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Odour-mediated host seeking and discrimination in mosquitoes

Chemistry, neurobiology and behaviour

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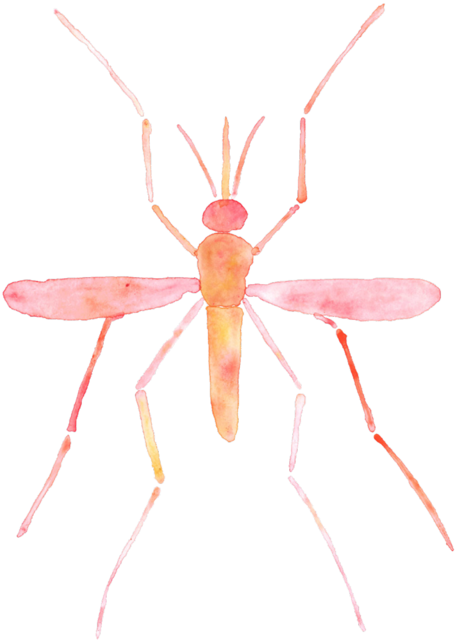

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Abstract

The majority of the world's population is at risk of one or more mosquito-borne diseases that are transmitted by blood-feeding female mosquitoes, affecting both human health and economic development. Especially *Anopheles gambiae*, the principal malaria vector, and *Aedes aegypti*, the vector of dengue and yellow fever, are of primary concern due to their strong specialisation on human hosts, and the high number of casualties caused by the pathogens they transmit. Host seeking and discrimination are crucial for disease transmission, and are predominantly mediated by olfaction. Using a wind tunnel system and a custom analysis pipeline, this thesis confirms that the two mosquito species use volatile host cues, derived from breath and body, differentially, as carbon dioxide on its own drives host seeking in *Ae. aegypti*, but not in *An. gambiae* (paper I). To discriminate between host and non-host species (paper V), *Ae. aegypti* encode human identity by the relative activation of two glomeruli within the antennal lobe, the primary olfactory centre, of which one is tuned to long-chain aldehydes enriched in human odour. A synthetic blend mimicking the glomerular activation elicited host seeking in *Ae. aegypti* (paper II). Next to preferring human over non-human hosts, *Ae. aegypti* also prefer some human individuals to others, which was demonstrated to be affected by the ABO blood type and pregnancy or menstrual cycle phase. Analysis of the volatiles associated with individual volunteers, identified 1-octen-3-ol to be significantly associated with very high attractiveness (paper III). The molecular regulation of host seeking acquisition during *An. gambiae* female adult maturation was independent of odorant receptor gene *AgamOR39* expression (paper IV). The results presented in this thesis contribute to the understanding of mosquito host seeking and discrimination from multiple perspectives, which is a prerequisite to ultimately develop novel tools for mosquito monitoring and control.

Keywords: *Aedes aegypti*, *Anopheles gambiae*, carbon dioxide, GC-MS, host discrimination, host seeking, human odour, olfaction, 3D tracking

Die geruchsgesteuerte Wirtsfindung und Wirtsunterscheidung durch Stechmücken

Kurzfassung

Ein Großteil der Weltbevölkerung ist von einer oder mehreren durch Stechmücken übertragenen Krankheiten bedroht, mit schwerwiegenden Folgen für Gesundheit und wirtschaftliche Entwicklung. Insbesondere die Malariamücke *Anopheles gambiae* und die Gelbfiebermücke *Aedes aegypti* sind aufgrund ihrer starken Anpassung an den Menschen als Wirt und die durch sie verursachten hohen Infektionszahlen von besonderer Bedeutung. Das Finden und Unterscheiden von Wirten ist kritisch für die Übertragung der Pathogene und vorwiegend durch den Geruchssinn gesteuert. Diese Doktorarbeit bestätigt, dass beide Stechmückenarten volatile Geruchskomponenten von Körper und Atem der Wirte auf verschiedene Weise nutzen, da Kohlenstoffdioxid alleine die Wirtsfindung in *Ae. aegypti*, aber nicht *An. gambiae*, auslöst (I). Um zwischen Wirts- und Nichtwirtsarten zu unterscheiden (V), wird die menschliche Identität bei *Ae. aegypti* durch die relative Aktivierung zweier Glomeruli im Antennallobus kodiert. Einer der Glomeruli ist auf die Erkennung von langkettige Aldehyden eingestellt, die im menschlichen Geruch dominant sind. Eine synthetische Geruchsmischung, die das Aktivitätsmuster menschlichen Geruchs imitiert, löst die Wirtsfindung aus (II). *Aedes aegypti* zeigt nicht nur eine Präferenz für Menschen als Wirt, sondern bevorzugt auch einige menschliche Individuen gegenüber anderen, welches von der ABO-Blutgruppe und einer Schwangerschaft, bzw. der Zykluswoche beeinflusst wird. 1-Octen-3-ol ist signifikant mit sehr hoher Attraktivität assoziiert (III). Die molekulare Regulierung des Einsetzens des Wirtsfindungsverhaltens nach dem Schlüpfen ist unabhängig von der Expression des Geruchsrezeptorgens *AgamOR39* (IV). Die Ergebnisse dieser Doktorarbeit leisten einen multiperspektivischen Beitrag zum Verständnis der Wirtsfindung und -unterscheidung durch Stechmücken, eine Voraussetzung für die Entwicklung neuer Methoden zur Beobachtung und Bekämpfung ebendieser.

Schlagwörter: *Ae. aegypti*, *An. gambiae*, 3D-Tracking, GC-MS, Geruchssinn, Kohlenstoffdioxid, menschlicher Geruch, Wirtsfindung, Wirtsunterscheidung

Dedication

To my first and second family.

„Denn alles Fleisch, es ist wie Gras.“/*“Behold, all flesh is as the grass.”*
(1 Peter 1:24; Ein deutsches Requiem, op. 45, by Johannes Brahms)
On chemical parsimony and how to place the Disease Vector Group
within the Department of Plant Protection Biology

“The question is not what you look at, but what you see.”
(Henry David Thoreau)

Contents

List of publications

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

I. **Annika Hinze**, Jörgen Lantz, Sharon R. Hill, Rickard Ignell (2021). Mosquito host seeking in 3D using a versatile climate-controlled wind tunnel system. *Frontiers in Behavioral Neuroscience*, 15, 643693.

II. Zhilei Zhao, Jessica L. Zung*, **Annika Hinze***, Alexis L. Kriete, Azwad Iqbal, Meg A. Younger, Benjamin J. Matthews, Dorit Merhof, Stephan Thiberge, Rickard Ignell, Martin Strauch, Carolyn S. McBride (2022). Mosquito brains encode unique features of human odour to drive host seeking. *Nature*, 605, pp. 706-712.

III. **Annika Hinze***, Julien Pelletier*, Majid Ghaninia, Sharon R. Hill, Rickard Ignell. Acquisition of host seeking in *Anopheles gambiae* is not regulated by OR39. (manuscript)

IV. **Annika Hinze***, Anaïs K. Tallon*, Betelehem Wondwosen*, Mengistu Dawit, Sharon R. Hill, Björn Bohman, Rickard Ignell. Cyphers and cycles – identifying the potential chemical basis of the differential attraction of mosquitoes to human odour. (manuscript)

V. **Annika Hinze**, Sharon R. Hill, Rickard Ignell (2022). Odour-mediated host selection and discrimination in mosquitoes. In: *Sensory Ecology of Disease Vectors* (Wageningen Academic Publishers).

*Equal contribution.
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The contribution of Annika Hinze to the papers included in this thesis was as follows:

I. Designed the study together with the co-authors. Established the experimental workflow, performed all data collection and all data analysis. Wrote the manuscript with the input of the co-authors.

II. Designed the part on mosquito behaviour in the wind tunnel together with the co-authors. Performed all data collection for this part and data analysis. Contributed to the final manuscript.

III. Designed the study together with the co-authors. Performed all data collection and analysis on mosquito behaviour. Performed the statistical analysis. Wrote the manuscript with the input of the co-authors.

IV. Designed the study together with the co-authors. Performed human headspace collection and relative attractiveness tests together with AT and performed the chemical analysis. Contributed to data analysis. Wrote the manuscript with the input of the co-authors.

V. Wrote the manuscript with the input of the co-authors.

Abbreviations

AL	antennal lobe
CO_2	carbon dioxide
Cas9	CRISPR-associated protein 9
CRISPR	clustered regularly interspaced short palindromic repeats
dpe	days post-emergence
GC-EAD	combined gas chromatography and electroantennographic detection
GC-MS	combined gas chromatography and mass spectrometry
GDP	gross domestic product
GR	gustatory receptor
IR	ionotropic receptor
OBP	odorant binding protein
OR	odorant receptor
orco	odorant receptor co-receptor
OSN	olfactory sensory neuron
VOI	volume of interest
VOC	volatile organic compound
WHO	World Health Organization
3D	three-dimensional / three dimensions

1. Introduction

There are more than 3 500 mosquito species and subspecies described worldwide, inhabiting all continents of the world, except Antarctica, but only a handful are dangerous to humans, due to their preference for human hosts and the pathogens they may carry (Knight and Stone, 1977; Clements, 1999; Foster and Walker, 2009). Current estimates from the World Health Organization (WHO) indicate that 80% of the world's population are at risk of one or more vector-borne diseases, predominantly transmitted by mosquitoes (WHO, 2017). The largest share of infections occurs due to *Anopheles* mosquitoes vectoring malaria parasites, which resulted in 241 million estimated malaria cases and 627 000 deaths in 2020 (WHO, 2021). Dengue, transmitted by *Aedes aegypti* and, to a lesser extent, *Aedes albopictus*, is less fatal than malaria, but is estimated to infect 104 to 390 million people annually, and is listed as a neglected tropical disease by the WHO (Bhatt *et al.*, 2013; WHO, 2020). Next to malaria and dengue, major vector-borne diseases transmitted by mosquitoes are lymphatic filariasis, chikungunya, Zika, yellow fever and Japanese encephalitis, resulting together in more than 700 000 deaths per year (GatesNotes, 2014; WHO, 2017).

Apart from affecting human health directly, mosquito-borne diseases also have a large socioeconomic impact, by forgone income during sickness, private and non-private medical costs, as well as other factors relating to, *e.g.*, schooling, fertility decisions and migration (Sachs and Malaney, 2002). On the macroeconomic level, growth in countries with intense malaria lagged by 1.3% per person per year in comparison to countries without malaria (Gallup and Sachs, 2001), resulting in a difference in GDP per capita growth rate that may be as high as six times (McCarthy *et al.*, 2000).

The substantial upscaling in the distribution of insecticide-treated mosquito nets, indoor residual spraying and implementation of environmental management has contributed successfully to controlling the vectors, and thereby reducing the burden of mosquito-borne disease, which has led to, *e.g.* a 24% decrease in malaria case incidence per population at risk between 2005 and 2015 (WHO, 2017, 2021; Dhiman, 2019). Despite the progress, the reduction in malaria cases has stagnated since 2015 and malaria remains endemic in many countries, whereas the incidence of dengue has increased exponentially since 1990; by more than 300% within the last decade (Stanaway *et al.*, 2016; Dhiman, 2019; WHO, 2020). The Covid-19 pandemic has significantly hampered vector control efforts and chemoprevention campaigns and disrupted the public health system, leading to an increase in both malaria incidence by 6%, and casualties by 12%, in comparison to the year before (WHO, 2021). Additional emerging challenges include the behavioural and physiological resistance to control interventions, such as insecticides and anti-malarial drugs, urbanisation, altered land use pattern, increased global trade and climate change (Bhatt *et al.*, 2013; Elbers *et al.*, 2015; Liu, 2015; Cobo, 2016; WHO, 2017, 2018; Conrad and Rosenthal, 2019; Colón-González *et al.*, 2021).

In the face of present and arising challenges, a thorough understanding of vector biology, sensory ecology and behaviour is key for the implementation of an integrated strategy for monitoring and controlling mosquito populations (World Health Organization, 2017; Shaw and Catteruccia, 2019; Wilson *et al.*, 2020). Host localisation and discrimination by female mosquitoes is a crucial determinant of disease transmission, and predominantly mediated by olfaction (DeGennaro *et al.*, 2013; Takken and Verhulst, 2013; Cardé, 2015; Ignell and Hill, 2020). This thesis investigates multiple aspects of odour-mediated host seeking and discrimination in the disease vector mosquitoes *Anopheles gambiae sensu stricto* (hereafter *An. gambiae*) and *Ae. aegypti aegypti* (hereafter *Ae. aegypti*), from chemistry to neural coding, behaviour and modulation. The results advance our understanding of a behaviour crucial for disease transmission, and may ultimately contribute to the improvement and development of novel tools for mosquito monitoring and control.

2. Background

2.1 Blood feeding

2.1.1 Vectorial capacity

Female mosquitoes of most species are anautogenous, *i.e.*, they require a blood meal to obtain proteins for egg production (Lehane, 2005). Following adult emergence, and a brief period of exclusive feeding on sugar sources, a female mosquito will take multiple blood meals over the course of her life (Clements, 1992; Foster, 1995, 2022). Several pathogens evolved to hijack the blood-feeding behaviour of mosquitoes (*e.g.*, Stanczyk *et al.*, 2022), and particularly those species that are specialised on feeding from a limited number of hosts are efficient – and dangerous – vectors (Lyimo and Ferguson, 2009). Upon taking a blood meal from an infected host, the pathogen infects the mosquito by initially replicating in the midgut epipthelial cells, then entering the haemolymph system, and ultimately reaching the salivary glands and other tissues (Hardy *et al.*, 1983; Beier, 1998). Spreading the pathogen occurs when an infectious mosquito takes a blood meal and injects her saliva, containing for instance molecules preventing blood clotting and vasoconstriction, but also the pathogen, into the host (Clements, 1992; Ribeiro and Francischetti, 2003).

Vector competence, *i.e.*, the ability of a vector to transmit a pathogen, is dependent on both extrinsic and intrinsic factors, such as the density of mosquito and host populations, environmental conditions, mosquito host preference and the susceptibility of the mosquito to become infected after ingesting an infectious blood meal (Hardy *et al.*, 1983). Disease transmission, however, is even more complex and shaped by several factors, including vector competence, that are summarised by the vectorial capacity, V, which is classically estimated as:

$$V = \frac{ma^2p^n}{-\ln(p)},$$

in which m corresponds to the density of female mosquitoes in relation to the host, a to the daily blood-feeding rate per mosquito, p to the probability of a mosquito surviving throughout one day and n to the extrinsic incubation period, *i.e.*, the time from ingestion of an infectious blood meal to transmission (Macdonald, 1961; Kramer and Ciota, 2015; Brady *et al.*, 2016). The vectorial capacity thus describes the number of potentially infectious bites arising from an infectious host on a single day (Garrett-Jones, 1964; Brady *et al.*, 2016).

Although this classical model is sufficiently accurate and widely used to assess the impact of different vector interventions on disease transmission, some of the basic assumptions may not be met in a natural context, and the model has thus been updated and adapted multiple times to accommodate additional factors (Reiner *et al.*, 2013; Brady *et al.*, 2016). Transmission dynamics are further influenced by, *e.g.*, spatial and temporal heterogeneities in host movement and pathogen-variant distribution, as well as by inter-individual differences in host attractiveness, resulting in a highly complex interaction between hosts, vectors and pathogens (Knols *et al.*, 1995; Kelly, 2001; Endy *et al.*, 2002; Stoddard *et al.*, 2009; Reiner *et al.*, 2013; Verhulst *et al.*, 2013; **paper IV**). Consequently, 80% of disease transmission are ascribable to 20% of a host population (Woolhouse *et al.*, 1997).

2.1.2 The evolution of haematophagy in mosquitoes

Haematophagy, *i.e.,* the obligatory or facultative feeding on blood, is thought to have arisen multiple independent times within the insect class, and may have followed one of two evolutionary pathways, requiring a number of morphological, physiological and behavioural adaptations (reviewed in Waage, 1979; Lehane, 2005).

First, haematophagy may have arisen from insects with chewing mouthparts that lived in close association with their host, likely feeding on organic debris, using host nests as shelter or host dung for oviposition (Waage, 1979; Lehane, 2005). With an intermediate step of feeding on dead particles of host skin, fur or feathers, selection most likely favoured digestive adaptations to make use of the host material. Occasional feeding directly from the host and further adaptations may have facilitated the subsequent establishment of regular blood feeding in the diet of the insect. Pool-feeding insects, such as lice and tsetse flies, are thought to have followed this evolutionary pathway. Second, blood feeding may have evolved when insects already equipped with piercing-sucking mouthparts to feed on plants or predate on other insects, accidentally fed on a vertebrate host. Due to the high protein content, selection favoured digestive, physiological and behavioural adaptations that permitted host location and the digestion of blood (Waage, 1979; Lehane, 2005).

In adult female mosquitoes, the mouthpart morphology suggests that the evolution of haematophagy most probably followed the second evolutionary pathway (Waage, 1979; Lehane, 2005). Here, two scenarios could explain the ancestral occurrence of piercing-sucking mouthparts: ancestors may have fed either on plant fluids (phytophagy) or on both plant fluids and insect haemolymph (entomophagy; Mattingly, 1965; Waage, 1979; Borkent, 2012; Peach and Gries, 2020; Peach and Matthews, 2022). Tus, the elongated mouthparts of ancestral mosquitoes may have arisen to reach the base of flowers to feed on nectar (Foster, 1995; Larson *et al.*, 2001) or to pierce the cuticle of plant parts, seeds, fruits (Mattingly, 1965; Lehane, 2005) or other insects (Waage, 1979; Lehane, 2005). Adult mosquitoes today feed readily on plant fluids, such as floral and extrafloral nectar, plant sap, honeydew and fruit juice (Nayar and Sauerman Jr, 1971; Foster, 1995; Peach and Gries, 2020), and, interestingly, also remnants of the hypothesised ancestral attraction to and feeding on insect haemolymph can be observed (Howard *et al.*, 1912; Harris *et al.*, 1969; Martel *et al.*, 2011; George *et al.*, 2014).

The host shift from either plants and/or other insects to vertebrate hosts was likely facilitated by the substantial overlap in floral, larval, and vertebrate odour cues (Ignell and Hill, 2020; Hill and Ignell, 2021; Peach and Matthews, 2022; Ignell *et al.*, *in preparation*; see *2.4.1 Chemical parsimony*), allowing to reshape the chemosensory detection pathways already established in other contexts.

2.2 Odour-mediated host-seeking in mosquitoes

Olfaction is a sensory modality involved in many mosquito behaviours, and crucial for locating and assessing the quality of multiple resources, including nectar sources, hosts and oviposition sites (reviewed in Takken and Knols, 1999; Ignell and Hill, 2020; Hill and Ignell, 2021; Konopka *et al*., 2021). This thesis focusses on the role of olfaction in mosquito host-seeking behaviour.

As an odour cue does not contain intrinsic directional information, flying insects rely on the ambient wind direction for identifying the direction to the odour source (Murlis *et al*., 1992; Cardé, 2021). The mechanism by which mosquitoes, and many other flying insects, track odour plumes (reviewed in, *e.g.*, Cardé and Willis, 2008; Cardé, 2021), is referred to as *odour-mediated optomotor-guided positive anemotaxis* (Kennedy, 1940). The term describes the odour-elicited directed upwind movement of an animal in response to an air current ("anemotaxis", from Ancient Greek *ánemos*, wind, and *táxis*, arrangement or order, which in biology refers to directed movement), in which wind direction is extracted from the optomotor response, *i.e.*, the image flow on the retina of the animal (Kennedy, 1940). Even though the successful localisation of a host is a multimodal process, odour-mediated optomotor anemotaxis is at the core of approaching a host at a distance where host odours are the only cues available.

2.2.1 The baseline of mosquito flight: saccades, optic flow and ambient wind direction

The basic requirements for tracking an odour plume by odour-mediated optomotor anemotaxis are the abilities to navigate in three-dimensional (3D) space, and to estimate the wind direction. Visual cues are of utmost importance, which is true for both diurnal and nocturnal mosquito species, of which the latter use the light from moon and stars for visual orientation (Gibson, 1995).

Most insects lack stereoscopic vision, especially in flight, as there is very little binocular disparity, *i.e.*, the projections of an object onto the retinas of both eyes are too similar due to the small interocular distance and a relatively large distance to objects in the environment (Srinivasan, 1992). To extract 3D information, insects use the displacement of the retinal image generated by apparent self-motion, referred to as *optic flow* (Gibson, 1950). In translational (forward) motion, the image of objects close to the insect move with higher velocity on the retina than objects further away. However, 3D information is lost in rotational motion, as the image of objects, irrespective of the distance, move with the same retinal velocity (Koenderink and van Doorn, 1987). To optimise the extraction of 3D information, insects use a saccadic flight strategy, in which they condense rotations to short bouts, or saccades, and maximise the stretches of translational motion (Collett and Land, 1975a, 1975b; Geiger and Poggio, 1977).

Insects can detect and measure air currents with their antennae and various types of mechanosensory hairs distributed over their body (Gewecke, 1974). However, during flight, a stable reference point is missing, and only the apparent wind, which is the sum of the ambient wind and the self-motion induced wind, can be measured. To extract information on ambient wind direction, mosquitoes and many other insects use visual information by the optic flow (Kennedy, 1940): in straight upwind or downwind flight, the image flow on the retina is in opposite direction to the direction of motion, while in flight diverging from this heading, the ambient wind induces a transverse component. In many insects, the estimation of own motion, ambient wind direction and velocity, as well as distance to the ground is likely achieved by a combination of optic flow and mechanosensory detection of apparent wind, as behavioural and computational studies suggest (Budick *et al.*, 2007; Taylor and Krapp, 2007; Bhandawat *et al.*, 2010; Rutkowski *et al.*, 2011). Moreover, frequent, large magnitude saccades may be an important, active strategy to estimate ambient wind direction accurately (van Breugel *et al.*, 2022). While this has been proposed based on the flight mechanics of *Drosophila melanogaster*, it is most likely that also mosquitoes optimise their flight trajectories to maximise 3D vision and estimation of ambient wind direction. The sharp turns, and longer stretches of forward flight, form the basis of host seeking flight in mosquitoes (*e.g.*, Dekker *et al.*, 2005; Dekker and Cardé, 2011).

2.2.2 Odour-mediated optomotor anemotaxis: cast-and-surge

The dominant model of odour plume tracking, or odour-mediated optomotor-guided positive anemotaxis, in insects is a sequence of cast-and-surge reflexes (Baker, 1990), a flight mechanism which is shared by mosquitoes (Dekker *et al.*, 2005; Dekker and Cardé, 2011; van Breugel *et al.*, 2015). Upon encountering a host plume, including CO_2, host body odour or a combination of both, the mosquito surges upwind and casts, *i.e.*, performs flight manoeuvres in crosswind direction, when losing contact with the plume (Dekker and Cardé, 2011). In contrast to pheromone tracking in male moths, which is mostly limited to the horizontal plane (Cardé, 2021), mosquitoes zigzag in both horizontal and vertical crosswind direction

(Dekker and Cardé, 2011). While this basic cast-and-surge host-seeking mechanism is common to all mosquito species, details may vary species-specifically, *e.g.*, due to differences in host spectrum, light conditions during the circadian host-seeking peak, or weighing of the input of other sensory modalities (*e.g.,* Klowden, 2007b; Cribellier *et al*., 2022).

2.2.3 Multimodal integration of host cues during host seeking

Host seeking in mosquitoes is a multimodal behavioural process of locating a suitable host, relying on the integration of chemosensory, visual and hygrothermal cues (DeGennaro *et al*., 2013; Spitzen *et al*., 2013; McMeniman *et al*., 2014; van Breugel *et al*., 2015; Hawkes and Gibson, 2016; **paper I** / Hinze *et al*., 2021; Alonso San Alberto *et al*., 2022; Laursen *et al*., 2022; reviewed in Cardé, 2015; Raji and DeGennaro, 2017; Buehlmann *et al*., 2020). Host odour cues, volatile organic compounds (VOCs) and CO_2, released from or associated with host body and breath, play a primary role, as they elicit the stereotypical zigzagging flight mechanism of host seeking (Kennedy, 1940; Dekker and Cardé, 2011; van Breugel *et al*., 2015). Host-derived VOCs, in combination with other sensory cues, have been demonstrated experimentally to be sufficient to drive host-seeking flight (Spitzen *et al*., 2013; McMeniman *et al*., 2014; van Breugel *et al*., 2015; Hawkes and Gibson, 2016; **paper I** / Hinze *et al*., 2021). However, the detection of specific groups of volatile compounds has not been shown to be necessary to generate this behaviour, as genetically disrupting one of the pathways of odour detection reduce, but does not eliminate, host attraction (DeGennaro *et al*., 2013; McMeniman *et al*., 2014; Raji *et al*., 2019; Sun *et al*., 2020). The integration of multiple sensory modalities and the resulting redundancy is at the core of the robustness and accuracy of mosquito host seeking (Buehlmann *et al*., 2020).

Mosquitoes encounter host cues in a sequential, but often overlapping, manner dependent on the proximity to the host. Carbon dioxide, exhaled by breathing humans and other animals (Gillies, 1980), is considered a long-range cue, which mosquitoes may detect at distances of more than 50 m from a host (Zollner *et al*., 2004; Lorenz *et al*., 2013), and which was shown to activate and gate the behavioural response to host VOCs, visual objects and thermal cues (Dekker and Cardé, 2011; McMeniman *et al*., 2014; van Breugel *et al*., 2015; **paper I** / Hinze *et al*., 2021; Alonso San Alberto *et al*., 2022; see also *2.4.3 Carbon dioxide*). At intermediate distances, mosquitoes encounter VOCs from the body of the host, which not only guide the mosquito to the location of the host, but also provide information about host identity (reviewed in Lyimo and Ferguson, 2009; Takken and Verhulst, 2013; Wolff and Riffell, 2018; **paper V** / Hinze *et al.*, 2022; see *2.5 Host specialisation and discrimination*). At this stage, host-derived VOCs seem to become more important than CO_2, as mosquitoes presented with both cues in non-overlapping plumes follow the host VOC plume (Lacey *et al.*, 2014). Mosquitoes have a low visual acuity in comparison to other insects (Land, 1997; Kawada *et al.*, 2006), but may sight hosts of the size of a human at a distance of about 5-10 m (Bidlingmayer and Hem, 1980), providing additional directional information. In the presence of CO_2, both achromatic contrast and chromaticity are important visual features for host-seeking mosquitoes (van Breugel *et al.*, 2015; Alonso San Alberto *et al.*, 2022). In close-range proximity, heat and water vapour released from the host body, but also VOCs with low volatility, may provide additional information for the selection of the landing site and for initiating landing on the host (de Jong and Knols, 1995b; Healy and Copland, 2000; Healy *et al.*, 2002; Spitzen *et al.*, 2013; McMeniman *et al.*, 2014; van Breugel *et al.*, 2015; Bello and Cardé, 2022; Laursen *et al.*, 2022).

