm height) in darkness to allow the eye to adapt to low light levels[61,62]. The mosquitoes were transferred to the release cage and were given five minutes to habituate to the wind tunnel environment, after which the cage was gently opened to avoid disturbing the mosquitoes and operated remotely to avoid introducing human odours into the arena. In assays requiring it, the odour treatment started immediately after the release cage was opened. Thirty minutes later the bioassay was terminated, the release cage door was closed, and the number of mosquitoes recovered in different parts of the wind tunnel was counted as follows: number in the release cage, number in the wind tunnel beside the release cage and landing target, and number on the landing target. The target surface was divided into four equal quadrants and the number of mosquitoes caught on each quadrant was recorded for each replicate. The number of mosquitoes considered activated was designated as the number found in the wind tunnel plus the number found on the landing target.

Twelve different treatments were tested (Table 2), representing all possible combinations of the following factors: visual variables (either transparent or visually conspicuous), odour variables (presence or absence of host odour) and thermal variables (the target was either at ambient temperature at 25 °C, human skin temperature at 35°C[46,63], or a high temperature at 45 °C). The twelve treatments were tested in a randomized order, between and within days, to exclude the effect of testing sequence.

Prior to the commencement of sets of replicates with no odour cue, the wind tunnel surfaces were washed with deionised water, then wiped with 100% ethanol, and left to dry. All fabric components of the wind tunnel were washed at high temperature with a fragrance-free detergent (Surcare, UK). Furthermore, clean surgical gloves were worn at all times when touching the equipment, to minimize contamination of human skin odour[26].

Secondary factors such as mosquito age, weather, and atmospheric pressure were introduced as co-variants in statistical analysis and found to not have a significant impact in either the activation rate or number landing on the target. Thus, they were excluded from further analysis.

Statistical analysis. Statistical analysis and data visualisation were performed using R (version 3.6.0, R Development Core Team, 2013). The R packages used were "multcomp" for Tukey tests[64], and "MASS" for negative binomial generalised linear models (GLMs)[65].

The data were analysed using GLMs, which can accommodate non-Gaussian data, with trap visibility, trap surface temperature, and presence or absence of host odour as factors. For each replicate, data on activation was taken as the proportion of mosquitoes activated over the total amount of mosquitoes released, for which a GLM with quasibinomial errors and a logit link was used. The landing analysis was carried out on the number of mosquitoes that landed on the target surface, and for that a GLM with negative binomial errors and a log link was used. A three-way analysis of deviance was used to assess differences in numbers of mosquitoes found in different parts of the wind tunnel following different treatments. Multiple comparisons of means, using Tukey contrast to allow for corrections against inflation of Type I errors were carried out to compare results from treatments that

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had different combinations of factors. Landing proportion on different quadrants was analysed using a one-way analysis of deviance and then further assessed with a Chi-square test to pair-compare the number caught on different sections of the landing target.

To determine whether the effect of two or more stimuli on the landing was synergistic or additive, we needed to compare the observed results with a hypothetical data set that reflected an additive effect. To do this, the recorded numbers of landings on treatments where two or more stimuli where presented together were compared with the predicted numbers of mosquitoes that were expected to land if each cue acted in an additive manner. The expected additive landing numbers were derived from assays where each singular component was tested, with these results added to give a predicted additive effect. For example, to create a simulated data point for the additive effect of all three cues presented together, the number of mosquitoes recovered from an assay where only the visual cue was offered was added to the number of landings from an assay where only odour was provided and the number of landings from an assay where only the thermal cue was presented. To select which data points to use to create the simulated data set, the assays were grouped together according to closest chronological proximity. This limited the effects of any potential covariants that fell outside of experimental control. In this way, a simulated data set of ten points was constructed for each of the four potential treatment combinations (i.e. a simulated data set for all three cues, for the visual plus odour cues, for the visual plus thermal cues, and for the thermal plus odour cues). All data (recorded from experimental observation and predicted from the simulated data sets) were tested in a GLM with negative binomial errors and a log link. An analysis of deviance was used to assess if the recorded mean number of landed mosquitoes in treatments where cues were presented together was greater than the mean number of mosquitoes expected to land if the total effect of those stimuli was the additive sum of the effect of the individual stimuli.