2.3 Host odour detection: the olfactory system of mosquitoes

Mosquitoes are equipped with a highly sophisticated olfactory system, tuned to ecologically-relevant cues, to meet the challenges of detecting, identifying and interpreting volatile cues present in their environment in a correct and precise manner (reviewed in Konopka *et al.*, 2021; Wheelwright *et al.*, 2021).

The peripheral olfactory organs of the mosquito, the antennae, the maxillary palps and the labella, are covered in diverse classes of hair-like sensilla, which each house the dendrites of olfactory sensory neurons (OSNs; Suh *et al.*, 2014; Wheelwright *et al.*, 2021). Odorants that reach a sensillum diffuse through the sensillum pores or spokes, traverse the lymph and reach the olfactory receptors expressed in the membrane of OSNs. Odorant binding proteins (OBPs) that are dissolved in the aqueous lymph, bind to the typically hydrophobic odour molecules and are presumably involved in odorant recognition, transport through the lymph, and possibly gating at high odorant concentrations (Leal, 2013; Pelosi *et al.*, 2018).

Each OSN expresses one, or several (Herre *et al.*, 2022; Ye *et al.*, 2022), olfactory receptors of three classes; odorant receptors (ORs), ionotropic receptors (IRs) and gustatory receptors (GRs; Suh *et al.*, 2014; Karner *et al.*, 2015). The receptors function as transmembrane ligand-gated ion channels and are composed of several subunits. Both ORs and IRs form multimeric channels from an obligate, highly conserved co-receptor part and a divergent part tuned to specific odour molecules. While each insect OR complex is a heterotetramer (Butterwick *et al.*, 2018), consisting of the ligand-specific tuning receptor and the conserved co-receptor, orco (Larsson *et al.*, 2004), tuning IRs form a receptor complex with one or up to three of three co-receptors, IR8a, IR25a, or IR76b (Benton *et al.*, 2009; Abuin *et al.*, 2011). The GRs involved in olfaction form a heterotrimeric complex comprised of three subunits, GR1, GR2 and GR3 in culicines and GR22, GR23 and GR24 in anophelines (Lu *et al.*, 2007; Syed and Leal, 2007; Robertson and Kent, 2009; Liu *et al.*, 2020). While mosquito ORs are tuned to VOCs from a wide range of chemical classes, such as aldehydes, alcohols, aromatics, esters, ketones and terpenes (Carey *et al.*, 2010; Wang *et al.*, 2010; Omondi *et al.*, 2019), IRs detect carboxylic acids and amines (Pitts *et al.*, 2017; Raji *et al.*, 2019). Compounds from all of these classes have been found in the odour of human and non-human hosts (*e.g.*, Bernier *et al.*, 1999, 2000, 2008; Penn *et al.*, 2007; De Obaldia *et al.*, 2022; **paper II** / Zhao *et al.*, 2022; **paper IV**). Concerning olfaction, the GR pathway is limited to the detection of carbon dioxide and acetone, compounds present in exhaled breath (Lu *et al.*, 2007; McMeniman *et al.*, 2014; Ghaninia *et al.*, 2019)

The response profile of an OSN is defined by the specificity and sensitivity of the tuning receptor(s) expressed, and it conveys that signal to the primary olfactory centres, the antennal lobe (AL) and the suboesophageal zone (Ghaninia *et al.*, 2007a, 2007b; Riabinina *et al.*, 2016). Canonically, OSNs expressing the same type of tuning receptor converge onto shared structures within the AL, called glomeruli, which are relaying centres of information, where axons of OSNs, dendrites of projection neurons and local interneurons converge (Vosshall *et al.*, 2000; Jefferis *et al.*, 2007). Two recent publications by Herre *et al.* (2022) and Ye *et al.* (2022) challenged the one receptor to one neuron dogma by demonstrating the co-expression of multiple chemosensory receptor genes from different receptor classes in

OSN subsets of both *Ae. aegypti* and *An. coluzzii*. From the AL, projection neurons convey signals to higher brain centres, the mushroom bodies and the lateral horn (Ignell *et al.*, 2005). Here, and in subsequent brain regions, the olfactory information is processed, temporally integrated with information from other sensory modalities, and translated into perceptions that guide behaviour (*e.g.*, Heisenberg, 2003; Tanaka *et al.*, 2004; Galizia and Rössler, 2010; Strutz *et al.*, 2014). Although higher olfactory processing has not been studied in mosquitoes, the basic olfactory circuitry is conserved among insect species, allowing careful generalisations from, *e.g.*, the fruit fly *D. melanogaster* (Strausfeld and Hildebrand, 1999; Zhao and McBride, 2020).

2.4 Host odorants and blends

2.4.1 Chemical parsimony

Odour from human and non-human hosts, emitted from and associated with breath and body, is composed of hundreds to thousands of different volatile compounds (Bernier *et al.*, 1999, 2000, 2008; Birkett *et al.*, 2004; Curran *et al.*, 2005; Penn *et al.*, 2007; Gallagher *et al.*, 2008; Jaleta *et al.*, 2016), of which mosquitoes detect only a fraction due to limitations imposed by the diversity, selectivity and sensitivity of their chemosensory receptor repertoire (Carey *et al.*, 2010; Wang *et al.*, 2010; Omondi *et al.*, 2019).

Of the host compounds detected, most, if not all, are shared between potential host species and there is a substantial overlap with the odour profile of nectar sources and oviposition sites, making host discrimination an interesting sensory challenge (Syed, 2015; Dekel *et al.*, 2019; Ignell and Hill, 2020; Hill and Ignell, 2021; **paper V** / Hinze *et al.*, 2022; Peach and Matthews, 2022; see *2.5.1 Discrimination between potential host species*). The use of the same compound as a cue in multiple behavioural contexts, serving different functions, is termed *chemical parsimony* (Blum, 1996). Sulcatone, for example, a break down product of human sebum (Wisthaler and Weschler, 2010), is one of the major compounds of human emanations (Harraca *et al.*, 2012; Omondi *et al.*, 2019; **paper II** / Zhao *et al.*, 2022; **paper IV**). However, this ketone has also been detected in the odour of various other species, such as sheep, cattle, guinea pigs and chicken (Jaleta *et al.*, 2016; **paper II** / Zhao *et al.*, 2022), and is commonly emanated by flowers (Knudsen *et al.*, 2006; Dekel *et al.*, 2019 and references therein). While sulcatone is a vertebrate and floral cue for both haematophagic and non-haematophagic mosquito species (Dekel *et al.*, 2019; Omondi *et al.*, 2019), it is aversive in concentrations that exceed the natural range (Logan *et al.*, 2008). Likewise, CO_2 may act not only as a cue signalling the presence of a breathing host (Gillies, 1980), but also to indicate the profitability of a floral resource (Thom *et al.*, 2004).

Due to the generic nature of most odorants, odorant recognition is contextual, *i.e.*, dependent on the qualitative and quantitative background a generic compound is embedded in (Bruce and Pickett, 2011; Majeed *et al.*, 2016; Wondwosen *et al.*, 2016; Nyasembe *et al.*, 2018; Ghaninia *et al.*, 2019; Omondi *et al.*, 2019; Lahondère *et al.*, 2020). For example, while (*R*)-1-octen-3-ol is present at different relative abundances in human and cattle odour, it has not been detected in the odour of chicken (Majeed *et al.*, 2016). Manipulating the (*R*)-1-octen-3-ol abundance to match the ratio of other host species significantly alters host recognition in *An. coluzzii*, *Ae. aegypti* and *Cx. quinquefasciatus* (Majeed *et al.*, 2016).

While the term chemical parsimony is predominantly used in respect to olfactory signalling to describe the occurrence and use of generic compounds in multiple contexts, it also extends to the biosynthesis of volatile compounds, since the number of pathways to produce a given VOC is restricted and thus may occur in evolutionary distant species (Blum, 1996; Hill and Ignell, 2021). Whether VOCs emanating from various resources used by mosquitoes originate from similar biosynthetic pathways remains to be investigated.

2.4.2 Activation and attraction

Most behavioural assays investigate the effect of individual host compounds, or host compound blends, on mosquito host seeking from an endpoint-perspective, since flight tracking is laborious, expensive and, dependent on the research question, may not add informative value. Even though omitting the navigational strategy of host seeking, they are well suited and sufficient to identify potential attractive or repellent compounds and blends for mosquito monitoring and control (Coutinho-Abreu *et al.*, 2022 and references therein). In this context, *activation* and *attraction* are two terms commonly used – and sometimes confused – to score mosquito behaviour in response to odour cues. *Activation* is the first step of host seeking, followed by upwind orientation and landing, that is defined as a change in behavioural state from resting to flight (Healy and Copland, 1995; Clements, 1999; Dekker *et al.*, 2005; Lacey and Cardé, 2011; Cardé, 2015). *Attraction* is commonly used to score the second stage of host seeking, and describes a directed orientation towards the odour source (Dethier *et al.*, 1960; Shorey, 1977). It is important to point out that, even though an odour can be an attractant, *i.e.*, it elicits attraction when above threshold, the upwind orientation itself is not guided by the odour, but by visual feedback: an olfactory cue does not contain intrinsic directional information, but wind direction can be estimated by the optic flow (Kennedy, 1940; see *2.2.1 The baseline of mosquito flight: saccades, optic flow and ambient wind direction*). Moreover, the term disregards that multiple sensory modalities and stimuli may contribute to the observed behaviour (Kennedy, 1978).

Attraction to a certain compound or blend is not only dependent on its identity, but also its quantity. While mosquitoes are attracted to a blend at a dose corresponding to what they would encounter in their environment, the same blend in concentrations exceeding the natural range may be neutral or even no longer preferred (Smallegange *et al.*, 2005; Wondwosen *et al.*, 2016; Ghaninia *et al.*, 2019), likely by activating OSNs non-specifically, sensory habituation or adaptation (Stengl *et al.*, 1992; Hallem and Carlson, 2006; Carey *et al.*, 2010; Andersson *et al.*, 2015). Natural repellents, such as lemongrass oil and eugenol, but also 1-octen-3-ol at high concentrations, were demonstrated to have a similar mode of action in *An. coluzzii* (Afify *et al.*, 2019; Afify and Potter, 2020). To investigate the impact of a compound or blend on the (host-seeking) behaviour of mosquitoes, it is thus crucial to adjust the stimulus to the natural range (Ignell and Hill, 2020).

2.4.3 Carbon dioxide

End-expiratory breath from vertebrate hosts contains approximately 40 000 ppm CO_2 (Gillies, 1980; Clements, 1999), and filaments of elevated CO_2 may be detected by mosquitoes up to 50 m downwind of a host (Zollner *et al.*, 2004; Lorenz *et al.*, 2013). Mosquitoes are highly sensitive to CO_2, as their CO_2-sensitive OSNs respond to concentrations from 150 ppm above ambient level and may discriminate fluctuations in CO_2 concentration as small as 50 ppm (Grant *et al.*, 1995; Majeed *et al.*, 2017). Attraction to CO_2 is highly dependent on the plume structure, with sustained upwind flight only elicited by intermittent, turbulent, but not by homogenous plumes (Gillies, 1980; Geier *et al.*, 1999b; Dekker *et al.*, 2001, 2005; Dekker and Cardé, 2011).

Carbon dioxide has been most intensely studied since its role in mosquito host seeking became known a century ago (Rudolfs, 1922), but its importance varies from species to species (Snow, 1970; Dekker and Takken, 1998) and is still not fully understood. In most haematophagous species studied, CO_2 is an activator and contributes to the attraction to the hosts by gating the behavioural and physiological response to olfactory, visual and thermal host cues (Dekker *et al.*, 2005; McMeniman *et al.*, 2014; van Breugel *et al.*, 2015; Webster *et al.*, 2015; Vinauger *et al.*, 2019; see also *2.2.3 Multimodal integration of host cues during host seeking*). In some species, such as *Ae. aegypti*, CO_2 on its own acts not only as a persistent activator (Sorrells *et al.*, 2022), but also elicits upwind orientation, *i.e.* attraction (Dekker *et al.*, 2005; Dekker and Cardé, 2011). However, other species, such as *An. gambiae*, respond either weakly or with neither activation nor attraction to CO_2 as a sole cue (de Jong and Knols, 1995a; Takken *et al.*, 1997; Spitzen *et al.*, 2008; **paper I** / Hinze *et al.*, 2021). Conflicting studies, showing activation and/or attraction to CO_2 alone in *An. gambiae*, exist (Healy and Copland, 1995; Lorenz *et al.*, 2013), partially explained by a potential contamination by host VOCs (Webster *et al.*, 2015).

The role of CO_2 as a sole cue in host seeking is hypothesised to be dependent on the degree of host specialisation of a mosquito species, since CO_2 on its own indicates the presence, but not the identity of a potential host (Mboera and Takken, 1997; Takken *et al.*, 1997). For less specialised mosquito species, which feed more randomly on vertebrates present in their environment, CO_2 is an informative cue, whereas specialist mosquito species need additional odour cues to identify the breathing vertebrate as either host or non-host (see *2.5.1 Discrimination between potential host species*). For example, *Anopheles stephensi,* a less specialised mosquito species with a zoophilic host preference, but not the specialised *An. gambiae*, is attracted to CO_2 as a sole cue (Takken *et al.*, 1997). However, while this differential role of CO_2 alone is likely applicable for the *Anopheles* species studied so far, it is not true for *Ae. aegypti*, and the knowledge about the host preference of most other mosquito species, as well as the role of CO_2 in regulating this behaviour, is insufficient.

2.4.4 Volatile organic compounds

Next to CO_2, the spectrum of host odour detected by mosquitoes is composed of many different VOCs, and a few volatile inorganic compounds, such as ammonia, originating from the host itself and skin microbiota, which, *e.g.*, convert sweat compounds into carboxylic acids (Braks and Takken, 1999; Smallegange *et al.*, 2011; Verhulst *et al.*, 2011; Dormont *et al.*, 2013). Major bioactive compounds associated with human volatile emissions include sulcatone, octanal, nonanal, decanal and geranylacetone (Gallagher *et al.*, 2008; Curran *et al.*, 2010; Harraca *et al.*, 2012; Omondi *et al.*, 2019; **paper II** / Zhao *et al.*, 2022; **paper IV**), whereas emissions from, *e.g.*, cattle and sheep include a high proportion of aromatic compounds and short-chain aldehydes, such as hexanal and heptanal, respectively (Birkett *et al.*, 2004; **paper II** / Zhao *et al.*, 2022). It has to be pointed out, however, that comparative studies of odour profiles from different potential host species are rare, and that the quality and quantity of VOCs collected is shaped by the sampling method used (Tholl *et al.*, 2006; Dormont *et al.*, 2013; Omondi *et al.*, 2022).

Host-seeking mosquitoes are attracted to many different blends and compounds, often in a species-specific manner (reviewed in Smallegange and Takken, 2010; Dormont *et al.*, 2021; Coutinho-Abreu *et al.*, 2022). The VOCs most intensely studied are 1-octen-3-ol, lactic acid, ammonia and acetone, partly because early research primarily focussed on isolated compounds known to be present in aged human sweat (Dormont *et al.*, 2021). This research, supported by more recent studies, also led to the conclusion that blends of compounds, in general, are more attractive than individual VOCs (Bosch *et al.*, 2000; Smallegange *et al.*, 2005, 2009; Okumu *et al.*, 2010; Mukabana *et al.*, 2012; Omondi *et al.*, 2019). Lactic acid alone, for instance, is not attractive to most mosquito species, but synergises with CO_2, ammonia and 1-octen-3-ol (Acree *et al.*, 1968; Dekker *et al.*, 2002; Smallegange *et al.*, 2005; Hoel *et al.*, 2007), and is used as a constituent of many synthetic blends for mosquito surveillance and, possibly, control (Smallegange *et al.*, 2005, 2009; Okumu *et al.*, 2010; Mukabana *et al.*, 2012; Homan *et al.*, 2016).

Similar to CO_2, the attractiveness of host-derived VOCs is dependent on the plume structure. Human odour was attractive to *Ae. aegypti* and *An. gambiae* when presented as a homogenous plume, but less mosquitoes were attracted when the plume was turbulent and intermittent (Geier *et al.*, 1999a; Dekker *et al.*, 2001, 2005).

2.5 Host specialisation and discrimination

2.5.1 Discrimination between potential host species[1]

Most haematophagous mosquito species are assumed to be generalists, which feed opportunistically on hosts that are abundant and available in their habitat (Clements, 1999; Lehane, 2005; Lyimo and Ferguson, 2009; Takken and Verhulst, 2013). However, several species, including many considered disease vectors for pathogens infecting humans and other animals, are specialised on feeding on a limited host range (Clements, 1999; Lehane, 2005; Lyimo and Ferguson, 2009; Takken and Verhulst, 2013). Due to the severe consequences of mosquito-borne diseases for human health (see *1. Introduction*), anthropophilic species have been at the focus of research, which implies that the host preference of many mosquito species is unknown, thus questioning the above widespread statement that most mosquito species are generalists.

Discrimination among potential host species is primarily mediated by olfaction, and is based on qualitative and quantitative differences in host odour profiles (*e.g.*, Rudolfs, 1922; Costantini *et al.*, 1998; DeGennaro *et al.*, 2013; McBride *et al.*, 2014; **paper II** / Zhao *et al.*, 2022; reviewed in Lyimo and Ferguson, 2009; Takken and Verhulst, 2013; Wolff and Riffell, 2018; **paper V** / Hinze *et al.*, 2022). Several generic VOCs, with species-specific abundance, have been highlighted as candidate signature compounds for driving discrimination between human and non-human hosts, including lactic acid, ammonia, (*R*)-1-octen-3-ol, sulcatone and long-chain aldehydes (Braks *et al.*, 2001; Steib *et al.*, 2001; Dekker *et al.*, 2002; Cook *et al.*, 2011; McBride *et al.*, 2014; Majeed *et al.*, 2016; **paper II** / Zhao *et al.*, 2022). Despite the growing knowledge on candidate VOCs mediating host discrimination, the molecular and neuronal mechanism is only beginning to be understood.

Fuelled by recent advancement in the genetic toolkit of mosquitoes (*e.g.*, Reegan *et al.*, 2016; Matinyan and Potter, 2022), comparative transcriptomic

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[1] This topic has been reviewed in more detail in **paper V** (Hinze *et al.*, 2022).

studies identified several chemosensory genes, encoding for ORs, IRs and OBPs, to be differentially expressed in closely related species with divergent host preference (Rinker *et al.*, 2013; Neafsey *et al.*, 2015; Athrey *et al.*, 2017). Furthermore, by knocking out the OR co-receptor gene, *orco*, DeGennaro *et al.* (2013) demonstrated that the OR-pathway is necessary for host discrimination. Female *Ae. aegypti* lacking *orco* expression were attracted to host odours, but lost their preference for human over non-human odour. Another study targeted genetic differences between two forms of *Ae. aegypti*, an anthropophilic and a zoophilic form, and found a strong association between mosquito host preference, and expression and sensitivity of the sulcatone-detecting receptor AaegOR4 (McBride *et al.*, 2014).

The neuronal mechanism of host discrimination is mostly unknown, but a recent study by Zhao *et al.* (2022; **paper II**) demonstrated that human identity is encoded by the distinctive activation pattern of two glomeruli, one broadly-tuned, and the other responding to human-enriched VOCs, in the AL of female *Ae. aegypti*. The human-sensitive glomerulus is strongly activated by human odour, but responds only weakly, or not at all, to other animal odours, and is tuned to the long-chain aldehydes, decanal and undecanal, likely derived from human sebum (Wisthaler and Weschler, 2010). Mimicking the relative activation specific to human odour using a synthetic blend was sufficient to drive host seeking (**paper II** / Zhao *et al.*, 2022).

Host preference does not follow phylogeny (Zhou *et al.*, 2014; Wolff and Riffell, 2018), which demonstrates the plasticity and adaptability of the mosquito olfactory system throughout evolution that have resulted in the occupation of similar ecological niches several times independently. Anthropophily, for instance, which is found in several distantly related lineages within the Culicidae, is hypothesised to have evolved only within the past 10 000 years when humans became sedentary, subsequently more numerous, and thus a predictable resource (Besansky *et al.*, 1994, 2004; Ayala and Coluzzi, 2005; Rose *et al.*, 2020). Host specialisation, in general, may have evolved at the peripheral olfactory level by changes in the sensitivity, selectivity and proportion of OSNs responding to a to a given host odorant, the complete loss or gain of OSN types, and/or equivalent adaptations in higher brain centres, such as changes in tuning and connectivity of projection and local interneurons (van den Broek and den Otter, 1999; Rinker *et al.*, 2013; McBride *et al.*, 2014; Majeed *et al.*, 2016, 2017; Ghaninia *et al.*, 2019; Zhao and McBride, 2020 and references therein).

The evolutionary driver(s) of host specialisation, *i.e.*, the fitness benefits associated with feeding from a limited range of hosts, may be associated with the host itself or the ecological niche of the host (Petersen 1977; Lyimo and Ferguson, 2009; Rose *et al.*, 2020, 2022). In the case of anthropophily, while reports on the nutritional benefits of human blood are inconclusive (Woke, 1937; Nayar and Sauerman Jr, 1977; Harrington *et al.*, 2001; Lyimo *et al.*, 2012), additional fitness benefits, such as reduced mortality due to low host defensive behaviour, may exist (Lyimo and Ferguson, 2009). In addition, two recent studies by Rose *et al.* (2020, 2022) provide evidence that anthropophily in *Ae. aegypti* may have arisen as a by-product of human modification of their environment, resulting in a dependency and adaptation to human-made water storage when the climate in the Sahel became more arid circa 5 000 years ago (Petersen 1977; Tabachnick 1991). Mosquito host preference, and thereby host discrimination, may thus be the cause or outcome of specialisation (Futuyma and Moreno, 1988).

2.5.2 Discrimination within a host species: humans

Mosquitoes do not only discriminate between potential host species, but they also show a preference for some human individuals over others, which is likewise dependent on differences in VOC profiles (Brouwer, 1959; Lindsay *et al.*, 1993; Knols *et al.*, 1995; Mukabana *et al.*, 2002; Qiu *et al.*, 2006b; Verhulst *et al.*, 2013; De Obaldia *et al.*, 2022; **paper IV**). The olfactory mechanism underlying this discriminatory ability, however, is less clear since mosquitoes lacking expression of either *orco* or any of the IR co-receptor genes retain their differential attractiveness to humans (De Obaldia *et al.*, 2022). The abundance of several VOCs has been suggested to be involved in shaping individual attractiveness to mosquitoes, including select carboxylic acids, lactic acid, 2-ethyl-1-hexanol, 1-octen-3-ol, limonene, octanal, decanal, sulcatone and geranylacetone (Acree *et al.*, 1968; Logan *et al.*, 2008; Verhulst *et al.*, 2013; Leal *et al.*, 2017; De Obaldia *et al.*, 2022; **paper IV**).

Interestingly, several of these compounds have also been suggested to be involved in inter-specific host discrimination (Steib *et al.*, 2001; Dekker *et al.*, 2002; Cook *et al.*, 2011; McBride *et al.*, 2014; Majeed *et al.*, 2016; **paper II** / Zhao *et al.*, 2022), raising the question why mosquitoes ultimately are able to discriminate among humans (Kelly, 2001). On one hand, the differential attractiveness towards some individuals may be a by-product of another discrimination task, *i.e.*, highly attractive individuals are “more” human, *e.g.*, by taking an extreme position on a non-human to human scale, or by an odour composition close to the human average (McBride *et al.*, 2014; De Obaldia *et al.*, 2022; **paper II** / Zhao *et al.*, 2022). This implies that individuals preferred by an anthropophilic species would not be preferred by a zoophilic species, a hypothesis that remains to be tested. On the other hand, and not necessarily mutually exclusive, there may be a direct fitness benefit associated with preferring one person to another. Blood quality, and the ABO blood type, have been suggested as drivers for human discrimination, but there is thus far no consensus on the preferred ABO blood type (Wood, 1974; Shirai *et al.*, 2004; Anjomruz *et al.*, 2014; Khan *et al.*, 2022), and limited evidence of any fitness benefits of ABO-specific feeding (Khan *et al.*, 2022).

2.6 Modulation of odour-mediated host seeking and host choice

The olfactory system of an insect is plastic, and modulated according to intrinsic factors, such as life stage or physiological state, and extrinsic factors, *i.e.*, upon interaction with the environment (Gadenne *et al.*, 2016). Mosquito host seeking is modulated by age, mating status, circadian rhythm, feeding state, infection, as well as experience (Gadenne *et al.*, 2016; Hill and Ignell, 2021). In general, changes in behavioural response to host odours are correlated with the up- or downregulation of chemosensory gene expression levels and changes in OSN sensitivity, as well as regulated by neuromodulators (Gadenne *et al.*, 2016; Hill and Ignell, 2021).

After adult emergence, the maturation of the olfactory system of mosquitoes continues for a brief period, during which females do not respond to, or may even avoid, host odour (Davies, 1984a; Clements, 1999; Foster and Takken, 2004; Bohbot *et al.*, 2013; Omondi *et al.*, 2019; Tallon *et al.*, 2019). The acquisition of host-seeking competence is correlated with changes in the expression level of a subset of chemosensory genes and a gain in the sensitivity of OSNs tuned to CO_2 and salient host VOCs, such as lactic acid and 1-octen-3-ol (Grant and O’Connell, 2007; Bohbot *et al.*, 2013; Omondi *et al.*, 2015, 2019; Das De *et al.*, 2018; Tallon *et al.*, 2019). So far, it is unknown how age and adult maturation modulate central olfactory processes in mosquitoes, but studies in other insects report, *e.g.*, an increase in the synaptic density in the AL and a refinement of synaptic connectivity in the mushroom bodies (Winnington *et al.*, 1996; Devaud *et al.*, 2003; Groh *et al.*, 2012). Similar mechanisms may also play a role in the onset of host-seeking behaviour in mosquitoes.

Mating in female mosquitoes occurs one to three days post-adult emergence (Clements, 1999). Upon contact to conspecific males, the expression of several chemosensory-related genes, especially OBPs, is downregulated, which may facilitate mating by decreasing the general sensitivity to host odours (Alonso *et al.*, 2019). Following mating, most of these genes increase to roughly previous expression levels (Alonso *et al.*, 2019).

The daily rhythm of host-seeking activity is species-specific (Jones and Gubbins, 1978; Jones, 1981; Yee and Foster, 1992; Rund et al., 2013a), in which, during the peak of activity, the peripheral sensory system is more sensitive to attractive host compounds (Rund *et al.*, 2011, 2013a; Eilerts *et al.*, 2018). The expression of multiple chemosensory-related genes, coding for orco and several OBPs, underlie a circadian rhythm of expression, which is regulated by the circadian clock and/or the presence of light (Rund *et al.*, 2011, 2013a, 2013b; Das De *et al.*, 2018; Benoit and Vinauger, 2022).

After taking a complete blood meal, the behavioural and physiological response to host odours is inhibited, triggered by the expansion of the abdomen due to the uptake of blood, and not fully restored until after oviposition (Klowden and Lea, 1979a, 1979b; Davies, 1984b; Brown *et al.*, 1994; Takken *et al.*, 2001; Qiu *et al.*, 2006a). Subsequent to blood feeding, modulatory neuropeptides are released and the abundance of chemosensory genes are regulated (Klowden and Lea, 1979b; Brown *et al.*, 1994; Fox *et al.*, 2001; Marinotti *et al.*, 2006; Bonizzoni *et al.*, 2011; Liesch *et al.*, 2013; Christ *et al.*, 2017; Duvall *et al.*, 2019; Hill *et al.*, 2021). Taking a full sugar meal suppresses host seeking as well, but for a shorter duration, as the digestion of sugar is faster (Foster, 1995).

Pathogen infection occurs by taking a blood meal from an infected host and affects mosquito host-seeking behaviour, which has been interpreted as manipulation by the pathogen to increase disease transmission (Koella *et al.*, 2002; Hurd, 2003; but see Cator *et al.*, 2013). Dengue-infected female *Ae. aegypti*, for instance, show an increased behavioural and physiological sensitivity to human odour at an infection stage in which the virus is disseminated into the salivary glands, which is concomitant with an increased expression of neural signalling genes in the antennae (Tallon *et al*., 2020). A similarly enhanced attraction to human hosts during the infectious stage has been reported for *Plasmodium*-infected *An. stephensi* (Anderson *et al*., 1999). On a side note, not only does vector infection modulate mosquito host-seeking behaviour, but so does host infection by mosquito-borne pathogens, which affects host choice by making infected hosts more attractive than non-infected hosts (Lacroix *et al*., 2005; De Moraes *et al*., 2014; Yan *et al*., 2018; Zhang *et al*., 2022).

Experience may also modulate host seeking, which has been studied in the context of feeding experience, response to (host) odours and host choice (reviewed in Vinauger *et al*., 2016; Wolff and Riffell, 2018). Both positive and negative feeding experiences of taking a blood meal from preferred or non-preferred hosts were demonstrated to modulate innate host preference, but the mechanism is unclear (Mwandawiro *et al*., 2000; Vantaux *et al*., 2014). Moreover, mosquitoes were demonstrated to learn olfactory cues in an associative manner (Tomberlin *et al*., 2006; Chilaka *et al*., 2012; Menda *et al*., 2013), but learning seems to be dependent on the identity or innate valence of an odour, as some, but not all, odours can be learned in classical conditioning experiments (Vinauger *et al*., 2014, 2018; Wolff *et al*., 2019).

Host choice is plastic and, next to feeding experience and associative learning, may be affected by host density, accessibility and physiological state (reviewed in Lyimo and Ferguson, 2009; Takken and Verhulst, 2013; Wolff and Riffell, 2018). *Culex pipiens*, for example, feeds preferentially on the American robin, but switches to mammalian hosts, including humans, when birds migrate south in late summer and early fall (Edman and Taylor, 1968; Kilpatrick *et al*., 2006; Thiemann *et al*., 2011), and the highly anthropophilic *An. gambiae* feeds readily on cattle when humans are not available (Lefèvre *et al*., 2009). Similarly, *Ae. albopictus* that exhibits a zoophilic host preference, feeds to a large extend on humans when they are abundant, for instance in densely populated urban areas (Fikrig and Harrington, 2021; Fikrig *et al*., 2022). Furthermore, even the preference of mosquitoes for one human over another may be shaped by past feeding experience and learning (Vinauger *et al*., 2018). Interestingly, 1-octen-3-ol, which may be involved in inter-individual discrimination in naïve *Ae. aegypti* (**paper IV**), was shown to be a compound that can be associated with both an appetitive or an aversive context, suggesting it may be involved in learning attractive or defensive hosts (Vinauger *et al*., 2018; Wolff *et al*., 2019).

3. Aim and objectives

The overall aim of this thesis was to contribute to the body of knowledge on odour-mediated host seeking in disease vector mosquitoes, with a focus on identifying the mechanisms regulating this behaviour.

The first objective was to establish the workflow of a new experimental setup, the wind tunnel system, and to investigate the role of CO_2 as a sole cue in the host seeking of female *An. gambiae* (**paper I /** Hinze *et al*., 2021).

The second objective was to test whether a synthetic blend, mimicking the encoding of human odour within the antennal lobe, is sufficient to drive host seeking in female *Ae. aegypti* (**paper II** / Zhao *et al*., 2022).

The third objective was to investigate whether *AgamOR39* regulates the acquisition of host-seeking behaviour during adult maturation in female *An. gambiae* (**paper III**).

The fourth objective was to identify the underlying chemistry of the observed differential attractiveness of individual humans to host-seeking *Ae. aegypti* (**paper IV**).

4. Methodology

In this chapter, I will briefly present the two mosquito species and the methods used for the different projects. The papers may contain additional methods performed by collaborators, such as electrophysiology and functional imaging, which I will not elaborate here.

4.1 Mosquitoes: *Anopheles gambiae* and *Aedes aegypti*

Two different species of disease vector mosquitoes have been used throughout the studies, *An. gambiae* (*An. gambiae s.s.*) and *Ae. aegypti* (*Ae. aegypti aegypti*). Both species are closely associated with human habitations, highly anthropophilic in their host preference, and transmit a range of dangerous diseases (Scott and Takken, 2012; Takken and Verhulst, 2013; WHO, 2022). *Anopheles gambiae* is the principal vector of malaria and distributed in tropical and southern Africa, with a high abundance in rural habitats with regular rainfall (Coetzee *et al.*, 2000; della Torre *et al.*, 2005; Lehmann and Diabate, 2008; Kamdem *et al.*, 2012; WHO, 2022). Next to malaria, female mosquitoes may also transmit lymphatic filiarisis (WHO, 2022). The domestic *Ae. aegypti* originates in sub-Saharan Africa, from where it spread in consequence of global trade and human movement, likely starting with the slave trade in the 17th century, and is now distributed across tropical, subtropical and warm temperate regions of the globe (Brown *et al.*, 2014; Kraemer *et al.*, 2015; Powell *et al.*, 2018; Rose *et al.*, 2020, 2022). *Aedes aegypti* is mainly transmitting dengue, chikungunya, Zika, yellow fever and lymphatic filiarisis (WHO, 2022). As all mosquitoes, both species undergo complete metamorphosis, with aquatic larval and pupal stages and a terrestrial imago. The eggs of *Ae. aegypti* are resistant to desiccation and can be stored for long periods of time, which makes them an attractive species to breed in the laboratory (Clements, 1992). *Anopheles gambiae* needs to be reared continuously for colony maintenance. The peak activity periods of host seeking are different between the two species: while *An. gambiae* is nocturnal, *Ae. aegypti* is diurnal and crepuscular, with the peak activity period approximately four hours after and two hours before and after the subjective nightfall, respectively (Jones and Gubbins, 1978; Jones, 1981; Yee and Foster, 1992; Rund *et al.*, 2013a).

The mosquito strains used in the papers included in this thesis were the wild-type G3 strain and derived mutant lines for *An. gambiae* (**paper I** / Hinze *et al.*, 2021, **III**), as well as the Rockefeller and Orlando strains for *Ae. aegypti* (**paper II** / Zhao *et al.*, 2022, **IV**). As both species will thrive in humid tropical to subtropical climate (della Torre *et al.*, 2005; Lehmann and Diabate, 2008; Brown *et al.*, 2014), their rearing conditions were adjusted accordingly. The experiments were timed to match the period of their peak host-seeking activity (Jones and Gubbins, 1978; Jones, 1981; Yee and Foster, 1992; Rund *et al.*, 2013a).

4.2 Tracking and analysing mosquito flight

The 3D tracking of mosquito flight has been used to, *e.g.*, understand the host-seeking mechanism of different mosquito species, the integration of other modalities, escape dynamics, as well as to optimise mosquito control methods, such as baited traps and insecticide-treated nets (Cooperband and Cardé, 2006; Dekker and Cardé, 2011; Lacey and Cardé, 2011; Spitzen *et al.*, 2013, 2014; McMeniman *et al.*, 2014; Parker *et al.*, 2015; van Breugel *et al.*, 2015; Angarita-Jaimes *et al.*, 2016; Hawkes and Gibson, 2016; Cribellier *et al.*, 2018, 2020, 2022; Amos *et al.*, 2020). The recently established wind tunnel setup, 3D tracking and custom analysis pipeline allows for the study of different aspects of mosquito host seeking by analysing their flight manoeuvres in a climate-controlled environment in the absence of odour contamination by the observer (figure 1; **paper I** / Hinze *et al.*, 2021). The main advantage of tracking mosquito flight in a wind tunnel setup in comparison to field settings is that the location of an invisible odour plume can be approximated due to the laminar airflow. In contrast to other flight assays, such as tube olfactometers, a wind tunnel provides the physical space to allow for more natural behaviour.

This thesis investigated the response and flight behaviour of *An. gambiae* and *Ae. aegypti* females to different host volatiles and blends. As the two species differ in their circadian peak of host-seeking activity, the illumination by visible white light within the flight arena was adjusted accordingly. Mosquito flight was tracked by two cameras mounted above the wind tunnel that captured the reflection of infrared light on the wings and body of the mosquitoes (figure 1; see **paper I** / Hinze *et al.*, 2021 for a detailed description).

Classically, the response of a mosquito to an odour cue may be assessed by the sequential progression within the flight arena, from activation (here: leaving the release cage) to attraction (upwind orientation) and approaching the target (see *2.4.2 Activation and attraction*). The progression within the flight arena and, *e.g.*, contact with a target volume can be detected reliably from the tracked position of the mosquito (see **paper II** / Zhao *et al.*, 2022; see figure 4B). Additionally, mosquito flight parameters may be analysed in detail (**paper I** / Hinze *et al.*, 2021, **II** / Zhao *et al.*, 2022, **III**). The custom analysis scripts developed for the different projects within this thesis allow

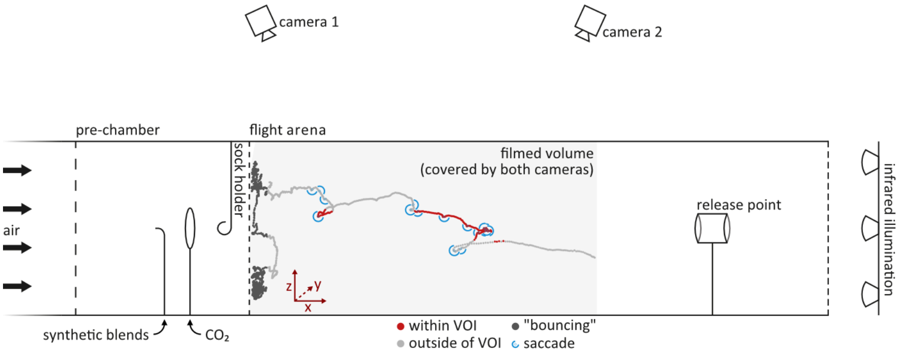

Figure 1: Schematic of the wind tunnel setup. A female *Anopheles gambiae* mosquito released at the release point enters the filmed volume (60 cm × 60 cm × 120 cm; grey background), where it flies to, and eventually lands on, the upwind screen, dividing the flight arena and the pre-chamber. Flight within the approximated volume of the plume (volume of interest; VOI; red), flight outside of the VOI (light grey) and ‘bouncing’ at the upwind screen (dark grey) is indicated. Saccades are denoted by blue semicircles. Cameras mounted above the wind tunnel record the reflections of infrared light on the wings and body of the mosquito. Odour cues may be presented by either a glass “tip” creating a homogenous plume, a glass “hoop” creating a turbulent plume (Dekker and Cardé, 2011) or by placing a used sock in a sock holder. Adapted from Hinze *et al.* (2021; **paper I**) and **paper III**.

for the quantification of flight parameters including duration, time within a select volume, *e.g.* the approximated volume of the odour plume (volume of interest; VOI) or the ‘bouncing’ at the upwind end, mean speed and speed in relation to a specific heading, as well as the frequency and consistency of turns (figure 1). Since mosquitoes leaving the release cage include both host-seeking and non-host-seeking individuals, an analysis recognising host-seeking flight was developed, by correlating mosquito flight parameters with visually identified host-seeking status (**paper II** / Zhao *et al.*, 2022). Visually, host-seeking flight is characterised by an increased speed, zigzagging in crosswind direction and sharp turns upon exiting the approximated VOI (*e.g.*, Dekker *et al.*, 2005; Dekker and Cardé, 2011). Using a kmeans clustering approach, host seeking in *Ae. aegypti* could be identified using the following parameters: flight duration (excluding bouncing), proportion of flight within the VOI, mean speed and mean tortuosity, as well as mean speed and proportion of crosswind flight. As the clustering was insensitive to short bouts of host seeking, the analysis was refined to 10 s segments of mosquito flight. Here, the proportion of flight spent within the VOI was the strongest predictor and a threshold was used for identifying host-seeking status (see **paper II** / Zhao *et al.*, 2022 for more details).

In short, tracking mosquito flight can be used to assess the effect of an odour cue on the behaviour of a mosquito by using a classical, although tracking-supported, approach, and by the analysis of mosquito flight manoeuvres in depth.

4.3 Odour collection and analysis

Human body odour was sampled by dynamic headspace extraction (Harraca *et al.*, 2012; **paper IV**). Individual undressed participants were placed in customised oven bags, where odourless synthetic air was introduced through a port at shoulder height. Odour-laden air was extracted from the bag through columns containing the adsorbent Porapak Q, which collects a large diversity of VOCs (Tholl *et al.*, 2006). The VOCs were then desorbed using an organic solvent, pentane, and the solution concentrated. Combined gas chromatography and mass spectrometry (GC-MS) was used to identify and quantify the VOCs extracted, in which the VOCs within a sample are separated by their affinity to the stationary phase of the GC, and identified by their relative retention time in comparison to a known standard and the electron impact fragmentation pattern (MS). Neat compounds have been injected to confirm the identity of putatively identified compounds. As human odour contains several hundreds of compounds (Bernier *et al*., 1999, 2000; Penn *et al*., 2007; Gallagher *et al*., 2008), the quantification was limited to the compounds that elicited an antennal response in *Ae. aegypti*, identified using combined GC and electroantennographic detection analysis of the human odour extracts.

5. Summary and discussion of results

5.1 Investigating odour-mediated host-seeking flight (papers I, II and III)

Both *An. gambiae* and *Ae. aegypti* modulate their flight in response to host-derived odour cues, but the extend, and the importance of the cues varies between the species (**paper I** / Hinze *et al*., 2021, **II** / Zhao *et al*., 2022, **III**). The role of CO_2 on its own, being universally released by all vertebrates, is hypothesised to be dependent on the degree of host specialisation of a given mosquito species, as a plume of CO_2 is informative only about the presence and location, but not the identity of a host (Gillies 1980; Mboera and Takken, 1997; Takken *et al*., 1997; Costantini *et al*., 1999). While the comparison of several *Anopheles* species provides evidence that the importance of CO_2 alone is correlated with the degree of specialisation in anopheline mosquitoes (Takken *et al*., 1997; Dekker and Takken, 1998), there is so far no clear consensus on the role of CO_2 in *An. gambiae*, despite its strong specialisation on humans (Takken and Verhulst, 2013).

The presence and concentration of a filamentous plume of CO_2, presented as the sole cue, did not affect the activation, attraction or the flight manoeuvres of *An. gambiae* females in the wind tunnel, unlike the combination of CO_2 and human body odour (figure 2A,B; **paper I** / Hinze *et al*., 2021). This is in line with previous studies demonstrating that CO_2 on its own does not play a role in *An. gambiae* host seeking (de Jong and Knols, 1995a; Takken *et al*., 1997; Spitzen *et al*., 2008). However, in other studies, this cue was sufficient to trigger activation and/or attraction (Healy and Copland, 1995; Dekker *et al*., 2001; Lorenz *et al*., 2013), which may be due to the unintentional presence of other host volatiles (Webster *et al*., 2015). The results presented in **paper I** (Hinze *et al*., 2021) do not resolve the disagreement, but support the specialist hypothesis: host seeking in *An. gambiae* is evoked only if CO_2 is combined with host odour, an interaction that is synergistic (see also Takken *et al*., 1997; Njiru *et al*., 2006). Although not specifically addressed, the results of experiments performed in *Ae. aegypti* (**paper II** / Zhao *et al*., 2022) are in congruence with the reported role of CO_2 alone as both an activator and an attractant in this species (*e.g.*, Dekker *et al*., 2005; Dekker and Cardé, 2011). Thus, as the specialist hypothesis does not hold true for *Ae. aegypti*, it is likely that CO_2 is differentially integrated in the two species.

The surge-and-cast mechanism of odour-mediated optomotor anemotaxis in response to the combination of CO_2 and human foot odour in *An. gambiae* was reflected by a larger proportion of flight spent within the approximated location of the plume (volume of interest; VOI) and a higher mean speed in crosswind direction, when compared to the controls (figure 2B; **paper I** / Hinze *et al*., 2021; see *4.2 Tracking and analysing mosquito flight*). The results presented in **paper III** did not analyse host-seeking flight of *An. gambiae* in the context of different stimuli, but rather different age groups and genotypes. Nevertheless, the flight parameters can be compared in the context of host seeking, as newly-emerged (teneral) females do not host

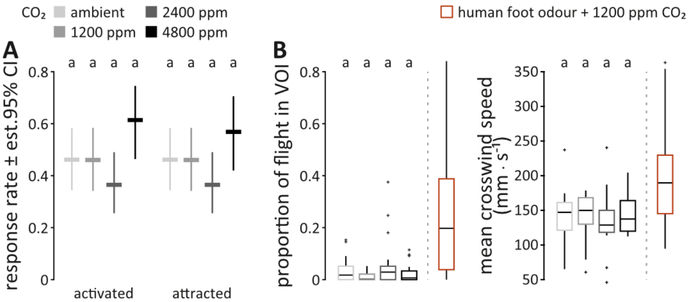

Figure 2: Carbon dioxide on its own does not play a role in *Anopheles gambiae* host seeking. (**A**) Proportion of mosquitoes activated (leaving the release cage) and attracted (entering the filmed volume) by ambient air and increasing concentrations of CO_2 (grey shades). (**B**) Flight parameters in response to increasing concentrations of CO_2: proportion of mosquito flight within the approximated volume of the plume (volume of interest; VOI) and mean flight speed in crosswind direction. The flight parameters in response to a combination of human foot odour and 1200 ppm CO_2 (red) are added for visual comparison, but please note that the data is from two separate experiments (dashed line). Data from **paper I** (Hinze *et al*., 2021).

seek, while mature females do (Davies, 1984a; Foster and Takken, 2004; Bohbot *et al.*, 2013; Omondi *et al.*, 2019; Tallon *et al.*, 2019). In comparison to teneral females, the flight manoeuvres of mature females in response to CO_2 and a synthetic blend mimicking human body odour (Omondi *et al.*, 2019) was more persistent, as reflected by a longer flight duration and a higher proportion of flight 'bouncing' at the upwind screen (figure 3B,C). Moreover, the flight of mature females was characterised by a higher turning frequency and turn consistency (figure 3D). Neither the proportion of flight time spent within the VOI, nor the crosswind speed, was significantly different between the two age groups, which may reflect the superiority of full human odour over a synthetic blend by, *e.g.*, containing a broader range of VOCs, including VOCs of low volatility that may act as close-range cues (Healy and Copland, 2000; Healy *et al.*, 2002; Bello and Cardé, 2022). However, it is difficult to draw robust conclusions, as the two cues were not directly compared and the analysis pipeline included more features in **paper III** than in **paper I** (Hinze *et al.*, 2021).

The host-seeking flight of *Ae. aegypti* in response to CO_2 and human foot odour is characterised by an increase in the proportion of flight spent within the VOI and an increased proportion and speed of crosswind flight (**paper II** / Zhao *et al.*, 2022; data not shown). For predicting host-seeking status at a high temporal resolution, *i.e.*, in segments of 10 s, the proportion of flight in the VOI had the highest predictive power and was thus used for consecutively classifying mosquito flight as host seeking or non-host seeking (**paper II** / Zhao *et al.*, 2022; see *4.2 Tracking and analysing mosquito flight*).

In conclusion, the newly-established wind tunnel setup, flight tracking and custom analysis pipeline allowed for the quantification of not only "classical" parameters, such as activation, attraction and spatial progression within the wind tunnel, but also for an in-depth analysis of mosquito host-seeking flight. The modulation of both *An. gambiae* and *Ae. aegypti* flight, when encountering an odour plume, is corresponding to previous reports (*e.g.*, Dekker *et al.*, 2005; Dekker and Cardé, 2011; Spitzen *et al.*, 2013; Lacey *et al.*, 2014; Hawkes and Gibson, 2016).

5.2 Age-dependent modulation of host seeking (paper III)

Host contact at times when a female mosquito is not able to take and/or process a blood meal, *e.g.*, before completing adult maturation or after a full blood meal, is heavily penalised due to the risk of being killed by the host and non-profitable energy expenditure (Webber and Edman, 1972; Kelly, 2001; Lehane, 2005; Klowden, 2007a). The high adaptive pressure has favoured an age- and state-dependent targeted regulation of the olfactory system (see *2.6 Modulation of odour-mediated host seeking and host choice*), which, in the case of adult maturation, is correlated with an increase in the attraction to human odour and its volatile components (Davies, 1984a; Foster and Takken, 2004; Bohbot *et al.*, 2013; Omondi *et al.*, 2019; Tallon *et al.*, 2019). During adult maturation, OSNs tuned to salient host compounds change in sensitivity, concomitant to alterations in the expression level of several chemosensory genes (Davies, 1984a; Grant and O'Connell, 2007; Bohbot *et al.*, 2013; Omondi *et al.*, 2015, 2019; Tallon *et al.*, 2019). The molecular mechanism underlying the observed increase in attraction to human odour is, however, unknown. Omondi *et al.* (2019) demonstrated a subset of VOCs, detected by the OR pathway, to be sufficient to replicate the observed behavioural shift in the response to human odour in *An. coluzzii*, and identified several candidate OR genes, whose expression is regulated in an age-dependent manner. Among them, *AcolOR39* was identified as a promising candidate, as the encoded receptor is tuned to one of the major compounds of human odour, sulcatone, and its main ligands, sulcatone and 1-hexanol, but not the synthetic human blend lacking these compounds, elicited aversion in newly-emerged females. As *AcolOR39* transcripts decrease significantly in abundance during adult maturation, the corresponding pathway was hypothesised to mediate aversion to key compounds of human odour in teneral females (Omondi *et al.*, 2019).

To investigate the role of the functional orthologous gene, *AgamOR39* (hereafter *OR39*), in the acquisition of host seeking in the closely related *An. gambiae*, we tested the neuronal and behavioural responses of wild-type females, and of females lacking *OR39* expression, to a blend of human-derived VOCs (**paper III**; Omondi *et al.*, 2019). *Anopheles gambiae* show a similar down-regulation of *OR39* expression during adult maturation to its sibling-species *An. coluzzii* (Machado *et al.*, *in preparation*). We thus hypothesised that the response of *OR39* CRISPR-Cas9 knockout teneral *An. gambiae* to the human odour blend is similar to that of mature wild-type females, owing to the release from behavioural aversion, presumably mediated by the OR39 pathway. The results demonstrate that mosquito flight behaviour in the wind tunnel is significantly affected by the age of the mosquito, as mature wild-type females exhibit a lower response threshold and a more persistent host-seeking behaviour than teneral females (figure 3A-C). Moreover, their flight is characterised by a higher turning frequency and turn consistency (figure 3D). Thus, *An. gambiae* display a similar age-dependent onset of host-seeking behaviour as previously demon-

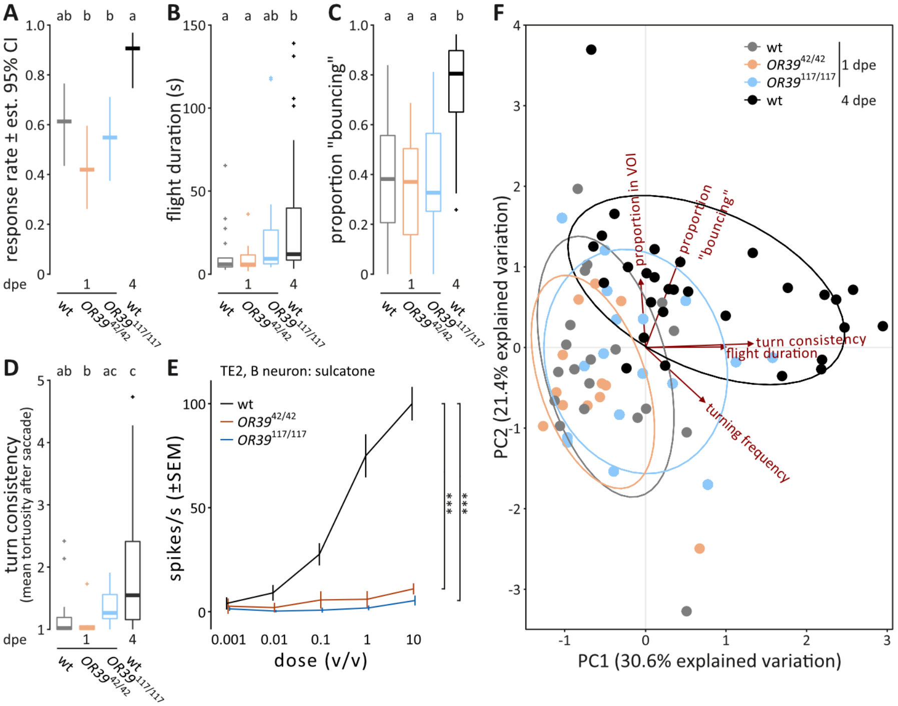

Figure 3: The role of *OR39* in the acquisition of host seeking in *Anopheles gambiae* females. (**A**) Response rate of wild-type (wt) and homozygous mutant (*OR39*$^{42/42}$, *OR39*$^{117/117}$) females of two age groups, teneral (1 day post-emergence; dpe) and mature (4 dpe), to CO_2 and a synthetic human blend (Omondi *et al*., 2019). (**B**) Flight duration, (**C**) proportion of flight time spent ‘bouncing’ at the upwind screen and (**D**) turn consistency, defined as the tortuosity of the flight path directly after a saccade. (**E**) Electrophysiological recording from the TE2-B neuron to increasing doses of sulcatone. (**F**) Principal component analysis (PCA) of select flight parameters: proportion of flight time spent within the approximated volume of the plume (volume of interest; VOI), proportion ‘bouncing’, turn consistency, flight duration and mean turning frequency. The PCA was optimised for maximal separation between the two wild-type age groups. Adapted from **paper III**.

strated in *An. coluzzii* (Omondi *et al.*, 2019). Electrophysiological analysis of sensilla housing OSNs sensitive to sulcatone in wild-type mosquitoes, and the paralogous OSNs in the two CRISPR-Cas9 generated knockout lines, revealed that the detection of sulcatone was abolished in mutant mosquitoes (figure 3E). Despite the non-functional OSN, and in contrast to our hypothesis, the lack of *OR39* expression did neither affect attraction, nor flight parameters, in teneral mosquitoes (figure 3A-D,F).