Data availability

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Author contributions

MC, conceived and designed the study, carried out the lab work, the statistical analysis, and drafted the manuscript. SB, RH, FH helped to conceive the study and the methodology, provided supervision and critically analysed the manuscript. RH and FH also provided funding. All authors reviewed the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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APPENDIX B: OVERVIEW OF TRACKS RECORDED IN CHAPTER 5

Table S1: General overview of the number of total tracks and the percentage of tracks removed from the analysis. *Note that aside from two replicates (first two entrances in the table), the rest of the replicates had a high tracking accuracy, with an acceptance rate for tracks of over 92%.*

Experiment tag number	Total recorded tracks	Accepted tracks	Percentage acceptance
2020_07_27_14_21_03 - exp 13 small vs small	1145	278	24.28%
2020_07_28_16_29_06 - exp 20 big vs big	328	236	71.95%
2020_08_20_14_33_07 - exp 18 vert(2) vs hor(1)	250	232	92.80%
2020_08_03_14_21_39 - exp 41 large (1) vs small (2)	112	104	92.86%
2020_09_09_18_03_01 - exp 63 - 1-2	130	123	94.62%
2020_08_21_17_46_35 - exp 28 vert vs vert	105	100	95.24%
2020_09_07_18_31_40 - exp 45 - 1-4	293	281	95.90%
2020_08_19_15_34_52 - exp 13 vert(2) vs hor(1)	225	217	96.44%
2020_09_05_18_36_18 - exp 37 - pos control	142	137	96.48%
2020_08_04_16_16_18 - exp 49 big vs big	115	111	96.52%
2020_09_10_14_28_56 - exp 66 - 1-4	104	101	97.12%
2020_08_17_16_46_42 - exp 3 vert vs vert	182	177	97.25%
2020_08_19_14_03_36 - exp 11 vert(2) vs hor(1)	114	111	97.37%
2020_08_19_16_16_55 - exp 14 vert vs vert	117	114	97.44%
2020_08_18_15_49_16 - exp 7 vert vs vert	120	117	97.50%
2020_07_30_17_09_03 - exp 32 small vs small	161	157	97.52%
2020_08_21_19_07_45 - exp 30 vert vs vert	172	168	97.67%
2020_08_21_14_34_39 - exp 24 vert(2) vs hor(1)	90	88	97.78%
2020_09_04_14_41_59 - exp 26 - 1-4	832	814	97.84%

Experiment tag number	Total recorded tracks	Accepted tracks	Percentage acceptance
2020_08_20_17_41_52 - exp 22 vert(1) vs hor(2)	154	151	98.05%
2020_08_20_18_30_03 - exp 23 vert vs vert	160	157	98.13%
2020_09_04_18_47_16 - exp 33 - neg control	430	422	98.14%
2020_09_08_15_37_24 - exp 50 - pos control	218	214	98.17%
2020_08_18_16_44_04 - exp 8 vert vs vert	277	272	98.19%
2020_07_30_16_27_38 - exp 31 large(1) vs small (2)	167	164	98.20%
2020_08_17_14_29_14 - exp 1 vert(1) vs hor(2)	168	165	98.21%
2020_09_07_19_09_53 - exp 46 - neg control	343	337	98.25%
2020_07_28_14_18_03 - exp 18 small vs small	235	231	98.30%
2020_09_07_14_35_35 - exp 39 - pos control	124	122	98.39%
2020_09_09_14_31_52 - exp 57 - 1-2	66	65	98.48%
2020_09_04_15_47_01 - exp 28 - 1-2	265	261	98.49%
2020_08_20_16_09_05 - exp 20 vert vs vert	133	131	98.50%
2020_09_08_14_23_02 - exp 48 - 1-8	404	398	98.51%
2020_09_04_17_38_30 - exp 31 - pos control	157	155	98.73%
2020_09_05_17_30_27 - exp 35 - pos control	158	156	98.73%
2020_07_28_18_12_43 - exp 22 small vs small	83	82	98.80%
2020_07_30_15_08_45 - exp 30 big vs big	686	678	98.83%
2020_09_03_17_00_48 - exp 21 - 1-8	177	175	98.87%
2020_09_10_15_04_36 - exp 67 - pos control	274	271	98.91%
2020_09_09_18_38_01 - exp 64 - 1-8	373	369	98.93%
2020_09_08_17_30_47 - exp 53 - neg control	187	185	98.93%
2020_09_04_17_03_54 - exp 30 - neg control	94	93	98.94%
2020_07_31_18_24_41 - exp 40 large (2) vs small (1)	299	296	99.00%
2020_08_04_17_52_04- exp 50 big vs big	100	99	99.00%
2020_08_21_15_19_09 - exp 25 vert(1) vs hor(2)	300	297	99.00%