In conclusion, at least in the context tested, the onset of host seeking in *An. gambiae* is likely not mediated by the age-dependent downregulation of *OR39* expression. However, this does not exclude the involvement of *OR39* as a contributing factor in the acquisition of host seeking, and it may be that OR39 plays a role in, *e.g.*, resource discrimination. In the current working hypothesis, OSNs expressing *OR39*, in concert with other OSNs tuned to sulcatone (Carey *et al.*, 2010), may act as a threshold detector for sulcatone, which will be explored in further behavioural experiments, including two-choice assays.

Despite reporting negative results, this study (**paper III**) is among the first to investigate the mechanism by which a single tuning OR can affect mosquito host-seeking behaviour. With the recent progress in the development of genetic tools in mosquitoes, the field is now ready to advance one step further and establish causality, not just correlation, between mosquito behaviour and, *e.g.*, regulation of chemosensory genes (Reegan *et al.*, 2016; Zhao and McBride, 2020; Matinyan and Potter, 2022).

5.3 Encoding of human odour in mosquito host discrimination (paper II)

Mosquitoes rely heavily on olfactory cues not only to locate a potential host, but also to discriminate between host species (reviewed in Takken and Verhulst, 2013; Wolff and Riffell, 2018; **paper V** / Hinze *et al.*, 2022). Host odour is composed of hundreds of VOCs, of which several of those detected by mosquitoes are shared across host species, with species-specific differences in their relative abundance (Braks *et al.*, 2001; Steib *et al.*, 2001; Dekker *et al.*, 2002; Cook *et al.*, 2011; McBride *et al.*, 2014; Majeed *et al.*, 2016; **paper II** / Zhao *et al.*, 2022). Several mosquito species, such as *Ae. aegypti*, evolved a strong innate odour-mediated preference for human hosts (Gouck, 1972; McBride *et al.*, 2014; McBride 2016; Powell *et al.*, 2018), but it is unclear how host identity is encoded on the neuronal level, enabling host recognition and thus discrimination.

Presented in **paper II** (Zhao *et al.*, 2022), the analysis of the volatile emissions from humans, other animals, as well as nectar sources, provides a comprehensive quantitative comparison across multiple ecologically relevant odour sources and identifies several compounds with differential abundance between hosts and non-hosts. Human odour is characterised by a higher relative abundance of sulcatone, geranylacetone, acetoin, decanal and undecanal, and a lower relative abundance of the short-chain aldehydes hexanal and heptanal. Calcium imaging in the AL of transgenic *Ae. aegypti* females expressing the calcium indicator GCaMP6f under the control of the *orco* locus, reveals that human and non-human odours evoke distinct, although overlapping, patterns of glomerular activity. The relative activation of three glomeruli, one broadly tuned, one predominantly responding to human odour, and one activated by non-human animal odour, condensed the complexity of the odour profiles to a comparatively simple code (figure 4A). The glomerulus activated by human odour (H glomerulus), but not, or only weakly, by non-human animal odour, is narrowly tuned to the long-chain aldehydes, decanal and undecanal, while the broadly-tuned glomerulus (B glomerulus) is activated by a range of compounds of both human and non-human origin. In a background of CO_2, a binary blend of decanal and 1-hexanol, which was designed and adjusted to mimic the glomerular activity elicited by human odour, was sufficient to drive host seeking, quantified by the sequential progression within the flight arena and an automated classification analysis (see *4.2 Tracking and analysing mosquito flight*), to an extent similar to human foot odour (figure 4B). Human identity is thus encoded by the relative activity of two glomeruli within the AL of *Ae. aegypti* (figure 4A), a coding principle first demonstrated in the context of plant seeking (Lahondère *et al.*, 2020).

Several open questions, however, remain. First, whether the relative activation of the three glomeruli are sufficient to drive host discrimination, *i.e.*, if the human-mimic blend is preferred to an animal-mimic blend, needs to be assessed. Second, it is unclear how the glomerular representation of human odour changes in the presence of CO_2. Third, it would be interesting to explore the evolutionary reason why the H glomerulus is tuned to long-chain aldehydes, even though sulcatone and geranylacetone are even more human-specific (**paper II** / Zhao *et al.*, 2022, extended data), and thus would provide a more precise coding of human identity. Moreover, a study by McBride *et al.* (2014), suggested sulcatone, and the increased sensitivity of its cognate receptor, AaegOR4, to govern the preference for human odour in the anthropophilic form of *Ae. aegypti*, although adding a human equivalent of sulcatone to the odour of non-preferred host did not alter host preference. The apparent discrepancy between the study by McBride *et al.* and the findings presented in **paper II** (Zhao *et al.*, 2022) may be explained by a higher evolutionary cost for repurposing the sulcatone pathway in comparison to the long-chain aldehyde pathway. Assuming that the ancestral zoophilic *Ae. aegypti* used the same combination of glomeruli for encoding the suitability of a host, it may be that the H glomerulus in today's anthropophilic form was previously tuned to aldehydes of shorter chain length, such as hexanal and heptanal, which are enriched in the odour of other

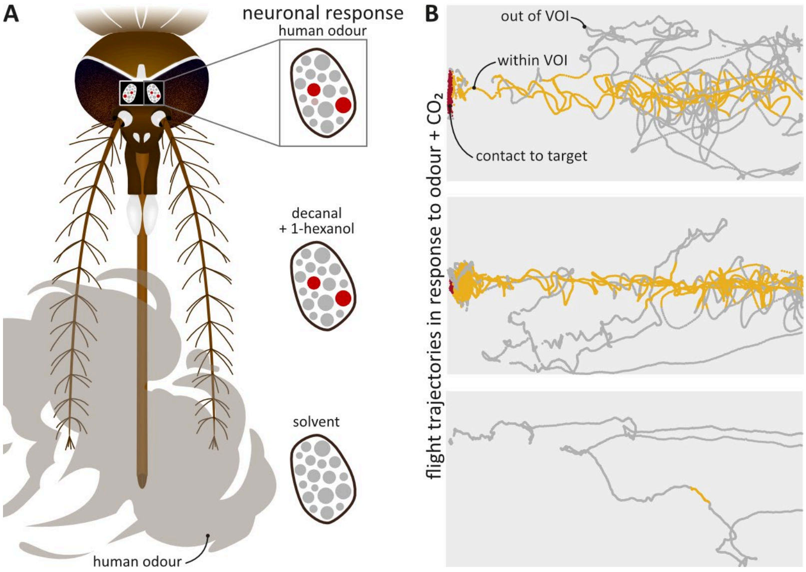

Figure 4: Encoding of human identity in female *Aedes aegypti*. (**A**) Schematic of glomerular activation within the antennal lobe (AL) in response to human odour, a blend mimicking the glomerular activation pattern of human odour, and the solvent of the blend. (**B**) Top view of representative flight trajectories in response to the odour given on the left and carbon dioxide. Flight within the approximated volume of the plume (volume of interest; VOI) is indicated in yellow and flight outside of the VOI is indicated in grey. Positions within the target volume at the upwind end of the flight arena are denoted in red. The blend mimicking the glomerular activation pattern, in combination with CO_2, is sufficient to elicit host seeking in *Ae. aegypti* females. Data from Zhao *et al.* (2022; **paper II**).

vertebrates (Bernier *et al.*, 2008; **paper II** / Zhao *et al.*, 2022). The shift in receptor tuning may have been less costly than changing the central neuronal connectivity to include OSNs expressing *AaegOR4* in the circuitry encoding host suitability (Zhao and McBride, 2020). Examples of receptor evolution in related species, resulting in a shift in receptor tuning and altered host preference, are known from other insects (*e.g.*, Prieto-Godino *et al.*, 2017; Auer *et al.*, 2020).

Furthermore, sulcatone, as well as geranylacetone, are, due to their putative origin from human-specific sebum (Wisthaler and Weschler, 2010), not only enriched in human odour in comparison to non-human odour, but they are common compounds of floral odour, and thus likely used by haematophagic and non-haematophagic mosquitoes in a plant-seeking context (Knudsen *et al.*, 2006; Dekel *et al.*, 2019). Nevertheless, the sensitisation of the AaegOR4 pathway, as an adaptation to the high sulcatone content of human odour (McBride *et al.*, 2014), may still contribute to the preference to human odour, even though human identity is encoded by the relative activation of the two glomeruli putatively encoding host suitability in ancestral *Ae. aegypti*. It would be informative to test this hypothesis by repeating the imaging experiment in the zoophilic form of *Ae. aegypti* to investigate the tuning and contribution of the aldehyde receptor in host discrimination.

In conclusion, while we are only beginning to understand how mosquitoes encode human odour, a prerequisite for host discrimination, current results suggest that host identity in *Ae. aegypti*, in both a blood feeding and nectar seeking context, is encoded by the relative activation of a limited number of glomeruli within the AL (Lahondère *et al.*, 2020; **paper II** / Zhao *et al.*, 2022).

5.4 The chemistry of human differential attractiveness (paper IV)

Apart from discriminating among potential host species, (anthropophilic) mosquitoes show an odour-mediated preference for some individual humans over others (Brouwer, 1959; Lindsay *et al.*, 1993; Knols *et al.*, 1995; Mukabana *et al.*, 2002; Qiu *et al.*, 2006b; De Obaldia *et al.*, 2022). Several compounds, including decanal, geranylacetone and sulcatone, were suggested to drive this inter-individual preference, but the underlying chemistry for the differential attractiveness of humans is poorly understood, and previous studies find only little common ground, are partially contradictory and some suffer from a low sample size (Logan *et al.*, 2008; Verhulst *et al.*, 2013; Leal *et al.*, 2017; De Obaldia *et al.*, 2022; **paper II** / Zhao *et al.*, 2022).

The work conducted in **paper IV** tested the relative attractiveness of 43 volunteers, identifying as women, to *Ae. aegypti* in a dual-choice landing assay (figure 5A), collected whole-body odour by dynamic headspace sampling and analysed the resulting odour extracts using GC-EAD and GC-MS (see *4.3 Odour collection and analysis*). The relative attractiveness of a volunteer was significantly affected by their ABO blood type and their menstrual cycle phase or pregnancy (figure 5A), but not by the use of hormonal contraception. The ABO blood type was previously suggested to affect individual attractiveness to mosquitoes, but the evidence is scarce and inconclusive, and the mechanism for the preference is unknown (Wood, 1974, 1976; Shirai *et al.*, 2004; Anjomruz *et al.*, 2014; Khan *et al.*, 2022). Even this study does not find a strong effect of the ABO blood type, as only the pairwise comparison was between participants with blood type A and AB was significant. While pregnancy is well established as a factor increasing individual attractiveness to malaria vectors (Lindsay *et al.*, 2000; Ansell *et al.*, 2002; Himeidan *et al.*, 2004), there was so far no report in *Ae. aegypti*. Moreover, the increased attractiveness has been predominantly ascribed to a higher breathing frequency, and thus a higher amount of exhaled CO_2 and breath-related compounds (Lindsay *et al.*, 2000; Ansell *et al.*, 2002), while the present study finds the body odour to play a role. As sulcatone, a derivative of human skin lipids (Wisthaler and Weschler, 2010), is associated with the high attractiveness of pregnant participants, the alterations of sebum composition and secretion during pregnancy may be a possible mechanism (Burton *et al.*, 1970; Shuster and Thody, 1974; Yang *et al.*, 2021). However, due to the low sample size for this group, additional experiments are needed to confirm the observed effect. Interestingly, there was also an effect of the menstrual cycle phase on individual attractiveness, with a significant difference between volunteers in the first and last week of their cycle. While it is well established that the odour of women changes with their menstrual cycle, as this is detectable by the human nose (Singh and Bronstad, 2001; Havlíček *et al.*, 2006; Gildersleeve *et al.*, 2012), the underlying changes in the VOC profile are unknown. Moreover, to our knowledge, there has so far only been one study addressing the effect of the menstrual cycle phase on mosquito attraction (Gilbert *et al*., 1966), mainly because menstruating persons have been excluded from studies investigating differential attractiveness, or their cycle phase has not been taken into account.

Of the 28 bioactive compounds identified in the collected volatile samples, the relative abundance of 1-octen-3-ol was significantly correlated

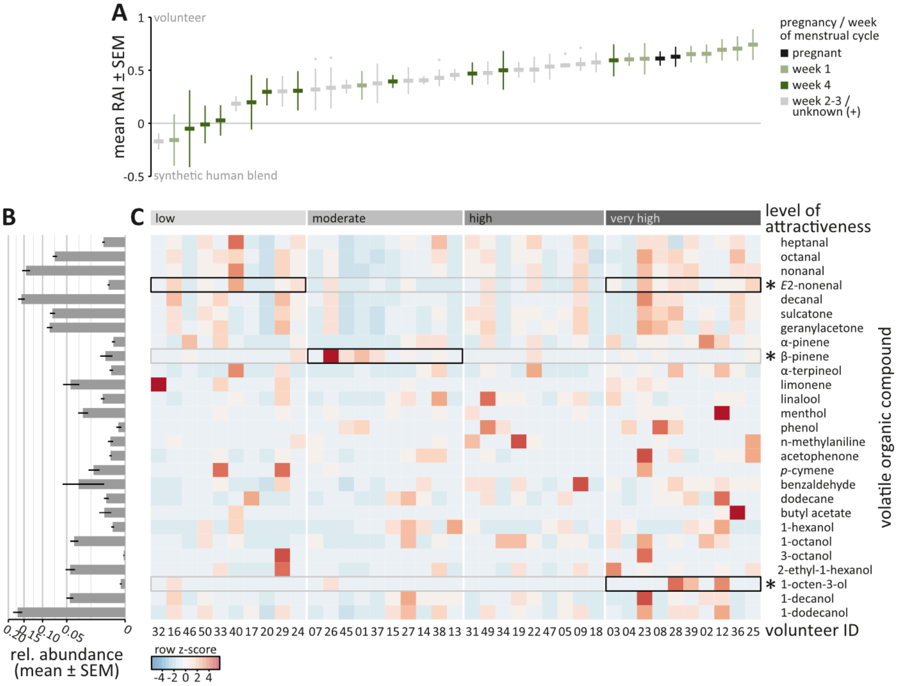

Figure 5: Differential attractiveness of female *Aedes aegypti* to individual human volunteers. (**A**) Relative attractiveness index (RAI). A positive value indicates that the odour of the volunteer is more attractive than the synthetic human blend, and a negative value that the blend is more attractive than the volunteer. Both pregnant participants and participants in the first week of their menstrual cycle were significantly more attractive than participants in their fourth week of their cycle. (**B**) Mean relative abundance of the bioactive compounds identified in the odour extracts, collected from human participants. Compound names are listed to the far right. (**C**) Heat plot displaying the divergence of VOC abundance from the mean. Blue colour refers to lower abundance and red to a higher abundance than the mean (row z-score). Level of attractiveness of the volunteers is shown above the plot. Asterisks and light grey boxes indicate VOCs with significantly higher similarity of VOC abundance within (black box) than between levels of attractiveness. Only 1-octen-3-ol was significantly more abundant in highly attractive participants. Modified from **paper IV**.

with very high attractiveness (figure 5C). Not all samples of highly attractive individuals, however, were characterised by a high relative abundance of the compound, suggesting multiple odour-driven mechanisms for differential attractiveness. This pattern was also suggested in a recent study by De Obaldia *et al.* (2022), which identified select carboxylic acids to be enriched in many, but not all attractive volunteers. Future experiments will establish whether an increased abundance of 1-octen-3-ol in the background of a synthetic blend containing the other bioactive VOCs is sufficient to drive mosquito preference. Here, it is also important to note that the VOCs collected by a given method are limited by the odour collection method used (*e.g.*, Tholl *et al.*, 2006; Omondi *et al.*, 2022), and other compounds important for defining differential attractiveness may have been missed.

1-Octen-3-ol is the second most investigated mosquito attractant after CO_2, and, despite its well-known attractive effect on *Aedes* and *Anopheles* mosquitoes, it is only rarely identified in human body volatile extracts (reviewed in Dormont *et al.*, 2021), likely due to its low relative abundance (figure 5B; Omondi *et al.*, 2019). Next to its occurrence in human odour, 1-octen-3-ol is a generic compound present in the odour of other vertebrates, as well as of flowers and fungi (Cronin and Ward, 1971; Knudsen *et al.*, 2006; Majeed *et al.*, 2016; Dickschat, 2017; **paper II** / Zhao *et al.*, 2022). Thus, 1-octen-3-ol is likely involved in multiple contexts, including host seeking, host discrimination and nectar seeking (Takken and Kline, 1989; Dekel *et al.*, 2016; Majeed *et al.*, 2016).

The olfactory mechanism by which mosquitoes differentiate between individuals, resulting in inter-individual preference, is still unclear. One possibility is that the odour of preferred individuals may be “more” human, *e.g.*, by eliciting a stronger response in the pathway encoding human identity (**paper II** / Zhao *et al.*, 2022; see *5.3 Encoding of human odour in mosquito host discrimination (paper II)*). This may either be achieved by a higher relative abundance of long-chain aldehydes that activate the human-specific glomerulus, or by an activation pattern, *i.e.*, odour profile, that is closer to the human average (**paper II / **Zhao *et al.*, 2022). We found no evidence that the pathway encoding human identity may play a role in the differential attractiveness to humans, as neither decanal was enriched or intermediate, nor was the ratio of decanal to 1-hexanol larger or intermediate in volunteers more attractive to mosquitoes (**paper IV**; supplementary material). While 1-octen-3-ol was demonstrated to weakly activate both glomeruli involved in the encoding of human identity (**paper II** / Zhao *et al*., 2022; extended data), the effect is most likely negligible due to the low abundance of the compound in human odour (Omondi *et al*., 2019; **paper IV**).

To explore if intra-specific host preference is an untargeted effect of encoding human *vs* non-human identity, *e.g.*, by the pathway identified by Zhao *et al*. (2022; **paper II**), it will be critical to compare the intra-specific host preference among species of different inter-specific host preferences. Similar to not knowing if different mosquito species prefer the same human individuals, there are only few studies investigating intra-species variations in the attractiveness of individuals of the same non-human species. A recent preprint addressing the innate host preference of *Ae. albopictus*, demonstrated a preference for the same guinea pig over another across different colonies of *Ae. albopictus* and *Ae. aegypti* (Fikrig *et al*., 2022). Further research is required to establish whether differential attractiveness to some individuals of a species over others is a general principle in mosquito attraction. Understanding differential attractiveness to mosquitoes is crucial to assess its implications for both individual health and heterogeneity in disease transmission (Woolhouse *et al*., 1997; Kelly, 2001).

6. Concluding remarks and perspectives

Mosquito host seeking and discrimination is the critical determinant of the interaction of humans and mosquitoes. Understanding the underlying chemistry, behaviour and neurobiology is crucial for shaping that interaction and combatting mosquito-borne disease transmission. This thesis combined approaches from multiple disciplines and explored diverse aspects of host seeking, from the importance of individual volatile cues, to the role of chemosensory gene expression in its regulation, the encoding of host identity and the chemical basis of differential human attractiveness. Due to major advancements within the past few years, especially the development of CRISPR-Cas9 and other genetic tools, the field is catching up with the progress made in the insect model species, *D. melanogaster*, and now allows for the investigation of the mechanisms of odour-guided behaviours.

Many open questions remain in order to understand the chemical and neuronal basis of host seeking and discrimination in mosquitoes. First, there is a need for quantitative studies comparing odour profiles across multiple host and non-host species, including compounds that are technically difficult to collect and quantify. Second, the knowledge concerning the chemoreceptor repertoire of mosquitoes is insufficient, as only one receptor class in a single species has been deorphanised systematically thus far. To be able to understand the detection and coding of olfactory cues at the molecular level, the receptors involved, and their tuning to ecologically-relevant odour doses, need to be identified. Moreover, recent findings challenge the canonical organisation of the olfactory system as one receptor – one neuron – one glomerulus, increasing the potential complexity of odour coding and integration. Third, the neuronal architecture within the antennal lobe beyond the OSNs is unknown, and it is thus unclear how a complex blend, such as human odour, may be condensed to the activation of only a few glomeruli.

Likewise, the translation of the simple encoding of human identity at higher olfactory levels needs to be explored. Lastly, the focus of research has almost exclusively been on those mosquito species specialised on human hosts, and a few of their closest relatives. Thus, it is unknown if a simple representation of host identity is transferrable to other mosquito species. The host seeking and discrimination of most of the 3 500 mosquito species identified so far is unknown, providing a rich diversity for further investigation.

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Popular science summary

Mosquitoes are not only a nuisance, but pose a serious threat to human health and economic development by passing on a slew of dangerous diseases, including malaria, dengue, yellow fever and Japanese encephalitis. Infection occurs when a female mosquito, which carries pathogens in her saliva, bites a human to take a blood meal for obtaining nutrients needed for the development of her eggs. During the process of biting, she injects her saliva, which prevents blood clogging, into the host and thereby also her infectious load. Despite enormous efforts over the past decades to control mosquito populations and the diseases they transmit, mosquitoes kill more than 700 000 people, and impact the lives of millions, every year. In order to combat mosquito-borne disease by novel strategies targeting the flying vehicle, we need to understand their biology in more detail. This thesis investigated several aspects of the host-seeking behaviour of female mosquitoes, which is critical for locating a suitable host for a blood meal.

Female mosquitoes locate hosts, and assess their suitability, predominantly by using their sense of smell. They detect volatile compounds from host breath and body odour using odour-sensitive neurons situated in their antennae. Carbon dioxide (CO_2) is of special interest, as it is a strong host cue, but exhaled by all vertebrates and therefore does not contain information on the suitability of the source for mosquitoes specialised on feeding on a few species. Among them are the malaria mosquito, *Anopheles gambiae*, and the yellow fever mosquito, *Aedes aegypti*, which are specialised on humans. Consequently, it is assumed that the role of CO_2 in host seeking varies with the degree of mosquito specialisation, a hypothesis to which we could add evidence for in *An. gambiae*. Only in combination with other host odours does CO_2 make sense for this highly human-specialised mosquito.

The ability to tell host species apart, such as humans from livestock that live in the same area, is undeniably an important prerequisite to the strong preference for humans in some mosquito species, and is a key factor in disease transmission. Host odour, which is characteristic for a given host species in comparison to another, is unique. Interestingly, this is mainly due to different ratios of shared volatile odorants and not to the existence of host species-specific odour components. How the mosquito brain solves this challenge of many shared volatiles in preferred and non-preferred hosts, and encodes host identity, was, however, unknown. We found that human odour elicits a distinct activation pattern of surprisingly small proportions within the first olfactory centre of the brain, the antennal lobe, of *Ae. aegypti*, which is different from the pattern elicited by any other animal odour. Mimicking this pattern using a mixture of synthetic odorants elicits the characteristic zigzagging host-seeking flight of mosquitoes, suggesting that human identity is encoded by the relative activation of only few brain areas.

Not only do some mosquito species prefer humans to other animals, but they also prefer some human individuals to others. We found that the ABO blood group, as well as pregnancy and the menstrual cycle phase affect the attractiveness of individual volunteers to *Ae. aegypti*. The mushroom alcohol, 1-octen-3-ol, which, despite its name is not only found in mushrooms, is more dominant in the odour of highly attractive individuals.

The propensity of host seeking in female mosquitoes is not static over time, but modulated throughout their life. In contrast to mature females, newly-emerged female mosquitoes do not prefer human odour, which is most likely due to the up- and/or downregulation of genes involved in olfaction, but the mechanism is unknown. We tested a candidate gene, *AgamOR39*, identified in previous research in *An. gambiae*, which codes for an odorant receptor binding to sulcatone, one of the major compounds of human odour. Through CRISPR-Cas9 gene editing, we generated mutant mosquitoes, in which the sulcatone detection via this pathway was abolished, but their host-seeking behaviour was not affected. This implies that *AgamOR39* is not key for the onset of host seeking. Further research will investigate the role of this gene in more detail.

Taken together, this thesis sheds light on multiple aspects of odour-mediated host seeking and discrimination in mosquitoes. The findings could lead to ultimately moving the boundaries of knowledge on combatting mosquito-borne disease forward and reduce their burden on human health.

Populärvetenskaplig sammanfattning

Stickmyggor är inte bara en olägenhet utan utgör också ett allvarligt hot mot människors hälsa samt ekonomisk utveckling genom att de överför en rad farliga sjukdomar, däribland malaria, denguefeber, gula febern och japansk encefalit. Infektionen sker när en honmygga, som bär på patogener i sin saliv, biter en människa för att ta en blodmåltid för att få de näringsämnen som behövs för att utveckla sina ägg. Under bettet sprutar hon in sin saliv, som förhindrar att blodet koagulerar, i värden och därmed också de sjukdomsframkallande smittämnena. Trots enorma ansträngningar under de senaste årtiondena för att kontrollera populationer av smittspridande stickmyggor och de sjukdomar som de överför dödar myggor mer än 700 000 människor och påverkar miljontals människors liv varje år. För att kunna bekämpa myggöverförda sjukdomar med hjälp av nya strategier riktade mot det flygande fordonet måste vi förstå deras biologi i mer detalj. I den här avhandlingen undersöktes flera aspekter av honmyggors värdsökbeteende, som styr dem till en lämplig värd för en blodmåltid.

Honmyggor lokaliserar sina värdar och bedömer deras lämplighet främst med hjälp av luktsinnet. De urskiljer doftämnen som avges från människor och andra värddjur, när de andas eller direkt från deras kropp, med hjälp av luktkänsliga sinnesceller i sina antenner. Koldioxid (CO_2) är av särskilt intresse eftersom det är en stark signal för blodsugande insekter, men eftersom den finns i utandningsluften hos alla ryggradsdjur innehåller den i sig inte tillräcklig information om källans lämplighet för myggor som specialiserat sig på ett fåtal arter. Bland dessa finns malariamyggan *Anopheles gambiae* och gulafebernmyggan *Aedes aegypti* som båda är specialiserade att suga blod från människor. Följaktligen antar man att koldioxidens roll i värdsökningsbeteendet varierar med graden av specialisering, en hypotes som vi kan lägga fram bevis för hos *An. gambiae.*

Endast i kombination med andra värddofter är CO_2 meningsfullt för denna mycket människospecialiserade mygga.

Förmågan att skilja värdarter från varandra, t.ex. människor och boskap som lever i samma område, är en viktig förutsättning för att vissa myggarter ska livnära sig på blodet från människor och är en nyckelfaktor för överföringen av sjukdomar. Värdlukten, som är karakteristisk för en viss värdart i jämförelse med en annan, är unik. Intressant nog beror detta främst på olika proportioner av gemensamma flyktiga luktämnen och inte på förekomsten av värdartsspecifika luktkomponenter i värddoften. Hur mygghjärnan löser denna utmaning med många gemensamma flyktiga ämnen hos föredragna och icke föredragna värdar, och kodar värdidentitet, var dock okänt. Vi fann att människodoft framkallar ett distinkt aktiveringsmönster, trots små proportionella skillnader jämfört med andra värddjursdofter, i det primära luktcentret i hjärnan, antennloben, hos *Ae. aegypti*. Om man kemiskt efterliknar detta mönster med en blandning av syntetiska doftämnen kan man framkalla myggornas karakteristiska sicksackande värdsökande flykt, vilket tyder på att människans identitet kodas av den relativa aktiveringen av endast ett fåtal hjärnområden.

Vissa myggarter föredrar inte bara människor framför andra djur, utan de föredrar också vissa människor framför andra. Vi fann att ABO-blodgruppen samt graviditet och menstruationscykelfasen påverkar enskilda personers attraktionskraft för *Ae. aegypti*. Svampalkohol, 1-octen-3-ol, som trots sitt namn inte bara finns i svampar, är mer dominerande i lukten hos mycket attraktiva individer.

Mygghonors benägenhet att söka värd är inte statisk över tid, utan moduleras under hela deras liv. I motsats till fullvuxna honor föredrar nykläckta honmyggor inte människolukt, vilket sannolikt beror på en upp- och/eller nedreglering av gener som styr funktionen av luktsinnet, men mekanismen är okänd. Vi testade en kandidatgen, *AgamOR39*, som identifierats i tidigare forskningsprojekt på *An. gambiae*, och som kodar för en luktreceptor som binder till sulkaton, en av de viktigaste föreningarna i mänsklig lukt. Genom CRISPR-Cas9-genredigering genererade vi muterade myggor, där sulkatondetektionen via denna väg var avskaffad, men deras värdsökningsbeteende påverkades inte. Detta tyder på att *AgamOR39* inte är avgörande för när värdsökandet inleds. Ytterligare forskning kommer att undersöka denna genens roll mer i detalj.

Sammantaget kastar den här avhandlingen ljus över flera aspekter av doftmedierad värdsökning och diskriminering hos myggor. Resultaten kan i slutändan leda till att kunskapsgränserna för bekämpning av myggburna sjukdomar flyttas framåt och att deras belastning på människors hälsa minskas.

Populärwissenschaftliche Zusammenfassung

Mücken sind nicht nur lästig, sondern eine ernsthafte Bedrohung für menschliche Gesundheit und wirtschaftliche Entwicklung. Die Plagegeister übertragen eine Reihe gefährlicher Krankheiten, wie beispielsweise Malaria, Denguefieber, Gelbfieber und Japanische Enzephalitis. Eine Infektion erfolgt, wenn eine infizierte weibliche Mücke einen Menschen sticht, denn der Speichel, den die Mücke dabei injiziert um die Blutgerinnung zu hemmen, kann zusätzlich Krankheitserreger enthalten. Trotz der enormen Anstrengungen der letzten Jahrzehnte die Mückenpopulationen und die von ihnen übertragenen Krankheiten einzudämmen, sterben jedes Jahr mehr als 700 000 Menschen an durch Mücken übertragene Krankheiten und das Leben von Millionen weiterer ist negativ beeinträchtigt. Um neue Bekämpfungsstrategien gegen die fliegenden Krankheitsüberträger zu entwickeln, ist es wichtig ihre Biologie genauer zu verstehen. In dieser Doktorarbeit wurde das Wirtsfindungsverhalten weiblicher Stechmücken, das entscheidend für die Übertragung der Krankheitserreger ist, von verschiedenen Perspektiven beleuchtet.

Weibliche Mücken finden ihre Wirte und beurteilen deren Eignung vor allem mithilfe ihres Geruchssinns. Mittels geruchsempfindlicher Neuronen in ihren Antennen spüren sie Geruchsstoffe auf, die Wirte über ihren Atem und Körper abgeben. Kohlenstoffdioxid (CO_2) ist hier von besonderem Interesse, da es ein starkes Wirtssignal ist, allerdings von allen Wirbeltieren ausgeatmet wird. Demzufolge enthält CO_2 keine Informationen über die Eignung der Geruchsquelle als Wirt für hoch spezialisierte Mückenarten, die nur wenige Wirtsarten bevorzugen. Zu diesen Mückenarten gehören die Malariamücke *Anopheles gambiae* und die Gelbfiebermücke *Aedes aegypti*, die stark auf den Menschen spezialisiert sind. Es wird angenommen, dass die Rolle von CO_2 bei der Wirtsfindung vom Grad der Spezialisierung der

Mücken abhängig ist, welches wir für *An. gambiae* bestätigen konnten. Nur in Kombination mit anderen Wirtsgerüchen ergibt CO_2 für diese hochspezialisierte Mücke Sinn.

Die Fähigkeit zwischen Wirtsarten wie z. B. Menschen und Schafen unterscheiden zu können, ist unumstritten eine wichtige Voraussetzung für die starke Bevorzugung von Menschen durch einigen Mückenarten und ein Schlüsselfaktor bei der Krankheitsübertragung. Der Geruch, der eine bestimmte Wirtsart charakterisiert, ist einzigartig. Interessanterweise ist dies hauptsächlich auf unterschiedliche Anteile von gemeinsamen, also unspezifischen, Geruchsstoffen zurückzuführen und nicht auf die Existenz wirtsartspezifischer Geruchskomponenten. Wie Stechmücken diese Herausforderung des unspezifischen Vorkommens vieler Geruchsstoffe im Geruch von sowohl bevorzugten als auch nicht bevorzugten Wirten löst und die Identität des Wirts im Gehirn kodiert, war jedoch unbekannt. Wir fanden heraus, dass menschlicher Geruch im primären Geruchszentrum des Gehirns von *Ae. aegypti*, dem Antennallobus, ein überraschend simples Aktivierungsmuster hervorruft, das sich vom Muster jedes anderen tierischen Geruchs unterscheidet. Die Nachahmung dieses Aktivierungsmusters mithilfe einer Mischung synthetischer Geruchsstoffe löst den charakteristischen Zickzackflug der Mücken bei der Wirtssuche aus, was darauf hindeutet, dass die menschliche Identität durch die relative Aktivierung nur weniger Hirnareale kodiert wird.

Einige Mücken bevorzugen nicht nur Menschen gegenüber anderen Tieren, sondern auch bestimmte menschliche Individuen gegenüber anderen. Wir fanden heraus, dass die ABO-Blutgruppe, sowie eine Schwangerschaft oder die Woche des Menstruationszyklus die Attraktivität einzelner Freiwilliger für die Gelbfiebermücke *Ae. aegypti* beeinflusst. 1-Octen-3-ol, ein Alkohol welcher in konzentrierter Form an Pilze erinnert, aber nicht nur dort vorkommt, ist im Geruch von durch Mücken bevorzugten Menschen dominanter.

Die Neigung zur Wirtssuche ist bei weiblichen Mücken nicht statisch, sondern ändert sich mehrfach im Laufe ihres Lebens. Im Gegensatz zu ausgewachsenen Weibchen bevorzugen frisch geschlüpfte Weibchen keinen menschlichen Geruch, was höchstwahrscheinlich auf die Hoch- und/oder Herunterregulierung von Genen zurückzuführen ist, die am Riechen beteiligt sind, der Mechanismus ist jedoch unbekannt. Wir haben in der Malariamücke *An. gambiae* die Rolle des Gens *AgamOR39* untersucht, welches in einer früheren Forschungsstudie als potentiell entscheidend identifiziert wurde. Das Gen kodiert für einen Geruchsrezeptor, der für einen der Hauptbestandteile menschlichen Geruchs, 6-Methyl-5-hepten-2-on (Sulkaton), spezifisch ist. Durch die CRISPR-Cas9 Methode haben wir das Gen *AgamOR39* und damit die Erkennung von Sulkaton durch den entsprechenden Rezeptor gezielt ausgeschaltet, allerdings wurde das Verhalten der Mücken bei der Wirtsfindung nicht beeinträchtigt. Dies deutet darauf hin, dass *AgamOR39* für das Einsetzen des Wirtsfindungsverhaltens nicht entscheidend ist. Weitere Versuche werden die Rolle dieses Gens näher untersuchen.

Die Ergebnisse dieser Arbeit tragen wichtige Erkenntnisse zum Verständnis der geruchsgesteuerten Wirtfindung und Wirtsunterscheidung durch Stechmücken bei und sind damit Teil des Wissensschatzes um neue Strategien zu entwickeln. Das Ziel ist, die hohe Belastung der menschlichen Gesundheit und Gesellschaft durch von Mücken übertragene Krankheiten zu verringern.

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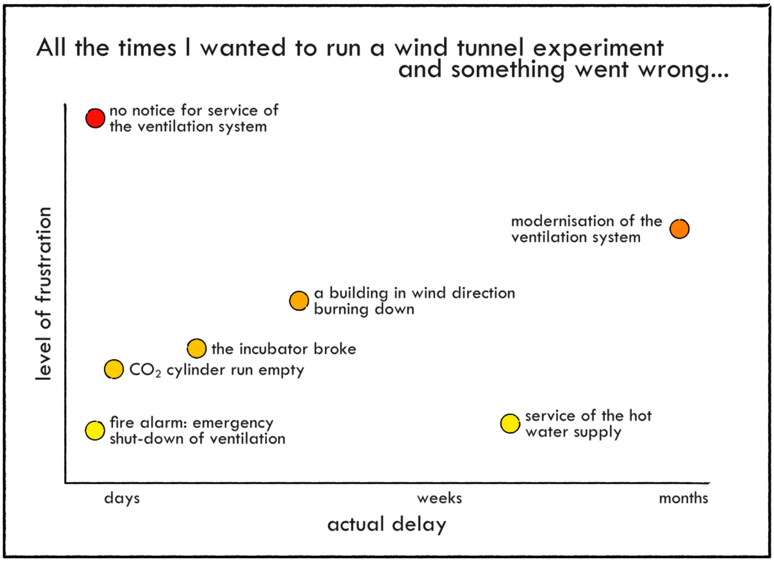

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METHODS
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Mosquito Host Seeking in 3D Using a Versatile Climate-Controlled Wind Tunnel System

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Future anthropogenic climate change is predicted to impact sensory-driven behaviors. Building on recent improvements in computational power and tracking technology, we have developed a versatile climate-controlled wind tunnel system, in which to study the effect of climate parameters, including temperature, precipitation, and elevated greenhouse gas levels, on odor-mediated behaviors in insects. To establish a baseline for future studies, we here analyzed the host-seeking behavior of the major malaria vector mosquito, *Anopheles gambiae sensu strico*, to human odor and carbon dioxide (CO_2), under tightly controlled climatic conditions, and isolated from potential background contamination by the presence of an experimenter. When presented with a combination of human foot odor and CO_2 (case study I), mosquitoes engaged in faster crosswind flight, spent more time in the filamentous odor plume and targeted the odor source more successfully. In contrast, female *An. gambiae s. s.* presented with different concentrations of CO_2 alone, did not display host-seeking behavior (case study II). These observations support previous findings on the role of human host-associated cues in host seeking and confirm the role of CO_2 as a synergist, but not a host-seeking cue on its own. Future studies are aimed at investigating the effect of climate change on odor-mediated behavior in mosquitoes and other insects. Moreover, the system will be used to investigate detection and processing of olfactory information in various behavioral contexts, by providing a fine-scale analysis of flight behavior.

Keywords: ***Anopheles gambiae*, host seeking, 3D tracking, carbon dioxide, olfaction, human odor, behavior**

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INTRODUCTION

Insects integrate cues of multiple sensory modalities to navigate in their environment in order to locate suitable food sources, mating partners, or oviposition sites (Buehlmann et al., 2020). Understanding insect flight behavior in response to their variable olfactory environment requires an experimental system that is able to mimic the required climatic conditions in a precise manner, while facilitating easy presentation of cues, observation, and analysis of flight behavior in detail. The versatile climate chamber and wind tunnel system presented in this study provides these features, and also facilitates tracking insect flight in 3 dimensions (3D).

Female mosquitoes rely predominantly on odors to find a blood meal, especially at longer distances from the host, while also using visual and thermal cues when nearing the target (Takken and Knols, 1999; Cardé, 2015; Raji and DeGennaro, 2017). Our understanding of host-seeking behavior in mosquitoes has expanded substantially due to advancements in video capture, tracking technology and computational power (Anderson and Perona, 2014; Spitzen and Takken, 2018; Manoukis and Collier, 2019). In the recent past, tracking mosquito behavior has allowed for analyses in greater detail, and provided new levels of understanding in host-seeking strategies, the different sensory cues involved and their integration (Dekker and Cardé, 2011; Lacey and Cardé, 2011; Lacey et al., 2014; McMeniman et al., 2014; van Breugel et al., 2015; Hawkes and Gibson, 2016). Moreover, behavioral responses to mosquito vector control tools that are targeting host-seeking behavior, such as insecticide-treated bed nets and baited traps, have been analyzed to improve their efficiency (Cooperband and Cardé, 2006; Spitzen et al., 2014; Parker et al., 2015, 2017; Angarita-Jaimes et al., 2016; Cribellier et al., 2018, 2020; Amos et al., 2020).

Based on tracking studies and behavioral observations, the long-range flight strategy of female anthropophilic mosquitoes, such as the African malaria vector, *Anopheles gambiae sensu lato*, and the yellow fever mosquito, *Aedes aegypti*, in response to human host odors has been characterized as "cast and surge," in which mosquitoes surge upwind upon contact with an odor-laden filament of air and perform crosswind flight if the trace is lost (Cardé and Willis, 2008; Dekker and Cardé, 2011; Spitzen et al., 2013). At intermediate distances, gated by the encounter of human-emanated carbon dioxide (CO_2) and body odors, mosquitoes approach high-contrast visual features (van Breugel et al., 2015; Hawkes and Gibson, 2016) and initiate landing in the presence of short-range host cues, such as body heat and humidity (McMeniman et al., 2014). While these basic characteristics are common to all host-seeking mosquito species, details, such as the relative importance of the respective cues, differ in respect to, e.g., host preference and daily flight activity patterns (Cooperband and Cardé, 2006; Dekker and Cardé, 2011; Spitzen et al., 2013; Hawkes and Gibson, 2016). In *An. gambiae*, for instance, the role of CO_2 in regulating host seeking is controversial. While some studies found CO_2 on its own to be a host-seeking cue, eliciting activation, orientation, or both (Healy and Copland, 1995; Lorenz et al., 2013), others did not find such an effect (de Jong and Knols, 1995; Takken et al., 1997; Spitzen et al., 2008). This discrepancy can partially be explained by differences in behavioral assays used and the mode of presentation of CO_2, but also contamination by odors from an experimenter cannot be excluded in some studies (Webster et al., 2015).

The improved wind tunnel system presented in this study is equipped with a highly-versatile automated climate-control that allows us to analyse the odor-mediated anemotaxis of *An. gambiae sensu stricto* in response to human host odors under stable and precise climatic conditions, while reducing background odors to a minimum. The case studies presented here investigate the role of human host cues in *An. gambiae s. s.* host seeking. Case study I confirms that, when presented with a salient odor, i.e., a combination of human odor and CO_2, mosquitoes spend more time in the filamentous odor plume, engage in faster crosswind flight maneuvers and find the source more reliably. Case study II supports previous findings that *An. gambiae s. s.* likely does not use CO_2 on its own as a cue in host seeking.

MATERIALS AND METHODS

Wind-Tunnel System

Hardware: Air Treatment, Climate Chamber, Wind Tunnel

Air for the wind-tunnel system was supplied from the ventilation system of the building, pre-filtered, with a low, constant pressure and a temperature of 20–22°C. Airflow within the wind tunnel system was regulated by two circular duct fans (F2: K 315 sileo, F3: KV 315 sileo; Systemair, Skinnskatteberg, Sweden) and a mechanical flow control valve (BDEP-4-025-1; FläktGroup, Herne, Germany), equipped with a modulating damper actuator (LM24A-SR; Belimo, Hinwil, Switzerland) that is adjusted from the control panel of the wind tunnel (**Figure 1**; F2 and the flow control valve are installed on the feeding line before the filter unit and thus not shown). Incoming air to the wind-tunnel system was pushed through a Camfil filter unit equipped with a pre-filter and 16 activated carbon filters (pre-filter: EcoPleat Eco 3GPF ePM1 55% 592x592x48-F7 ISO; carbon filters: CamCarb CM 2600 GC VOC; ducted filter housing: CamCube HF-CC 1010 1010AZ; Camfil, Stockholm, Sweden) before entering the mixing unit. In the mixing unit, turbulent intermixing of the fresh air from the filter unit and recirculating air from the climate chamber occurs and mixed air is passed into the climate chamber (**Figure 1**).

Within the climate chamber (stainless steel, l × w × h: 1,760 mm × 2,000 mm × 1,570 mm; **Figure 1**), air temperature and relative humidity (RH) can be adjusted up to 27.0°C and 70 % RH, respectively, regulated from the control panel. The lower limits of both parameters are determined by the air fed into the wind-tunnel system from the ventilation system of the building. Within the climate chamber, the air is humidified by five humidifiers (B 1/4 ML-1.5; Spraying Systems Co, Wheaton, IL, US) that are placed in the zone of recirculating air in the chamber and fed by the warm water supply of the building (**Figure 1B**). Connections for cold water, distilled water and pressurized air are installed and can be used for future applications, e.g., to adjust temperature and humidity to values different from those specified above. The climate chamber with its large inner dimensions is constructed such that it both facilitates turbulent intermixing of the air and permits easy servicing, as well as placing of additional equipment for e.g., raising ozone and CO_2 background levels. The climate chamber is built in a stainless steel tray (fold height 20 mm) and placed on a waterproofing membrane (Biltema, Helsingborg, Sweden) to protect the floor from humidity. Climate chamber walls are thermally insulated with styrofoam (thickness 40 mm) and covered with a waterproofing membrane. The recirculating duct, which is constantly passing a part of the warm, humidified air from the climate chamber back to the mixing unit (**Figure 1B**), is equipped with an in-line duct fan (F1: KV 315 sileo; Systemair) and a circular electric duct heater (H1: CV25-60-M; VEAB Heat Tech AB, Hässleholm, Sweden), regulated by the control panel. The climate chamber and the

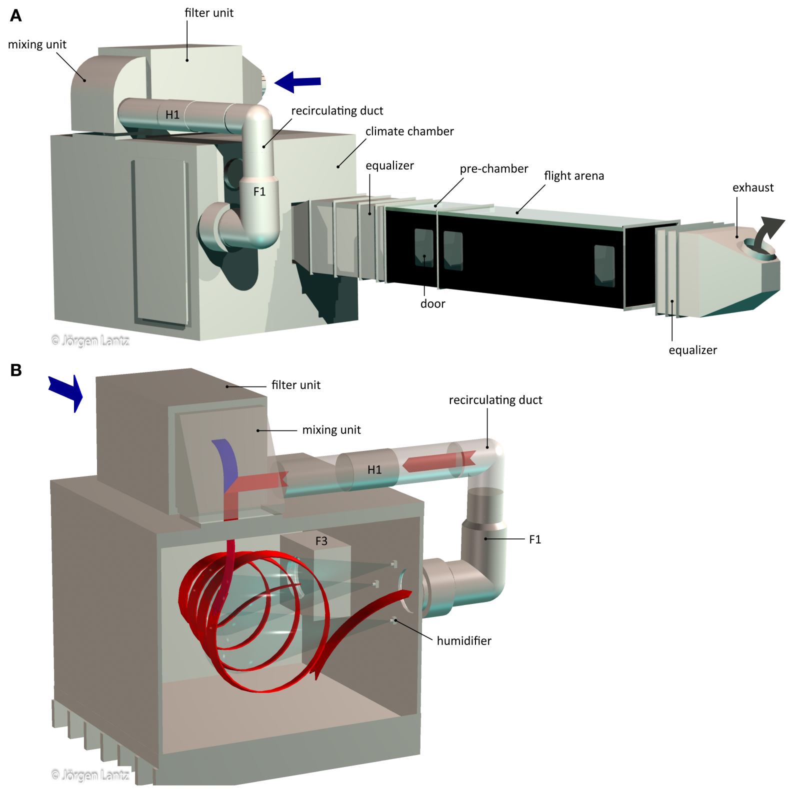

FIGURE 1 | Schematic of the wind tunnel system **(A)** and climate chamber **(B)**. Incoming air (blue) is filtered in the filter unit and passed on to the mixing unit where it is mixed with warm air (red) from the recirculating duct. In the climate chamber, the air is humidified by five humidifiers (three shown) and passed on through an equalizer toward the pre-chamber and flight arena. Both pre-chamber and flight arena can be accessed by doors. Air is removed by the exhaust. F1 and F3 indicate the fans and H1 the heater. F2 and the flow control valve are placed on the feeding line to the wind tunnel system and thus not shown.

majority of other parts are made from stainless steel, except fans, filter house and heater.

From the climate chamber to the pre-chamber and flight arena, the air passes through an equalizer, in which variations in temperature, humidity, and speed of the airflow are stabilized (6 perforated metal sheet units; 1,000 mm × 600 mm × 600 mm; **Figure 1A**). The near-laminar airflow entering the pre-chamber and flight arena is stable in temperature, RH, and speed (methods see below; **Figure 2**). Within the pre-chamber (600 mm × 600 mm × 600 mm), the air passing toward the adjacent flight arena (2,000 mm × 600 mm × 600 mm) can be manipulated, e.g., by introducing an odor source as demonstrated in this study. The bodies of both chambers are made from black polycarbonate (thickness 5 mm), each covered with a transparent, removable acrylic glass roof (thickness 6 mm). The matt surface of the black polycarbonate body of the flight arena limits light reflections. Three doors enable access to the setup with minimal disturbance to the airflow (**Figure 1A**). The flight arena is closed off on both sides by black mosquito netting (mesh size 1.4 × 1.6 mm, plastic-covered fiberglass; Biltema), set in a black metal frame. Downwind of the wind tunnel, the air exits through an exhaust equipped with an equalizer unit (3 perforated metal sheet units; 400 mm × 610 mm × 610 mm, placed 280 mm away from the wind tunnel. The airspeed of the exhaust is adjusted to ca. $0.5\ \mathrm{m\ s^{-1}}$, which removes air from both the wind tunnel and the room in which it is placed.

Control Panel: Airspeed, Temperature and Relative Humidity

The coarse setting for the air pressure was pre-set upon installation of the wind tunnel system, where the fans F1, F2, and F3 were individually adjusted via three five-step transformers (Systemair 5000, type RE 1.5, Tuvfassons 7886-009; Tuvfassons, Sigtuna, Sweden; **Figure 1**). Upon operation of the wind tunnel system, wind speed can be finely regulated by an airflow damper, which is operated from the control panel. With the fixed pre-set

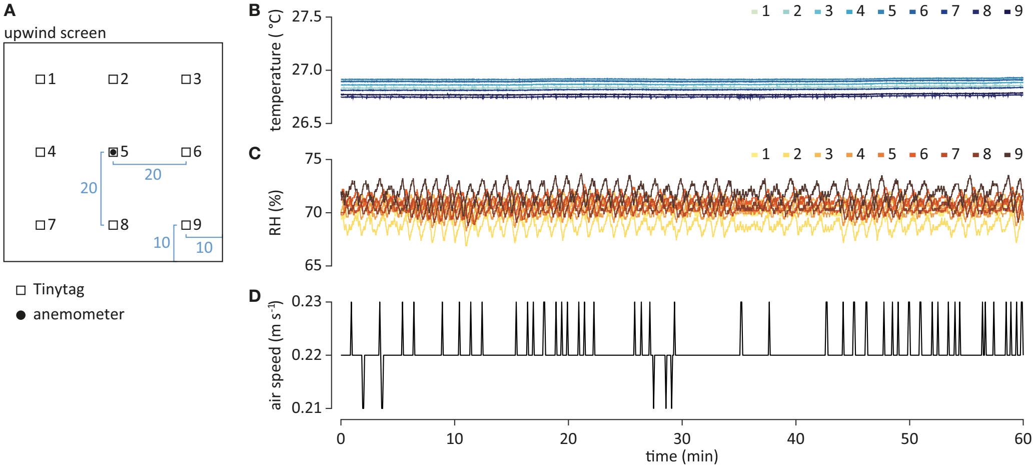

FIGURE 2 | Physical parameters of the wind tunnel system. **(A)** Schematic of the placement of the Tinytags (open square), for measurement of temperature and relative humidity (RH), and anemometer (filled circle), for wind speed, at the upwind screen. Measurements in cm. The distance to the upwind screen was 15 cm. Temperature **(B)**, RH **(C),** and air speed **(D)** over time.

adjustment of F1, F2, and F3, the airspeed can then be adjusted to up to 0.35 m s^{-1}.

Temperature and RH are controlled via the control panel, mainly by a custom-programmed PLC unit (Millenium 3 Essential CD20- 12I/8O S 24VDC; Crouzet, Valence, France; Jörgen Lantz Engineering Consulting Firm; **Supplementary Figure 1**). In short, the control panel is integrating set points, actual values and input of e.g., time of ventilation and drain flushing, limits for set points and actual values, conditions for starting the wind tunnel (e.g., air flow from the building), and the control of the flow adjustment damper.