Experiment tag number	Total recorded tracks	Accepted tracks	Percentage acceptance
2020_09_07_13_57_04 - exp 38 - neg control	306	303	99.02%
2020_09_09_15_42_26 - exp 59 - 1-4	102	101	99.02%
2020_09_05_16_57_24 - exp 34 - neg control	724	717	99.03%
2020_09_09_15_04_30 - exp 58 - 1-8	113	112	99.12%
2020_09_08_13_43_43 - exp 47 - 1-2	124	123	99.19%
2020_08_21_18_25_59 - exp 29 vert(1) vs hor(2)	126	125	99.21%
2020_09_08_18_04_23 - exp 54 - 1-8	257	255	99.22%
2020_09_05_18_02_20 - exp 36 - neg control	523	519	99.24%
2020_08_03_18_53_26 - exp 45 big vs big	137	136	99.27%
2020_09_03_18_46_33 - exp 24 - 1-2	274	272	99.27%
2020_09_09_16_52_18 - exp 61 - pos control	139	138	99.28%
2020_07_27_16_01_57 -exp 15 small vs small	149	148	99.33%
2020_09_07_16_32_24 - exp 42 - 1-2	153	152	99.35%
2020_09_08_14_58_08 - exp 49 - 1-2	153	152	99.35%
2020_08_03_15_02_55 - exp 42 small vs small	156	155	99.36%
2020_09_07_15_13_10 - exp 40 - 1-2	482	479	99.38%
2020_09_04_18_17_09 - exp 32 - neg control	342	340	99.42%
2020_09_07_17_50_32 - exp 44 - pos control	172	171	99.42%
2020_07_29_14_28_26 - exp 23 large vs large	177	176	99.44%
2020_07_28_17_23_45 - exp 21 big vs big	190	189	99.47%
2020_09_03_17_34_35 - exp 22 - pos control	392	390	99.49%
2020_09_08_18_41_08 - exp 55 - 1-4	202	201	99.50%
2020_07_29_15_33_06 - exp 24 large (2) vs small (1)	213	212	99.53%
2020_09_04_15_15_47 - exp 27 - pos control	234	233	99.57%
2020_09_09_16_16_30 - exp 60 - neg control	268	267	99.63%
2020_08_03_16_16_07 - exp 43 small vs small	295	294	99.66%
2020_08_17_15_51_07 - exp 2 hor vs hor	302	301	99.67%

Experiment tag number	Total recorded tracks	Accepted tracks	Percentage acceptance
2020_08_20_15_16_16 - exp 19 vert vs vert	322	321	99.69%
2020_09_03_14_31_09 - exp 17 - 1-8	697	695	99.71%
2020_07_29_18_56_48 - exp 28 big (2) vs small (1)	421	420	99.76%
2020_07_29_17_37_13 - exp 26 big (1) vs small (2)	432	431	99.77%
2020_09_03_16_20_44 - exp 20 - 1-2	548	547	99.82%
2020_07_27_15_10_09 -exp 14 big vs big	274	274	100.00%
2020_07_27_18_28_12 - exp 17 small vs small	127	127	100.00%
2020_07_28_15_06_09 - - exp 19 small vs small	753	753	100.00%
2020_07_29_16_25_52 - exp 25 big vs big	182	182	100.00%
2020_07_29_18_18_50 - exp 27 small vs small	349	349	100.00%
2020_07_30_14_24_51 - exp 29 big(2) vs small (1)	108	108	100.00%
2020_07_30_17_43_55 - exp 33 large (2) vs small (1)	209	209	100.00%
2020_07_30_18_24_56 - exp 34 large (2) vs small (1)	187	187	100.00%
2020_07_31_14_14_28 - exp 35 large(1) vs small	267	267	100.00%
2020_07_31_14_57_57 - exp 36 small vs small	118	118	100.00%
2020_07_31_15_36_13 - exp 37 large(1) vs small (2)	135	135	100.00%
2020_07_31_16_20_45 - exp 38 big vs big	159	159	100.00%
2020_08_03_17_08_07 - exp 44 big vs big	208	208	100.00%
2020_08_04_14_51_42 - exp 47 small vs small	158	158	100.00%
2020_08_04_15_27_40 - exp 48 large vs large	318	318	100.00%
2020_08_04_18_35_26 - exp 51 big (1) vs small (2)	214	214	100.00%
2020_08_17_18_07_17 - exp 4 vert(2) vs hor(1)	199	199	100.00%
2020_08_18_14_08_25 - exp 5 vert vs vert	226	226	100.00%
2020_08_18_14_55_21 - exp 6 vert(1) vs hor(2)	347	347	100.00%