Upon shutting down the wind tunnel, an ejector drain flush is automatically activated by the control panel that flushes remaining water from the climate chamber using pressurized air. Then, the wind tunnel is dehumidified by running at maximum speed (0.35 m s^{-1}) for 12 h. This removes the remaining water from the climate chamber and humidity from associated parts of the setup.

Quantification of Physical Parameters Within the Flight Arena

Air temperature and RH were quantified using Tinytag Plus 2 TGP-4500 data loggers (Intab, Stenkullen, Sweden), set to 1 Hz sampling rate. Tinytags were arranged in an array (**Figure 2A**), and placed 15 cm downwind of the upwind screen. Air speed was measured using a ThermoAir3 hot wire anemometer (Schiltknecht Messtechnik AG, Gossau, Switzerland), read every 5 s. The anemometer was placed mid-center in the flight arena, 15 cm downwind of the upwind screen (**Figure 2A**). Air temperature, RH and speed were recorded for an hour (**Figures 2B–D**).

Case Studies

Mosquitoes

Anopheles gambiae sensu stricto (G3 strain) were reared as previously described (Omondi et al., 2015). Adult mosquitoes were maintained in Bugdorm cages (30 × 30 × 30 cm; MegaView Science, Taichung City, Taiwan) at 27 ± 1°C and 65 ± 5% RH under a 12 h light: 12 h dark regimen, and provided with 10 % sucrose *ad libitum*. For colony maintenance, adult females were fed on donor sheep blood (Håtunalab, Bro, Sweden) using a membrane feeding system (Hemotek Ltd, Blackburn, UK). For oviposition, wet filter papers were provided, and eggs transferred to larval trays (24 × 18 × 7.5 cm, filled with 2 cm of distilled water) before hatching. Larvae were fed daily on Tetramin Baby fish food (Tetra GmbH, Melle, Germany). For experiments, pupae were collected and transferred to Bugdorm cages (17.5 × 17.5 × 17.5 cm) prior to eclosion. Experiments were conducted with non-blood-fed females at 4 days post-eclosion (4 dpe). Prior to the experiment, females were sugar starved either for 4–16 h without (case study II), or 15–23 h with *ad libitum* access to water (case study I), and then transferred to individual release cages (ø10 × 7 cm), at least 30 min before the start of the experiment, using a mouth aspirator. Host-seeking females were pre-selected by placing a gloved hand on the netting of the cage. All experiments were conducted within the peak activity period of host seeking, i.e., the first 4 h of the scotophase (e.g., Jones and Gubbins, 1978).

Flight Arena

Mosquito flight behavior was tracked in the wind tunnel setup described above (**Figures 1A, 3A**). The wind tunnel was adjusted to 27.0°C and 70% RH, and the wind speed was set to 0.22 m s^{-1}.

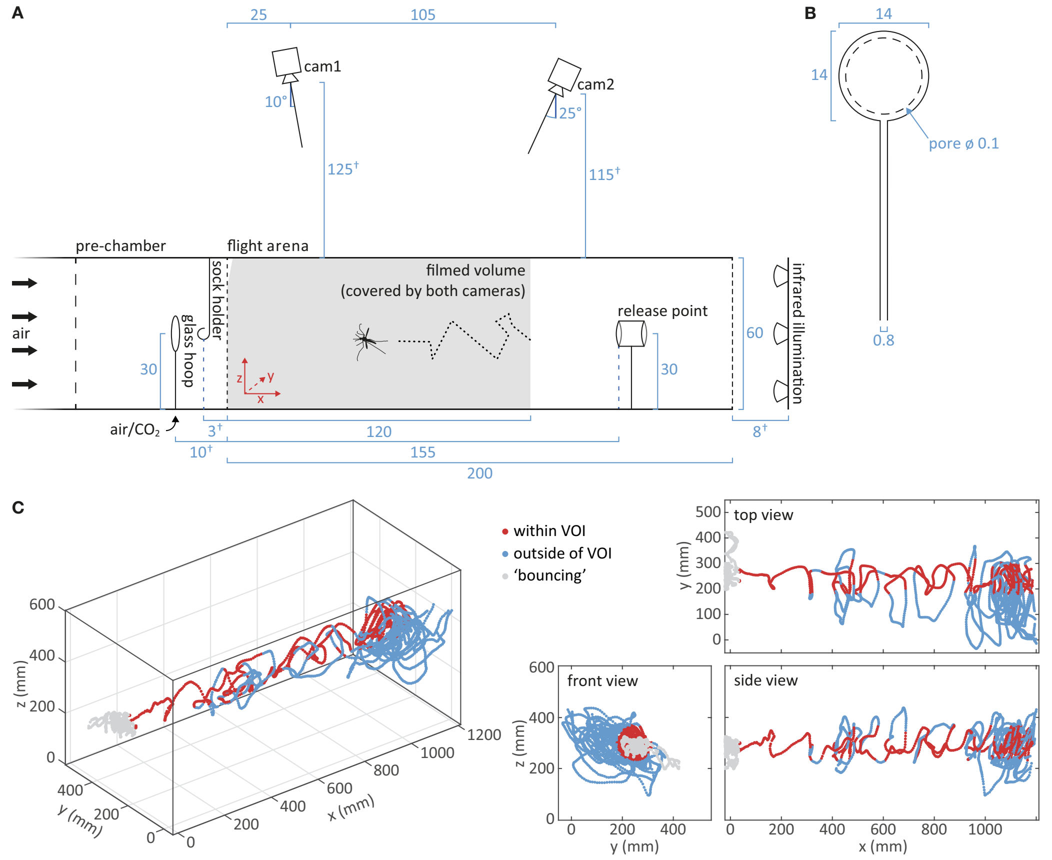

FIGURE 3 | Tracking of mosquito flight. **(A)** Schematic of the flight arena and the setup for video capture of mosquito flight. A mosquito, released at the release point, flies upwind toward the upwind screen. Cameras mounted above the flight arena record the reflection of the infrared light on the body of the mosquito. The filmed volume, i.e., the area covered by both cameras, is shown in gray. Distance measurements are given in cm. Dimensions drawn to scale, apart from measurements marked by †. **(B)** Schematic of the glass hoop used for turbulent presentation of CO$_2$ (Dekker and Cardé, 2011). Measurements are given in cm. **(C)** A representative flight trajectory of a single mosquito in response to carbon dioxide and human foot odor, viewed from different angles. Positions within the cylindrical volume of interest (VOI) are indicated in dark red, whereas positions outside the VOI are in light blue. "Bouncing" at the upwind screen is denoted in gray.

Odor Stimuli

Odor stimuli were delivered from two different devices, a glass hoop and a metal sock holder, placed within the pre-chamber of the wind-tunnel system (**Figures 3A,B**). Carbon dioxide of either ambient or elevated concentrations (1,200, 2,400, 4,800 ppm) were presented using a glass hoop with equidistant holes to create a turbulent plume (Dekker et al., 2001), which was positioned in the pre-chamber, 10 cm upwind of the upwind screen. For elevated CO$_2$ concentrations, pure, pressurized CO$_2$ (Strandmöllen AB, Ljungby, Sweden) was mixed in different proportions with carbon-filtered and humidified ambient air at a resulting flow rate of 1 l min^{-1}. The concentration of the resulting mix was measured using a LI-820 CO$_2$ analyser (LICOR Biosciences, Lincoln, NE, US) and adjusted to the desired concentration ± 50 ppm prior to entering the glass hoop. Compliance to a tolerance interval of ± 200 ppm was confirmed after each trial, as the pressure of the pure CO$_2$ showed minor shifts over time. Addition of CO$_2$ did not detectibly increase the flow rate of the air passing toward the glass hoop (BA-4AR flow meter; Kytola Instruments, Muurame, Finland). For the presentation of human foot odor, socks worn by the experimenter were used, in accordance with previous studies (e.g., Njiru et al., 2006; Verhulst et al., 2011; Robinson et al., 2018). Black cotton socks were worn for 19–21 h prior to the experiment, rolled up and then suspended from a metal hook between the glass hoop and the upwind screen (**Figure 3A**). Used socks provided an odor source for a maximum of 1 h, and were later washed with a low-perfumed washing detergent (ICA Skona, Solna, Sweden) before reuse. Here, cotton socks were chosen over nylon socks, as the foot odor collected on the former elicited responsiveness from a higher proportion of mosquitoes (enter the filmed volume; data not shown).

Odor stimuli presented in case study I were combinations of either air or 1,200 ppm CO_2, and human foot odor or corresponding controls, i.e., no sock and clean sock. The resulting treatment combinations were "air/air," "CO_2/air," "air/clean sock," "air/used sock," and "CO_2/used sock." In case study II, CO_2 on its own was presented at either ambient (400 ppm to 445 ppm), or elevated concentrations (1,200, 2,400, 4,800 ppm). The order of treatments was randomized over the experimental day.

Experimental Procedure

For each trial, a release cage containing a single female mosquito was placed at the release point close to the upwind end of the wind tunnel (**Figure 3A**). Mosquitoes were exposed to the odor stimulus during acclimatization to avoid disturbing the air current and the mosquitoes once the trial started. After an acclimatization period of 2 min, the video recording was started, and the door of the release cage gently opened. Individuals that did not enter the filmed volume of the flight arena (**Figure 3A**, gray area) within 3 min were removed from further analysis. Flight behavior of responding mosquitoes was recorded until landing on the upwind screen for at least 5 s, or for up to 10 min of continuous flight. After each trial, the response ("flight," "no flight") and landing site ("upwind screen," "other," "not landing") was visually observed. Each mosquito was only tested once. Surgical gloves were worn during the experiment, and equipment and mosquitoes were handled with great care to avoid contamination with human odor.

Video Capture and Flight Trajectory Reconstruction

Flight behavior was recorded from above the wind tunnel with two infrared light (IR) sensitive GigE cameras (acA1300-60gm; Basler AG, Puchheim, Germany; **Figure 3A**), equipped with 4.4–11 mm lenses (LMVZ4411; Kowa, Aichi, Japan), at 60 frames s^{-1} using Media Recorder 4.0 (Noldus Information Technology, Wageningen, The Netherlands). Illumination was provided by six IR arrays (850 nm; VAR2-i2-1 IR illuminators; VAR-i2-LENS-6025 diffuser lenses; Raytec, Ashington, UK) placed at the downwind end of the flight arena (**Figure 3A**). Cameras recorded the reflection of the IR light on the wings and body of the mosquito. An LED array, shielded with a paper screen, at the upwind end of the wind tunnel, provided diffuse visible white light of low intensity (<1 lux; LX-101 lux meter; Lutron Electronic Enterprises, Taiwan) for visual orientation of the mosquito. Cameras were mounted at an angle above the wind tunnel, resulting in a coverage of the entire volume of the upwind 120 cm of the wind tunnel. A narrow volume at the top of the upwind screen (triangular intersection, 2.2 × 11.5 cm) was shielded by the frame holding the netting, where mosquitoes could only be observed by one camera and therefore not be tracked in 3D (**Figure 3A**). Due to the mosquito's protruding abdomen and hind legs while sitting, landing could be tracked except the top 5 cm of the upwind screen (**Figures 4E**, **5E**).

EthoVision XT 14 (Noldus Information Technology) was used to convert the video files from both cameras to 2D position data. For all trials, the data was manually inspected during the process to exclude frames with identification errors. Data was generated without interpolation of missing samples or smoothing of the flight path. The 2D position data was then combined into a 3D flight path using Track3D (Noldus Information Technology; see Spitzen et al., 2013). The system was calibrated using a customized calibration frame and CentroidFinder software (Noldus Information Technology) at the start of the experimental series and if required, i.e., when the daily mean intersection error exceeded a threshold of 2.0 pixels. The following variables were calculated by Track3D and used in subsequent analysis: position in three dimensions (x, y, z), flight speed and heading angle in the vertical plane.

Analysis of Response Rate and Flight Trajectories

A mosquito was considered responsive if it entered the filmed volume within 3 min. Treatment factor effects were tested using a binomial generalized linear model (GLM), followed by a Chi-square test (R, version 3.5.1; R Core Team, 2018). *Post-hoc* pairwise comparisons of the treatment combinations were tested with the "emmeans" package (R), corrected using the Tukey method.

Obtained 3D trajectory data was processed and analyzed using customized Matlab (version R2020a; MathWorks, Natick, MA, US) and R scripts (version 3.5.1). In a first step, the analysis window of individual trajectories was defined and frames containing outliers were excluded. The start of the analysis window was determined by the mosquito entering the filmed volume, and the end by either the instance of landing or a maximum flight duration of 10 min. Landing was identified by detecting the time point at which the mean speed over 60 frames was below a threshold of 50 mm s^{-1} for three consecutive seconds, which was also confirmed by visual observation. Landing coordinates were determined for future analysis. In very few cases, the video recording was ended before the above criteria were fulfilled, and in these cases those files were excluded from further analysis. Data points where the mosquito's position was <6 cm away from the upwind screen were excluded from most further analyses since the physical boundary likely affected mosquito flight ("bouncing").

For analyzing mosquito flight in the volume where it may encounter odor filaments, a volume of interest (VOI) was defined, and approximated to be a cylinder in space, with a diameter of 14 cm, centered within the flight arena (**Figure 3C**), based on the shape and dimension visualized by smoke paper (Günther Schaidt SAFEX Chemie GmbH, Tangstedt, Germany; **Supplementary Figure 2**). The proportion of flight in the VOI was calculated by the number of frames with a position within the VOI divided by the total number of frames. A Dunn Kruskal-Wallis multiple comparison *post-hoc* test with Benjamini-Hochberg correction was used for pairwise comparison between the treatments ("FSA" package; R, version 3.5.1).

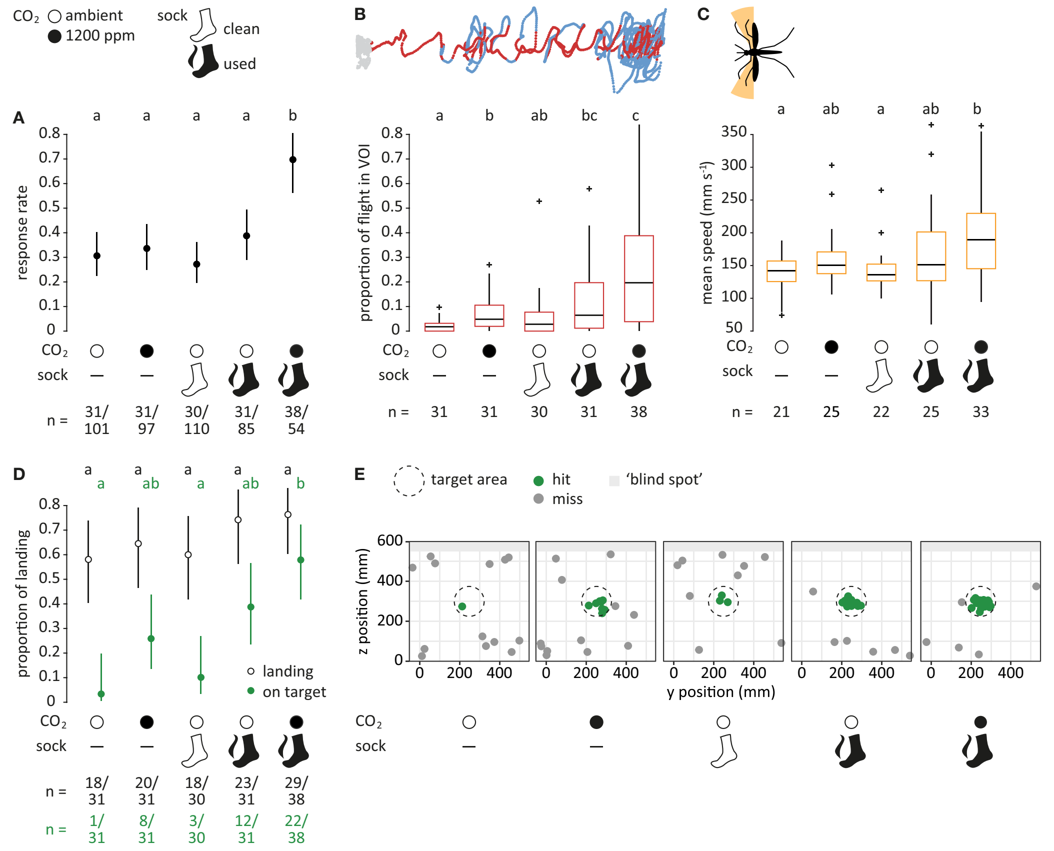

FIGURE 4 | Mosquito flight in response to human host cues. **(A)** Proportion of mosquitoes responding (dots), i.e., entering the filmed volume within 3 min, for each treatment. Bars denote 95% confidence intervals. Different letters denote significant differences between the treatments (pairwise comparison using "emmeans," corrected with the Tukey method). Sample sizes are given below the plot. **(B)** Proportion of flight in volume of interest (VOI), as shown in dark red in the top panel (see **Figure 3C**). Boxes represent upper and lower quartiles, whiskers denote 1.5 times interquartile distance, crosses outliers, and black horizontal lines the median. Different letters denote significant differences between the treatments (Dunn Kruskal-Wallis pairwise comparison test, Benjamini-Hochberg corrected). **(C)** Mean speed of crosswind flight for heading angles between 90° to 120° and 240° to 270° (orange area in the top panel). Only mosquitoes that were within the VOI at least once were taken into account. **(D)** Proportion of mosquitoes landing (black, open) and of mosquitoes landing on the target (green, filled) relative to the total number of responsive mosquitoes. **(E)** Landing location on the upwind screen. The defined target area (ø 15 cm) is denoted as a dashed circle. Green dots indicate landing positions within, and gray dots positions outside of the target area. Light gray area denotes the "blind spot" where tracking of landing was not possible.

Crosswind flight was quantified using the mosquito's heading angle, which is defined as the angle between the x-axis (direction of air movement) and the direction of mosquito flight in the vertical plane, in which 180° corresponds to straight upwind flight. The mean speed of crosswind flight per mosquito was calculated for heading angles between 90° to 120° and 240° to 270°. Only flight trajectories that were within the VOI at least once were considered for analysis. For pairwise comparison between the treatments, a Benjamini-Hochberg corrected Dunn Kruskal-Wallis multiple comparison *post-hoc* test was used.

Mosquito-landing response was analyzed by determining whether the landing coordinates were within a target area on the upwind screen. The target area was circular, 15 cm in diameter and centered downwind of the odor delivery devices. Treatment factor effect was tested using a binomial GLM and Chi-square test. For multiple pairwise comparisons between the treatments, the "emmeans" package was used (corrected using the Tukey method).

RESULTS

Case Study I—Human Host Cues

Response Rate

Of the 447 mosquitoes tested, 161 responded by entering the filmed volume within 3 min after opening the door of the release cages. Human host cues had a significant effect on the number of

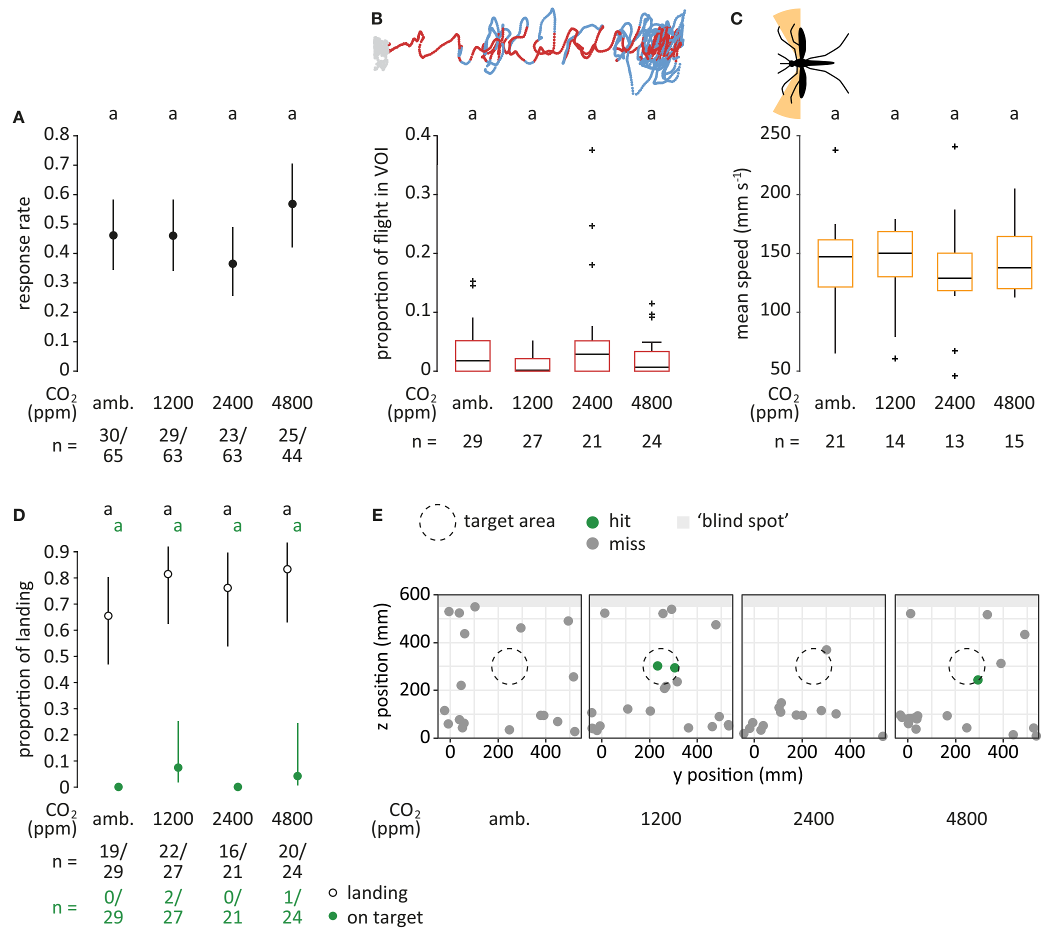

FIGURE 5 | Mosquito flight in response to different concentrations of carbon dioxide. **(A)** Proportion of mosquitoes responding (dots), i.e., entering the filmed volume within 3 min, for each treatment. "amb." for ambient. Bars denote 95% confidence intervals. Different letters denote significant differences between the treatments (pairwise comparison using 'emmeans,' corrected with the Tukey method). Sample sizes are given below the plot. **(B)** Proportion of flight in volume of interest (VOI), as shown in dark red in the top panel (see **Figure 3C**). Boxes represent upper and lower quartiles, whiskers denote 1.5 times interquartile distance, crosses outliers, and black horizontal lines the median. Different letters denote significant differences between the treatments (Dunn Kruskal-Wallis pairwise comparison test, Benjamini-Hochberg corrected). **(C)** Mean speed of crosswind flight for heading angles between 90° to 120° and 240° to 270° (orange area in the top panel). Only mosquitoes that were within the VOI at least once were taken into account. **(D)** Proportion of mosquitoes landing (black, open) and of mosquitoes landing on target (green, filled) relative to the total number of responsive mosquitoes. In treatments where no mosquitoes landed on target, CIs were infinite and thus not shown. **(E)** Landing location on the upwind screen. The defined target area (ø 15 cm) is denoted as a dashed circle. Green dots indicate landing positions within, and gray dots positions outside of the target area. Light gray area denotes the "blind spot" where tracking of landing was not possible.

mosquitoes responding, in which both factors, CO_2 and human foot odor, and their interaction, contributed significantly to the observed effect (Chi-square test, $p < 0.05$). A significantly larger proportion of mosquitoes (ca. 70%) entered the filmed volume when exposed to both CO_2 and human foot odor in comparison to all other treatments ($p < 0.05$; **Figure 4A**). No significant differences were observed among the other treatments.

Flight in Volume of Interest

When human host cues were present, mosquitoes spent a larger proportion of flight within the VOI ($p < 0.05$; **Figure 4B**). The highest proportion of flight within the VOI was elicited by the combination of CO_2 and human foot odor, which was significantly different from all other treatments except human foot odor alone (Dunn Kruskal-Wallis test, $p < 0.05$). Stimulation with either human host cue on its own resulted in a significant increase of flight inside the VOI in comparison to the air control ($p < 0.05$), whereas there was no difference between air and clean sock control ($p = 0.2$).

Crosswind Flight

When analyzing the mean speed of mosquito crosswind flight for mosquitoes that were in contact to the VOI at least once (**Figure 4C**), a significant difference between the combination of both human host cues and air control (Dunn Kruskal-Wallis test, $p = 0.01$) and clean sock control ($p = 0.01$) was detected. Mosquitoes that were exposed to both CO_2 and human foot odor flew on average 1.3× faster in comparison to the air control. In addition, there was a tendency of increased crosswind flight frequency for both the human foot odor and the combination of human food odor with CO_2 for larger distances to the source when pooling all mosquitoes (**Supplementary Figure 3**).

Landing and Landing Location

Of the 161 mosquitoes responding to the different treatments, 108 landed within the maximum recording time of 10 min. No significant difference was observed when comparing between treatments ($p > 0.05$; **Figure 4D**). However, the proportion of mosquitoes landing on target was significantly affected by the factors CO_2 and human foot odor (Chi-square test, $p < 0.001$), in which 57% of the responsive mosquitoes landed on the target area on the upwind screen in response to CO_2 and human foot odor, compared to 3% for the air and 10% for the clean sock control. These differences were significant among treatments ($p < 0.01$; **Figures 4D,E**).

Case Study II—Carbon Dioxide

Response Rate

In response to the four CO_2 treatments, 107 of 235 mosquitoes responded by entering the filmed volume. No significant effect of the concentration of CO_2 was observed (GLM, Chi-square test; $p = 0.2$; **Figure 5A**).

Flight in Volume of Interest

No effect of the concentration of CO_2 on the proportion of flight within the VOI was observed (Kruskal-Wallis test; $p = 0.2$; **Figure 5B**). The proportion of flight inside the VOI was generally low, ranging from 0.2% in response to 1,200 ppm to 2.9% for 2,400 ppm CO_2.

Crosswind Flight

No significant difference in crosswind flight speed was observed when comparing between the treatments (Kruskal-Wallis test; $p = 0.2$; **Figure 5C**). There was no tendency of increased crosswind flight frequency between the treatments (**Supplementary Figure 4**).

Landing and Landing Location

Within the maximum recording time of 10 min, 77 of 101 mosquitoes landed. No significant difference was observed for the total proportion of mosquitoes landing (GLM, Chi-square test; $p = 0.4$), nor for the proportion of mosquitoes landing "on target" ($p = 0.3$; **Figures 5D,E**).

DISCUSSION

The two case studies presented here demonstrate the functionality of the versatile climate-controlled wind tunnel system as an experimental setup for analyzing insect flight. Moreover, we provide new findings and confirm previous observations on odor-mediated optomotor anemotaxis in *An. gambiae s. s.* Case study I recapitulates the characteristics of female *An. gambiae s. s.* host-seeking behavior in response to human host cues, as previously described in other contexts by 3D tracking studies (Spitzen et al., 2013; Hawkes and Gibson, 2016). In the present study, the combination of CO_2 and human foot odor elicited a significant increase in mosquito responsiveness and host seeking, as reflected by a higher proportion of flight spent inside the volume where mosquitoes were more likely to encounter odor filaments. Moreover, mosquitoes tended to engage in more and faster crosswind flight in response to human odor cues. In addition, mosquitoes that responded to human host cues were also more prone to localize the odor source. This is consistent with previous studies on host seeking in both *An. gambiae* (Spitzen et al., 2013; Hawkes and Gibson, 2016) and other vector mosquito species (Cooperband and Cardé, 2006; Dekker and Cardé, 2011; Lacey and Cardé, 2011; van Breugel et al., 2015).

Similar to case study I, case study II took advantage of the isolated, thus human-odor-free, environment of the wind tunnel system and assessed the role of CO_2 on its own as a host-seeking cue in *An. gambiae s. s.* There is currently a lack of consensus about the role of CO_2 in eliciting activation, orientation and landing in *An. gambiae s. s.* females (de Jong and Knols, 1995; Healy and Copland, 1995; Takken et al., 1997; Spitzen et al., 2008; Lorenz et al., 2013; Webster et al., 2015). The concentrations of CO_2 used in the present study are within the physiologically dynamic range of the CO_2-sensitive neurons (Majeed et al., 2017), yet had no effect on responsiveness, crosswind flight, i.e., a measure for host seeking, or the accuracy of landing on the source. These findings are in accordance with previous observations in large-volume flight arenas, which demonstrate that *An. gambiae* do not rely on CO_2 on its own to locate a human host (de Jong and Knols, 1995; Takken et al., 1997; Spitzen et al., 2008; see however Omondi et al., 2015; Majeed et al., 2017). Carbon dioxide is emitted by all hosts and is thus considered a general cue, signaling the presence of a host, but not necessarily the presence of a human (e.g., Mboera and Takken, 1997). For the highly anthropophilic *An. gambiae s. s.*, CO_2 is hypothesized to only contain information in the context of human odor (e.g., Takken and Verhulst, 2013). Such contexts include, e.g., the identification of presently inhabited human dwellings, by integrating CO_2 with a persistent human odor-laden background, or, in the presence of multiple breathing hosts, the discrimination of host type, such as in dwellings shared by cattle and humans (Cardé and Willis, 2008; Webster et al., 2015). The latter has also been shown in mosquito species that demonstrate a wider breadth of host preference, in which the general host signal, emitted CO_2, can be used as a reliable cue for host localization and discrimination (Dekker and Takken, 1998; Majeed et al., 2017).

The two case studies demonstrate the potential of the climate-controlled wind tunnel system to investigate the odor-mediated behavior of insects, including species that are sensitive to background odor contamination. The ability to finely adjust temperature and humidity, and to maintain these physical parameters at stable levels, provides the means to analyse the impact of future changes in climatic conditions on insect flight behavior. These parameters significantly affect population dynamics and vectorial capacity (Reiter, 2001; Paaijmans et al., 2010; Shapiro et al., 2017; Tang et al., 2018), but their effect on host seeking and other odor-mediated behaviors has until now not received any attention. The wind tunnel system provides additional means to analyse the effect of future anthropogenic changes in climate conditions on odor-mediated behaviors, as it requires no major modification to, for example, elevate background levels of greenhouse gasses, such as CO_2 and ozone, as well as other atmospheric pollutants (Agrell et al., 2005; Majeed et al., 2014; Cook et al., 2020). Besides assessing the impact of future climatic changes on mosquito behavior, the future perspective for our laboratory is to evaluate the effectiveness of synthetic blends as attractants in mosquito control and monitoring devices. Moreover, we envision that fine-scale analysis of behavior will provide valuable information on how the peripheral and central olfactory systems detect and integrate olfactory information.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AH, SH, and RI conceived and designed the experiments. JL designed and constructed the wind tunnel system. AH established the experimental system, performed the experiments, and analyzed the results. AH drafted the manuscript and AH, SH, and RI critically revised the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.643693/full#supplementary-material

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Mosquito brains encode unique features of human odour to drive host seeking

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A globally invasive form of the mosquito *Aedes aegypti* specializes in biting humans, making it an efficient disease vector[1]. Host-seeking female mosquitoes strongly prefer human odour over the odour of animals[2,3], but exactly how they distinguish between the two is not known. Vertebrate odours are complex blends of volatile chemicals with many shared components[4–7], making discrimination an interesting sensory coding challenge. Here we show that human and animal odours evoke activity in distinct combinations of olfactory glomeruli within the *Ae. aegypti* antennal lobe. One glomerulus in particular is strongly activated by human odour but responds weakly, or not at all, to animal odour. This human-sensitive glomerulus is selectively tuned to the long-chain aldehydes decanal and undecanal, which we show are consistently enriched in human odour and which probably originate from unique human skin lipids. Using synthetic blends, we further demonstrate that signalling in the human-sensitive glomerulus significantly enhances long-range host-seeking behaviour in a wind tunnel, recapitulating preference for human over animal odours. Our research suggests that animal brains may distil complex odour stimuli of innate biological relevance into simple neural codes and reveals targets for the design of next-generation mosquito-control strategies.

The discrimination of odour cues is a challenging problem faced by animals in nature. Decades of olfactory research have revealed the principles by which animals may identify individual compounds or simple mixtures—using combinatorial codes for flexible, learned behaviours[8–11] or labelled lines for hard-wired, innate responses[12–15]. However, most natural odours are blends of tens to hundreds of compounds[4,16,17]. How animals evolve to efficiently recognize these more complex stimuli, especially those with important innate meaning, is poorly understood[18–21].

This problem is particularly relevant for *Ae. aegypti* mosquitoes, which have recently evolved to specialize in biting humans and therefore become the primary worldwide vectors of human arboviral disease[1,22]. Female mosquitoes can detect vertebrate animals using the carbon dioxide in breath and other general cues such as body heat, humidity and visual contrast[23]. However, they rely heavily on body odour for discrimination among species[24] and show a robust preference for human odour over the odour of animals[2,3] (Fig. 1a–d). The apparent ease with which they distinguish between these stimuli is notable as vertebrate body odours are complex blends of relatively common compounds that are frequently shared across species[4–7]. Female mosquitoes require a multicomponent blend for strong attraction[25,26] and may discriminate on the basis of the ratios in which different components are mixed. Understanding exactly which features of human body odour are used for discrimination and how these features are detected at the neural level would provide basic insights into olfactory coding and potential targets for use in vector control.

Tools for mosquito olfactory imaging

Mosquitoes detect most volatile chemical cues using receptors expressed in thousands of olfactory sensory neurons scattered across the antennae and maxillary palps[27]. Neurons that express the same complement of ligand-specific receptors are believed to send axons to a single olfactory glomerulus within the antennal lobe of the brain[28] (Fig. 1e), making this an ideal location to decipher the coding of human odour blends across sensory neuron classes[10,19] (Fig. 1f). We therefore developed tools to visualize odour-evoked responses in the axon terminals of olfactory sensory neurons at this critical junction. We focus in particular on the subset of neurons that express receptors in the odorant receptor (OR) family, as these have a critical role in fine-grained host discrimination: females carrying mutations in the conserved OR co-receptor *orco* are attracted to hosts, but discriminate only weakly between humans and animals[29].

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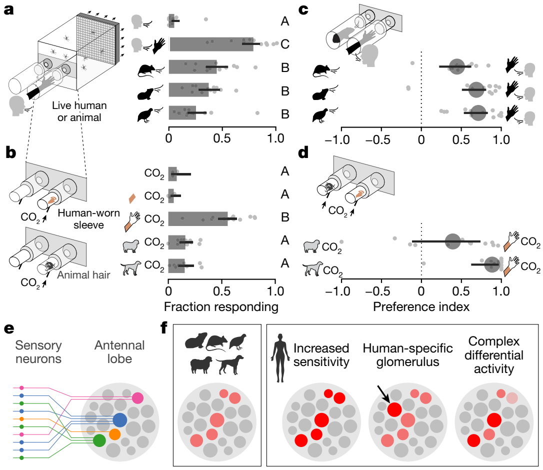

Fig. 1 | Preference of *Ae. aegypti* mosquitoes for human odour and possible coding mechanisms. a–d, The response of female *Ae. aegypti* mosquitoes to human and animal odours in no-choice (**a**, **b**) and choice (**c**, **d**) olfactometer trials. The bars (or circles) and lines represent the mean values and 95% confidence intervals from beta-binomial mixed models. $n = 9$–14 trials per treatment evenly spread across 6 humans, 2 rats, 2 guinea pigs, 1 quail, wool from 1 sheep and hair from 4 dogs. Upper-case letters (A, B, C) indicate groups with non-overlapping confidence intervals. The response to exhaled human breath (**a**, top), synthetic CO_2 (**b**, top) or unworn control sleeves (**b**, second from top) was minimal in the absence of human or animal odour. **e**, All olfactory sensory neurons that express the same receptor complex (same colour) send axons to a single glomerulus in the antennal lobe. **f**, Schematics of the several ways in which the neural activity evoked by human and animal odours in the antennal lobe may differ, enabling mosquitoes to discriminate between them. Shades of red indicate different levels of neural activity.

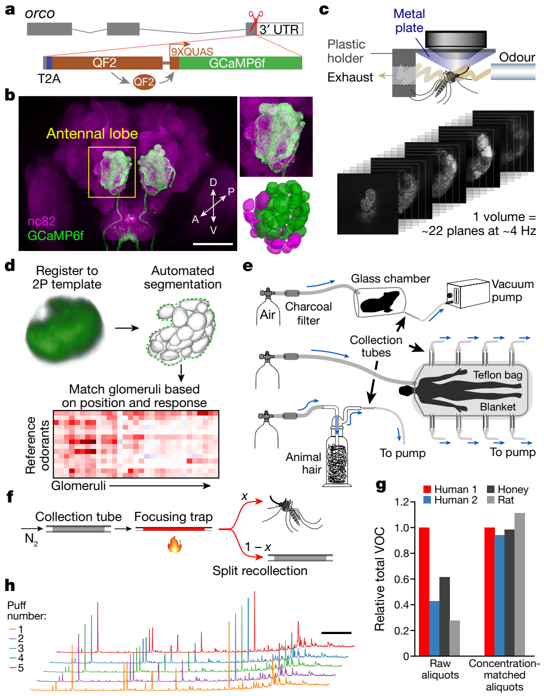

Fig. 2 | Reagents and methods for imaging *Ae. aegypti* olfactory circuits. a, The gene-targeting strategy used to drive GCaMP6f expression in Orco$^+$ sensory neurons while preserving *orco* function. **b**, Antibody staining of the *orco-T2A-QF2-QUAS-GCaMP6f* adult female brain, with a magnification of the antennal lobe (top right) and a 3D reconstruction of around 34 Orco$^+$ (green) and around 20 Orco$^-$ (magenta) glomeruli (bottom right). Scale bar, 100 µm. **c**, Schematic of mosquito preparation and stack of videos from fast volumetric imaging. **d**, The analysis pipeline. The final glomerulus-matching step can be completed manually or using an automated algorithm (Methods and Extended Data Fig. 2). **e**, Odour sampling set-ups for live animals/milkweed (top), humans (middle) and animal hair/honey (bottom). **f**, Schematic of two-stage thermal desorption for delivery of complex odour samples. The samples are transferred from collection tubes to a sorbent-filled focusing trap by slow heating and nitrogen flow. The focusing trap is then heated ballistically (to 220 °C in around 3 s) to release the samples in a short period of time (Extended Data Fig. 3a–d). The odour stream cools to room temperature during travel and is split such that an adjustable proportion flows to the mosquito, while the remainder can be recollected. **g**, Verification of the concentration-matching procedure for four representative odour samples (Methods and Extended Data Fig. 3f). Total volatile content was quantified using gas chromatography coupled with mass spectrometry (GC–MS) before (left) and after (right) matching. **h**, GC–MS chromatograms of five consecutive puffs of the same human sample demonstrating consistency of blend ratios and absolute abundance. Arbitrary *y* axis units are not shown. Scale bar, 2 min.

We used CRISPR–Cas9 to generate knockin mosquitoes expressing the calcium indicator GCaMP6f under the endogenous control of the *orco* locus[30] (Fig. 2a and Methods). Transgenic adult mosquitoes showed GCaMP6f expression in sensory neurons on the antenna and maxillary palp that project to approximately 34 out of 60 glomeruli in the dorsomedial antennal lobe (Fig. 2b and Extended Data Fig. 1; see the Methods for a discussion of variability among recent estimates of glomerulus number in *Ae. aegypti*). We also observed GCaMP6f in sensory neurons that project to the suboesophageal zone from the labellum[31] and, most likely, the legs (Extended Data Fig. 1j–l). Together with a two-photon microscope that was custom-designed for fast, volumetric imaging (Fig. 2c) and a novel analytical pipeline (Fig. 2d and Extended Data Fig. 2), the strain enabled us to capture odour-evoked responses in all Orco$^+$ glomeruli at around 4 Hz.

We next collected natural odours and developed methods to faithfully deliver these stimuli to mosquitoes during imaging. We sampled odour from humans ($n = 8$), rats ($n = 2$), guinea pigs ($n = 2$), quail ($n = 2$), sheep wool ($n = 1$), dog hair ($n = 4$) and two nectar-related stimuli that mosquitoes find attractive—milkweed flowers[32] and honey[29] (Fig. 2e). Individual human samples were kept separate, whereas those from animals were pooled by species to generate independent replicates for the human–animal comparison. For delivery, most studies use a solvent to elute odour extracts from sorbent collection tubes and then allow the solution to evaporate from a vial, septum or filter paper. However, the diverse odorants in a blend often require different solvents and will evaporate from solution at different rates based on volatility[33], changing the character of a blend over time. We therefore developed an odour-delivery system involving thermal desorption[34] that enabled us to deliver natural extracts directly from sorbent tubes to mosquitoes with precise quantitative control (Fig. 2f and Extended Data Fig. 3). Importantly, we were able to match the total odour concentration of diverse samples delivered to the same mosquito (Fig. 2g) and to deliver replicate puffs of the same sample to different mosquitoes, while maintaining the original blend ratios (Fig. 2h).

Human odour evokes unique neural responses

With these tools and odour samples, we set out to characterize the response of Orco$^+$ glomeruli to human and animal odours. There are

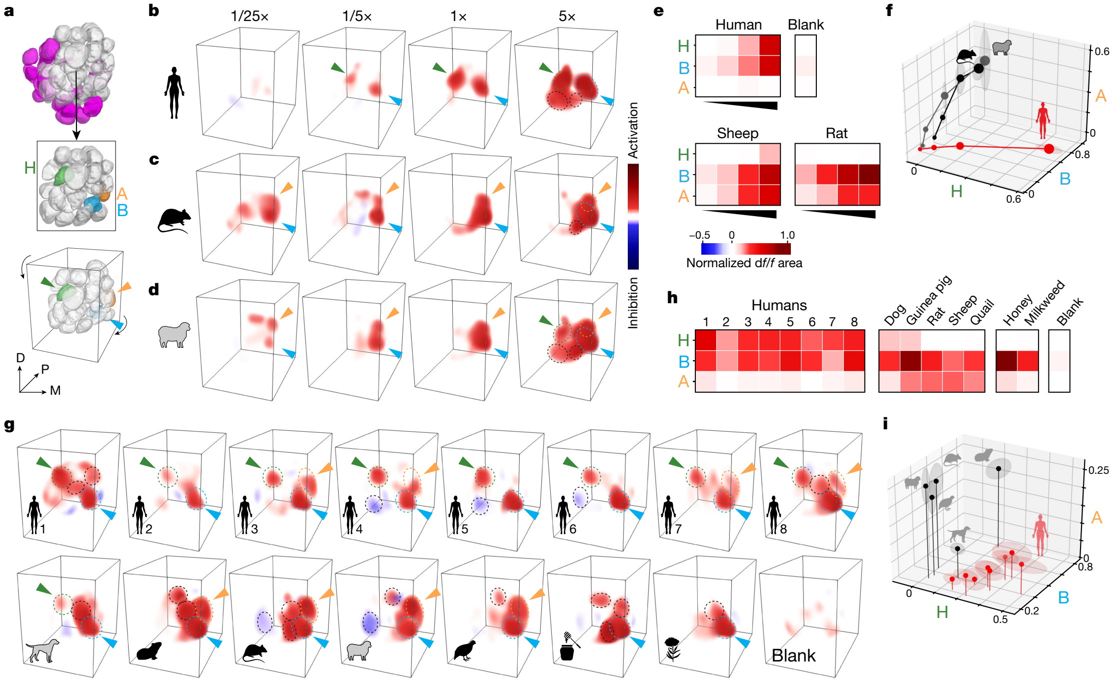

Fig. 3 | Human and animal odours activate unique combinations of antennal lobe glomeruli. a, Antennal lobe reconstructions highlighting Orco$^+$ glomeruli (top, grey), three focal glomeruli (middle, with a few anterior glomeruli removed to reveal B and A glomeruli) and the angle from which they are viewed in 3D renderings (bottom). A, animal-sensitive glomerulus; B, broadly tuned glomerulus; H, human-sensitive glomerulus. **b**–**d**, 3D renderings of the response of a single representative female mosquito to human (**b**), rat (**c**) and sheep (**d**) odours. The arrowheads indicate focal glomeruli from **a**. The dashed circles outline glomeruli responding strongly at 5× total concentration. **e**, **f**, The mean response of focal glomeruli to stimuli in **b**–**d** as heat maps (**e**) or the relative activation of each glomerulus (**f**). $n = 4$ mosquitoes. In **f**, the dot size indicates the dose, and the shading around the dots shows the s.e.m. **g**–**i**, 3D renderings (**g**), heat maps (**h**) and the relative activation of each glomerulus (**i**) as in **b**–**f**, but showing the response to the odour of 8 individual humans, 5 animal species and 2 nectar stimuli at 1× total concentration. $n = 5$ mosquitoes. Human participant numbers correspond to those in Fig. 4a, b. Neural responses were quantified by integrating the area under the *df*/*f* curve and normalizing to the highest response in each brain (Methods).

several ways in which the activity of key glomeruli might help female mosquitoes to discriminate, including increased sensitivity to human odour, exclusive activation by human odour or more-complex patterns (Fig. 1f). To investigate these possibilities, we first imaged responses to the odour of a single human and two animal species across a concentration gradient. We chose rat and sheep for the animals because they are common in human environments and provided ample odour in our extractions. All of the host odours were delivered at the same four total blend concentrations, ranging from 1/25× to 5×, where 1× approximately matches the odour of a whole human body funnelled to a mosquito in real time (Methods).

Three glomeruli dominated responses at low and middle doses (Fig. 3a–d). One was strongly activated by the odour of all three species (cyan arrowheads), whereas another responded strongly to human odour but was insensitive or only weakly sensitive to animals (green arrowheads). A third glomerulus was strongly activated by both animals, but not by humans (orange arrowheads). We tentatively refer to these as the broadly tuned (B), human-sensitive (H) and animal-sensitive (A) glomeruli, respectively. Although additional glomeruli were activated by the highest dose of each host blend (Fig. 3b–d), and there may be weak responses below the sensitivity threshold of our preparation, we were struck by the simplicity of this pattern. The relative activity of three glomeruli cleanly separated human and animal odours across the concentration gradient (Fig. 3e, f).

The preference of *Ae. aegypti* for humans over animals is robust to within-group variation, with most humans being preferred over most animals (Fig. 1a–d). We therefore examined whether the patterns of glomerular activity described above were similarly robust by imaging responses to odour from 7 additional humans (8 total), 3 more animal species (5 total) and the 2 nectar-related stimuli at a single concentration (1×) (Fig. 3g, h and Extended Data Fig. 4). The B glomerulus was again strongly activated by all of the odour extracts, including the two nectar odours, whereas the H and A glomeruli were most strongly activated by human and animal odours, respectively. The separation of human and animal odours on the basis of activity in three glomeruli is therefore robust to within-group variation (Fig. 3i). To ensure that we had not missed additional discriminatory signals among Orco$^+$ glomeruli, we also used an automated pipeline to match and quantify the response of as many glomeruli as possible across mosquitoes (Extended Data Fig. 2c). B, H and A glomeruli again explained most of the variation at 1× (Extended Data Fig. 2i–l). This analysis also revealed a fourth glomerulus just posterior to B that responded to all vertebrate odours (Extended Data Fig. 2i–l) and may be the target of well-known 1-octen-3-ol-sensing neurons that project to this region from the palp[35,36]. In summary, our results indicate that human and animal odours activate distinct combinations of glomeruli in the antennal lobe of *Ae. aegypti*, including both shared signals and those that are selectively tuned to either human or animal blends.

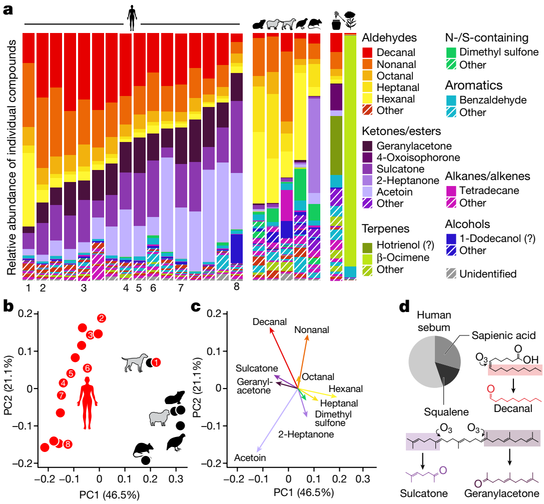

Fig. 4 | Human and animal odour blends differ in the relative concentration of key compounds. a, Odour profiles for humans, animals and nectar-related stimuli. Named compounds made up >10% of at least one sample or an average of >1% across samples. The question marks indicate tentative identifications (Methods). Animal samples were pooled by species before analysis. $n = 4$ dogs, 2 guinea pigs, 1 sheep, 2 rats and 2 quail. The numbers beneath the human samples indicate those used for imaging (Fig. 3g, h). **b**, Unscaled PCA of host odour data from **a**. **c**, The top ten loadings on the first two principal components from **b**. **d**, The proportion of human sebum made up of sapienic acid and squalene (data from ref. [50]). Oxidation of the two lipids produces volatile compounds that are enriched in human odour[41].

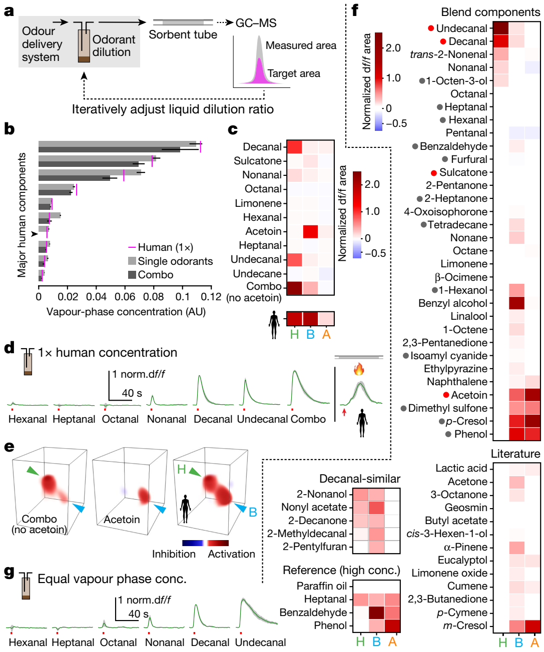

Fig. 5 | Tuning of focal glomeruli to major host odorants can explain the response to blends. a, The single-odorant delivery system and the procedure used to calibrate vapour-phase concentrations. **b**, Vapour-phase concentration of 3 s puffs of major human odorants (delivered singly or as a combo blend) calibrated to match those found in 1× human odour (magenta lines). Arbitrary units reflect the GC–MS peak area. Odorant names as in **c**. Acetoin was excluded from the mixture (black arrowhead). $n = 4$–5 puffs. **c**, The mean normalized response to stimuli from **b**. $n = 4$ mosquitoes. **d**, Time traces for the H glomerulus response to aldehydes and the combo blend from **b** and **c** (left) plus 1× human odour delivered by thermal desorption (right). **e**, 3D rendering of the response to the combo blend, acetoin and 1× human odour in a representative mosquito. The arrowheads point to H (green) and B (cyan). **f**, The mean normalized response to single-odorant stimuli delivered at equal vapour-phase concentrations (Extended Data Fig. 7d). The dots before the names indicate human-biased (red) and animal-biased (grey) compounds from our blends (Fig. 4 and Extended Data Fig. 5). $n = 4$–5 mosquitoes. **g**, Time traces for the H glomerulus response to aldehydes from **f**. The bars and black lines in **b** and green lines and grey shading in **d** and **g** indicate mean ± s.e.m.

Human odour is enriched for key compounds

The neural response to human odour must be traceable to chemical features of human odour blends. Human blends contain an array of common volatile compounds that originate from skin secretions, the skin microbiome or interactions between the two[4]. They differ consistently from animal blends in the relative abundance of at least two or three components, but quantitative, cross-species comparisons are rare and usually focus on a single compound[3,6,7,37,38]. We therefore lack a clear picture of the relative ratios and other chemical features mosquitoes may use to discriminate.

To help to fill this gap, we analysed the composition of the human, animal and nectar-related odour samples used for imaging, plus eight new human samples (Fig. 4a and Extended Data Fig. 5a–d). Importantly, we quantified the abundance of all compounds that made up at least 2% of any blend, excluding acids (sensed primarily by non-OR pathways[39,40]) and other highly polar or volatile compounds that cannot be quantified reliably within the same framework (Methods). Consistent with previous research, the vertebrate odours were dominated by aliphatic aldehydes[4,5,7], whereas nectar odours were enriched in terpenes[16] (Fig. 4a). As expected, human and animal odours shared almost all components (Extended Data Fig. 5c).