Experiment tag number	Total recorded tracks	Accepted tracks	Percentage acceptance
2020_08_18_17_39_36 - exp 9 vert(1) vs hor(2)	71	71	100.00%
2020_08_18_18_26_36 - exp 10 hor vs hor	228	228	100.00%
2020_08_19_14_51_14 - exp 12 hor vs hor	196	196	100.00%
2020_08_19_17_51_16 - exp 16 vert vs vert	221	221	100.00%
2020_08_20_13_51_03 - exp 17 vert(2) vs hor(1)	216	216	100.00%
2020_08_20_16_49_45 - exp 21 hor vs hor	133	133	100.00%
2020_08_21_16_07_35 - exp 26 hor vs hor	196	196	100.00%
2020_08_21_17_04_58 - exp 27 vert vs vert	147	147	100.00%
2020_09_03_15_11_03 - exp 18 - 1-4	32	32	100.00%
2020_09_03_15_45_30 - exp 19 - 1-4	251	251	100.00%
2020_09_03_18_12_32 - exp 23 - 1-4	374	374	100.00%
2020_09_04_16_23_21 - exp 29 - 1-8	44	44	100.00%
2020_09_07_15_49_54 - exp 41 - 1-8	290	290	100.00%
2020_09_07_17_11_17 - exp 43 - neg control	48	48	100.00%
2020_09_08_16_15_47 - exp 51 - 1-8	276	276	100.00%
2020_09_08_16_57_12 - exp 52 - 1-4	190	190	100.00%
2020_09_08_19_16_41 - exp 56 - 1-2	176	176	100.00%
2020_09_09_17_25_57 - exp 62 - 1-4	270	270	100.00%
2020_09_09_19_15_02 - exp 65 - 1-8	368	368	100.00%

APPENDIX C: LIST OF COMPARISONS OF ERYTHROCYTE DIAMETER

Table S2: Comparisons of erythrocyte diameter between different treatments. *The comparisons that are the main focus of the study are presented and discussed in the main text in Chapter 6 (Tukey's test, P<0.05).*

Treatment 1	Mean ± SEM diameter (µm)	Treatment 2	Mean ± SEM diameter (µm)	t-value	P-value
4 °C	4.82 ± 0.09	30 °C	4.86 ± 0.09	0.35	1.00
4 °C	4.82 ± 0.09	42 °C	4.91± 0.09	0.80	0.97
4 °C	4.82 ± 0.09	48 °C	4.83 ± 0.09	0.13	1.00
4 °C	4.82 ± 0.09	60 °C	4.73 ± 0.09	-0.63	0.99
30 °C	4.86 ± 0.09	42 °C	4.91± 0.09	0.45	1.00
30 °C	4.86 ± 0.09	48 °C	4.83 ± 0.09	-0.22	1.00
30 °C	4.86 ± 0.09	60 °C	4.73 ± 0.09	-0.98	1.00
42 °C	4.91± 0.09	48 °C	4.83 ± 0.09	-0.67	0.98
42 °C	4.91± 0.09	60 °C	4.73 ± 0.09	-1.43	0.71
48 °C	4.83 ± 0.09	60 °C	4.73 ± 0.09	-0.76	0.97

APPENDIX D: LIST OF COMPARISONS OF THE NUMBER OF ERYTHROCYTES COUNTED IN DIFFERENT TREATMENTS

Table S3: Comparisons of the number of erythrocytes counted in the haemocytometer unit in different treatments. *The asterisk denotes a significant difference at a level of at least P<0.05 (Tukey's test).*

Treatment 1	Mean ± SEM number of cells per unit	Treatment 2	Mean ± SEM number of cells per unit	t-value	P-value
4 °C	529 ±19.58	30 °C	562 ± 19.08	-1.10	0.87
4 °C	529 ±19.58	36 °C	551 ± 13.36	-1.72	0.48
4 °C	529 ±19.58	42 °C	551 ± 18.90	-1.49	0.64
4 °C	529 ±19.58	48 °C	533 ± 18.58	-2.19	0.22
4 °C	529 ±19.58	60 °C	21 ± 3.73	-18.77	<0.001*
30 °C	562 ± 19.08	36 °C	551 ± 13.36	-0.44	1.00
30 °C	562 ± 19.08	42 °C	551 ± 18.90	-0.39	1.00
30 °C	562 ± 19.08	48 °C	533 ± 18.58	-1.09	0.87
30 °C	562 ± 19.08	60 °C	21 ± 3.73	-18.45	<0.001*
36 °C	551 ± 13.36	42 °C	551 ± 18.90	-0.01	1.00
36 °C	551 ± 13.36	48 °C	533 ± 18.58	-0.81	0.96
36 °C	551 ± 13.36	60 °C	21 ± 3.73	-18.52	<0.001*
42 °C	551 ± 18.90	48 °C	533 ± 18.58	-0.70	0.98
42 °C	551 ± 18.90	60 °C	21 ± 3.73	-18.34	<0.001*
48 °C	533 ± 18.58	60 °C	21 ± 3.73	-18.14	<0.001*