Despite the overlap in blend components, human and animal samples differed consistently in blend ratios, leading to a clear separation in a principal component analysis (PCA) (Fig. 4b and Extended Data Fig. 5e). Loadings on the human–animal axis of the PCA showed that human odour was enriched in three ketones: sulcatone, geranylacetone and acetoin (Fig. 4c). Human odour also stood out for its high relative abundance of the long-chain aldehyde decanal (ten carbons) and low relative abundance of the short-chain aldehydes hexanal and heptanal (six and seven carbons) (Fig. 4a, c). Sulcatone, geranylacetone and decanal are widely recognized as abundant in human odour[4], but consistent enrichment compared with animal odours has been previously documented only for sulcatone[3]. Interestingly, these three compounds are oxidation products of squalene and sapienic acid[41], which are unique components of human sebum that may have a role in skin protection[42,43] (Fig. 4d).

The unscaled PCA gives the most weight to abundant compounds. When we extended our analysis to minor components using compound-specific comparisons (Extended Data Fig. 5f, g), we found that human odour is also enriched for a second long-chain aldehyde: undecanal (11 carbons). An independent analysis that considered all of the detected ions, rather than a subset of curated compounds, identified a largely overlapping set of human- and animal-enriched odorants (Extended Data Fig. 5h, i). Taken together, human odour can be distinguished from animal odours by the relative abundance of a diverse set of compounds, none of which are unique to humans, but which come together in characteristic ratios to produce a uniquely human bouquet.

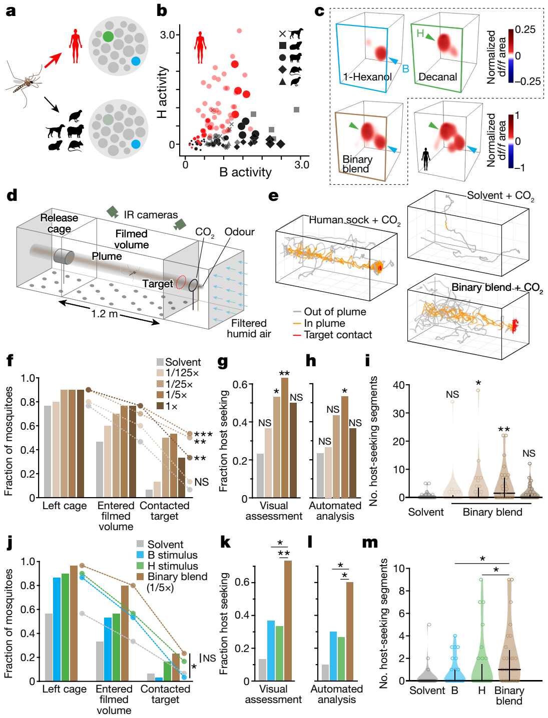

Fig. 6 | Activation of the H glomerulus enhances host-seeking behaviour. **a**, Human and animal odour can be reliably separated by a simple neural code, in which animal odour strongly activates the B glomerulus, but human odour strongly activates both the B and H glomeruli. **b**, Single-trial data from the blend-imaging experiments (Fig. 3) illustrating the separation of human and animal odour based on signalling in the B and H glomeruli. The dark symbols indicate variable doses (Fig. 3e); the light symbols indicate 1× dose (Fig. 3h). **c**, Neural responses to 1-hexanol, decanal and their binary mixture at concentrations calibrated to activate B and H glomeruli at approximately equal levels, as is the case for odour from a representative human. **d**, The wind-tunnel flight arena. **e**, Example single-mosquito flight trajectories. **f**–**m**, The response of female mosquitoes to increasing concentrations of the binary blend (**f**–**i**) or the 1/5× binary blend and its individual components (**j**–**m**). Responses quantified as the fraction of mosquitoes reaching various positions within the wind tunnel (**f**, **j**), the fraction showing at least a single bout of host-seeking flight according to a human observer (**g**, **k**) or an automated analysis (**h**, **l**; Methods and Extended Data Fig. 8), and the number of 10 s segments during which each mosquito showed host-seeking flight according to the automated analysis (**i**, **m**). The horizontal and vertical lines show the median values and quartiles. Statistical analysis was performed using survival analysis (**f** and **j**), χ^2 tests (**g**, **h**, **k** and **l**) and Mann–Whitney *U*-tests (**i**, **m**), comparing the binary blend with the solvent (**f**–**i**) or the binary blend with each individual component (**j**–**m**). $n = 30$ mosquitoes per treatment. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

H is tuned to long-chain aldehydes

To connect the unique pattern of neural activity evoked by human odour (Fig. 3) to its chemical composition (Fig. 4), we conducted additional imaging with synthetic odorants and blends delivered using standard approaches (Extended Data Fig. 6). We first examined whether the neural response to a representative human sample could be explained by the response to its major components delivered either individually or in combination (combo blend). We considered each of the 11 most abundant compounds in the human sample with two exceptions: geranylacetone was excluded because it is unstable under laboratory conditions, and acetoin was delivered singly but was absent from the combo as it requires a different solvent. We carefully calibrated the liquid dilution ratio of each stimulus (Fig. 5a) to generate vapour-phase concentrations that are characteristic of the human odour sample at 1× (Fig. 5b).

Decanal, undecanal and the combo stimulus (which included decanal and undecanal) all evoked strong and prolonged activity in the H glomerulus (Fig. 5c–e and Extended Data Fig. 7a). The B glomerulus was strongly activated by acetoin and modestly activated by the combo blend of non-acetoin compounds (Fig. 5c, e), probably the sum of a number of weak individual responses (Extended Data Fig. 7a). No human odour components evoked activity in the A glomerulus at physiological concentrations. Previous research implicated a sulcatone-sensitive receptor in *Ae. aegypti* preference for humans[3]. Although we did not see consistent activity in response to this compound at its concentration in 1× human odour (Fig. 5c), several glomeruli responded at higher doses (data not shown), suggesting that it may be more relevant to behaviour at close range. Taken together, the antennal lobe response to 1× human odour is largely explained by individual responses to a subset of perceptually dominant components, including long-chain aldehydes and acetoin (Fig. 5e).

The strong response of the H glomerulus to physiological concentrations of decanal and undecanal in human odour (Fig. 5d) suggests that it may be selectively tuned to long-chain aldehydes. To rigorously test this hypothesis and more broadly examine the tuning of all three focal glomeruli, we next imaged the response of the H, B and A glomeruli to a panel of 50 compounds all delivered at approximately the same vapour-phase concentration (Fig. 5f and Extended Data Fig. 7b–d; target concentration set to that of sulcatone in 1× human odour). The panel included compounds that were (1) identified in our odour extracts (≥0.1% abundance in any host blend); (2) suggested by the literature to be ecologically relevant for mosquitoes; or (3) structurally similar to decanal and undecanal (Methods).

As hypothesized, the H glomerulus responded selectively to long-chain aldehydes (Fig. 5f and Extended Data Fig. 7b). Both response amplitude and duration increased with aldehyde chain length, from the six-carbon hexanal that evoked no response to the eleven-carbon undecanal that evoked strong activity lasting >40 s beyond the 3 s puff (Fig. 5g). The compounds that were chosen for their chemical similarity to decanal and undecanal sometimes generated modest responses, but these were weaker than those evoked by the long-chain aldehydes themselves (Fig. 5f and Extended Data Fig. 7b). By contrast, the B glomerulus showed broad tuning. It responded to more than half of all of the compounds in the panel, including human-biased, animal-biased and unbiased odorants (Fig. 5f and Extended Data Fig. 7b), consistent with its broad response to all of the complex blends in our sample.

The A glomerulus was strongly activated by four compounds found in our host odour blends (Fig. 5f). One of these (acetoin) was human-biased (Fig. 4c), but present in 1× human odour at a concentration too low to evoke consistent activity in the A glomerulus (Fig. 5c). The other three (dimethyl sulfone, phenol, *p*-cresol) were animal-biased in our samples (Extended Data Fig. 5f, i). However, they were previously shown to be enriched in vertebrate faeces and urine[17,44], which were occasionally passed by the smaller animal species during odour extraction (Fig. 2e). Further work will therefore be needed to determine whether the A glomerulus truly provides an animal-biased signal that is useful for host discrimination. By contrast, it is clear that the H glomerulus is selectively activated by human odour due to its narrow tuning to long-chain aldehydes, and the B glomerulus responds to a wide array of natural blends due to broad tuning at the single-odorant level.

H activation enhances host seeking

Human odour evoked consistent activity in both B and H glomeruli, whereas animal odour evoked strong activity in the B glomerulus, but no activity or only weak activity in the H glomerulus (Fig. 6a). Although not the only host-responsive signals in the antennal lobe, these two glomeruli alone generate a simple neural code that can robustly separate human and animal blends at the single-trial level (Fig. 6b). We next asked whether female mosquitoes leverage these signals for host seeking and discrimination. To answer this, we characterized the behaviour of females exposed to a synthetic binary blend that is distinct from human odour, but formulated to evoke similar strong activity in B and H glomeruli (Fig. 6c and Methods). We specifically tested long-range attraction in a wind tunnel (Fig. 6d), reasoning that this stage of the host-seeking behavioural sequence is likely to rely on the olfactory responses that dominate at low to moderate host-odour concentrations. Signals evoked by more concentrated host odour, as well as thermal and visual cues, probably come into play at close range[23].

When combined with the mosquito activator carbon dioxide, the binary blend evoked a characteristic plume-tracking behaviour similar to that evoked by a human-worn sock but rarely observed in response to a solvent control[45] (Fig. 6e). This behaviour was dose dependent, peaking at a concentration that generated neural activity similar to 1/5× human odour (Fig. 6f–i and Extended Data Fig. 8). It also depended on activity in both B and H glomeruli, as revealed by testing of single components of the binary blend that activate either glomerulus individually (Fig. 6j–m and Extended Data Fig. 8). Most importantly, coactivation of the B and H glomeruli elicited stronger host seeking than activation of the B glomerulus alone (Fig. 6j–m), just as human odour elicits stronger host seeking than animal odour (Fig. 1a–d).

Discussion

Animal survival and reproduction often depend on the ability to discriminate among complex odour blends without previous experience. Here we investigated the innate preference of *Ae. aegypti* mosquitoes for human odour, offering insights into how such discrimination is achieved at the neural level. We show that human odour is enriched in long-chain aldehydes and that these aldehydes generate strong and prolonged activity in a selectively tuned olfactory glomerulus within the mosquito brain. Activation of this glomerulus alongside a second, broadly tuned glomerulus drives robust host seeking, resulting in a binary signal with the potential to explain preference for human over animal odour at long range. The simplicity of this pattern belies the complexity of the underlying stimuli and suggests that sparse coding may be a general feature of innate olfactory responses, even to multicomponent blends[18,19].

Although we have shown that activation of the H glomerulus enhances host seeking, current knowledge and genetic tools do not yet allow us to conduct the converse experiment—that is, to silence the H glomerulus and measure the extent to which it is required for host seeking and preference. We expect that the H glomerulus will be required for robust discrimination between humans and animals in at least some contexts. After all, the H glomerulus represents the most prominent human-biased signal in the OR–Orco pathway, which is itself required for such behaviour[29]. Nevertheless, other Orco$^+$ glomeruli may contribute, including those that respond only at high odour concentrations (Fig. 3b–d). It is also important to note that even *orco* mutants are strongly attracted to host odour and retain a weak preference for humans in olfactometer assays[29]. These residual responses must be largely mediated by the second major olfactory pathway in mosquitoes, made up of acid- and amine-sensing neurons that express ionotropic receptors[38–40,46]. Our own preliminary imaging in mosquitoes expressing GCaMP in all glomeruli revealed additional host-responsive signals in non-Orco regions of the antennal lobe, but no clear human-biased activity (Extended Data Fig. 9). A more complete characterization of ionotropic-receptor-based responses to human and animal odours is nevertheless an important area for future research.

Our research also sheds light on the compounds that mosquitoes may be using to discriminate among hosts. Most people associate human body odour with sweat, but the odorants we found to be important for host discrimination are probably derived from sebum (Fig. 4d), an oily substance that is secreted at the base of hair follicles. Sebum composition tends to be species-specific[47], and its output is temporally stable—as high at rest as when active[48]. These features make sebum derivatives reliable targets for human-seeking mosquitoes. Interestingly, sebum composition[49] and long-chain aldehyde levels (Fig. 4a) also vary among individual humans, albeit at a smaller scale than the difference between humans and animals. Moreover, among the handful of people who participated in our preference assays (Fig. 1), those with long-chain aldehyde levels close to the human average were more likely to be targeted by *Ae. aegypti* than those with lower or higher levels (Extended Data Fig. 10; see also the recent research linking preference for individual humans to sebum-derived acids[46]). This raises the intriguing possibility that the evolution of preference for humans over animals spills over to affect the choices mosquitoes make when targeting individual humans. In summary, our research provides new insights into mosquito preference for humans and the neural coding of complex olfactory stimuli that animal brains have evolved to discriminate.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions

and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-022-04675-4.

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Methods

Methods are provided in the Supplementary Information.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

All relevant data supporting the findings of this study are available from the corresponding authors on request. For odour-profile analysis, data are included in Supplementary Table 1. Source data are provided with this paper.

Code availability

Code used for analyses and all unique biological materials generated in this study are available from the corresponding authors on request. Control code for the odour-delivery system, design files for the two-photon mosquito holder, and code for the XCMS-based odour analysis are available at GitHub (https://github.com/mcbridelab/Zhao_2020_HumanOdorRepresentation). For the analysis pipeline for volumetric antennal-lobe imaging, code is available at GitHub (https://github.com/rwth-lfb/Zhao_et_al).

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Author contributions Z.Z. and C.S.M. conceived the project and designed and interpreted all of the experiments, with equal contribution from J.L.Z. on odour analyses and A.H. and R.I on wind-tunnel behaviour. Z.Z. performed the experiments in Figs. 2, 3 and 5 and helped to perform the experiments in Figs. 4 and 6. J.L.Z. helped to collect odour samples and analysed all odour data in Fig. 4 and Extended Data Fig. 5. A.H. performed behaviour experiments in Fig. 6 under the supervision of R.I.; A.L.K. helped to design and perform odour extractions in Fig. 4. A.I. performed the experiments in Fig. 1a–d. B.J.M. and M.A.Y. provided advice on sgRNA and donor plasmid design for targeting the *orco* locus. S.T. designed and built the two-photon microscope used for volumetric imaging. M.S. developed the automated analysis pipeline for volumetric imaging, which he discussed with D.M.; Z.Z. and C.S.M. wrote the paper with help from J.L.Z. and the other authors.

Competing interests Princeton University has filed a patent (US 62/705,910, status pending) for using synthetic blends that mimic the response to human odour in the mosquito brain as mosquito attractants, listing C.S.M. and Z.Z. as inventors. The other authors declare no competing interests.

Additional information
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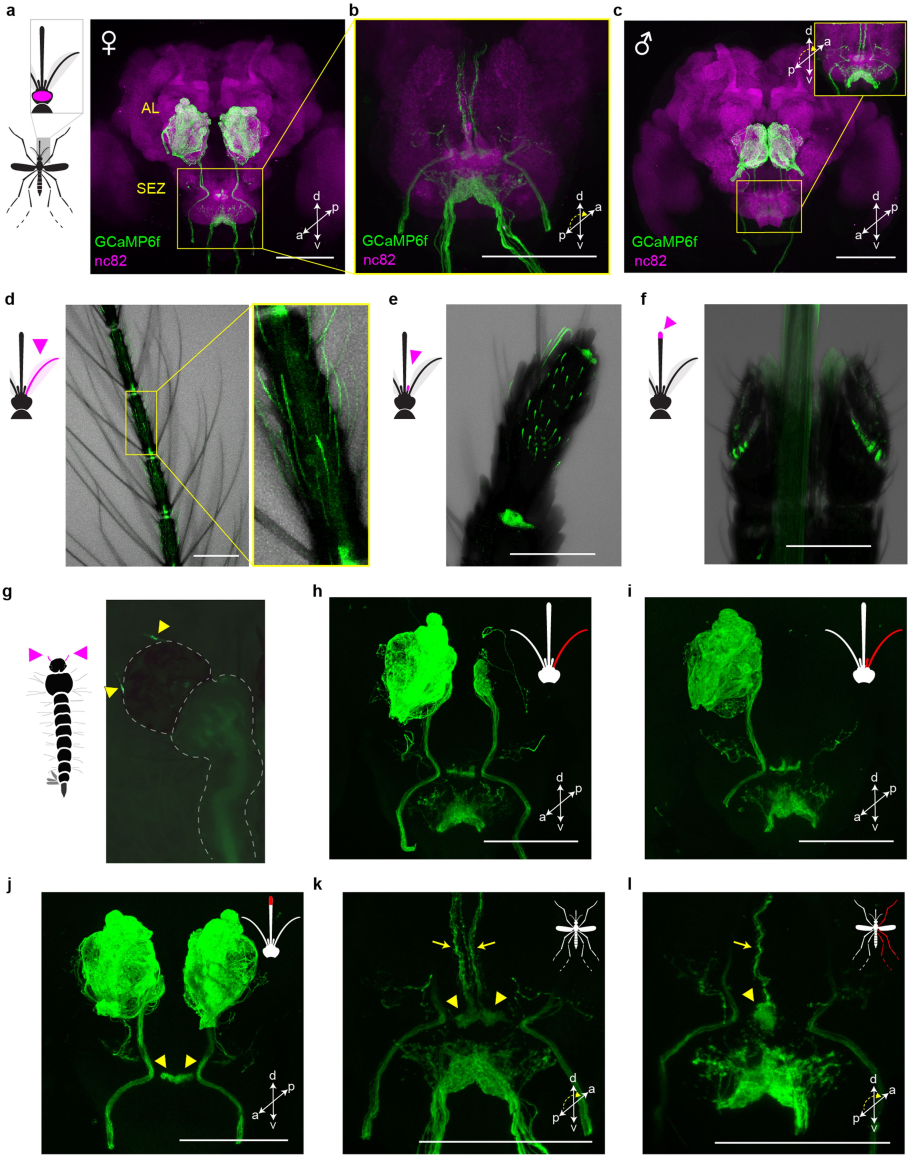
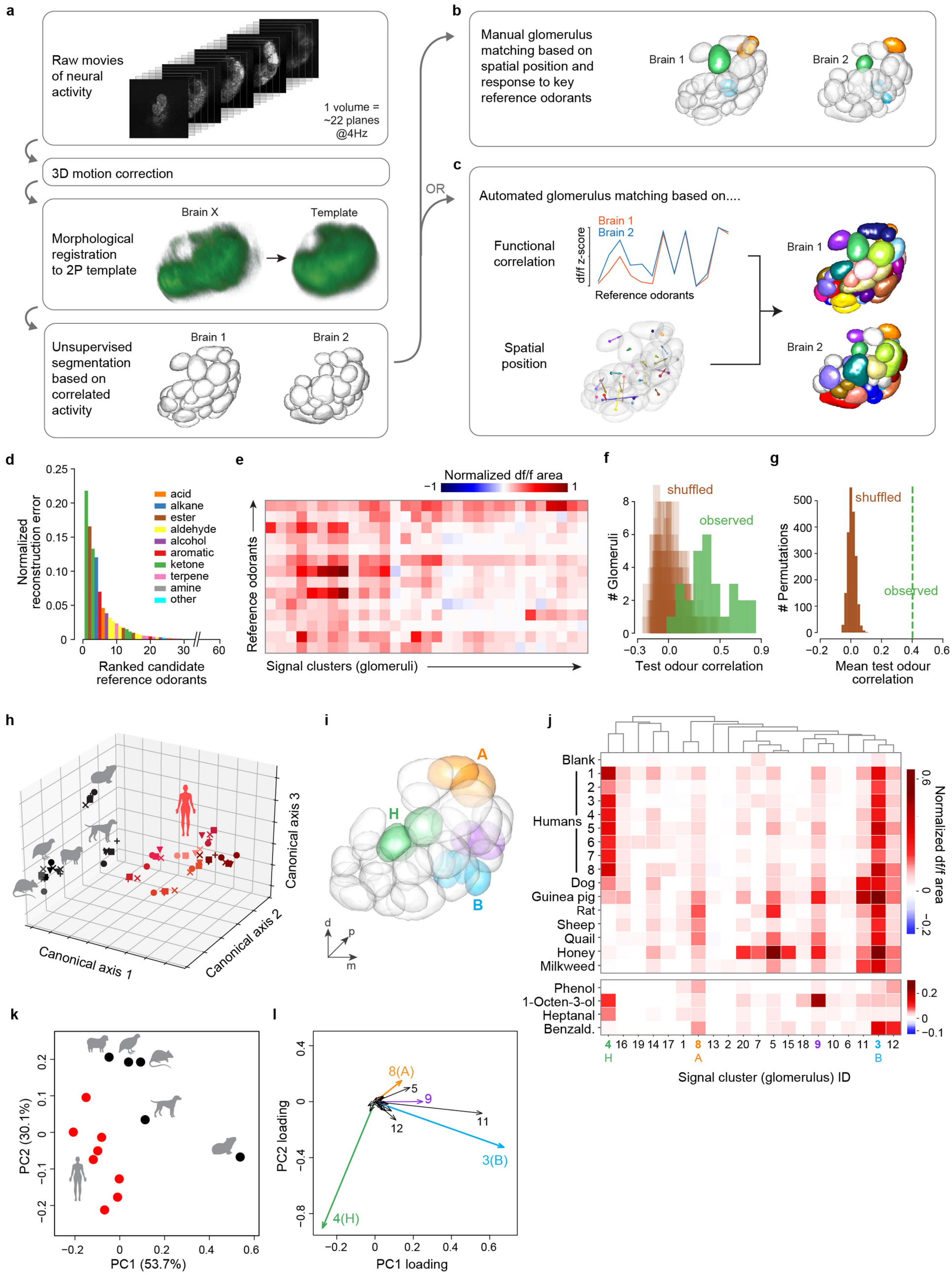
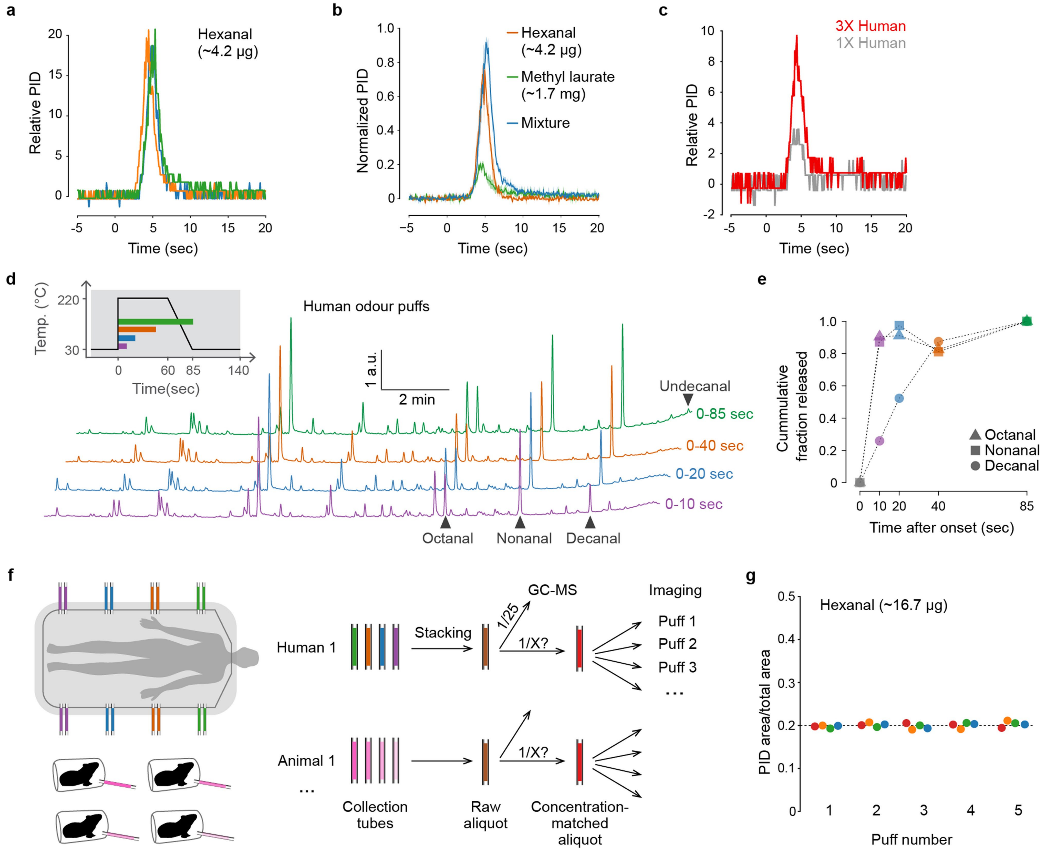


Extended Data Fig. 1 | *orco-T2A-QF2-QUAS-GCaMP6f* labels chemosensory neurons in peripheral organs that project to the brain. a–**c**, Antibody staining in female (**a**, **b**) and male (**c**) brains showing GCaMP in sensory neurons that innervate the antennal lobe (AL) and suboesophageal zone (SEZ). SEZ in (**b**) and the inset of (**c**) are viewed from posterior to better visualize GCaMP signal. **d**–**g**, Intrinsic GCaMP fluorescence in sensory neurons of adult female antenna (**d**), maxillary palp (**e**), labella (**f**) and larval antennae (**g**, arrowheads). Transmitted light image overlaid in (**d**–**f**). **h**–**l**, Antibody staining in brains of female mosquitoes with severed sensory organs (red in mosquito schematics). Severing right antenna only (**h**) led to loss of signal in all ipsilateral glomeruli except two in the posterior-medial region. Severing of both right antennae and right palp (**i**) led to loss of signal in all ipsilateral glomeruli. We therefore infer that Orco$^+$ AL glomeruli are innervated by sensory neurons in the ipsilateral antenna (n~32 glomeruli) and palp ($n = 2$ glomeruli)[51,52]. Severing the tip of the proboscis (including the labella) led to loss of signal throughout the ventral SEZ (**j**), consistent with work in *Anopheles gambiae* indicating that Orco$^+$ labellar neurons innervate this region[31]. However, labellum-less animals retained signal in an area of the dorsal SEZ recently termed the suboesophageal glomeruli (arrowheads in **j**) (https://www.mosquitobrains.org/). Signal in this dorsal region and corresponding ascending nerves was present in intact animals (**k**, arrowheads and arrows, respectively) but was lost when the ipsilateral legs were severed (**l**). This suggests that *Ae. aegypti* has Orco$^+$ neurons on the legs that project to the SEZ, consistent with electrophysiological responses to olfactory stimuli on legs[53]. All scale bars 100 µm.

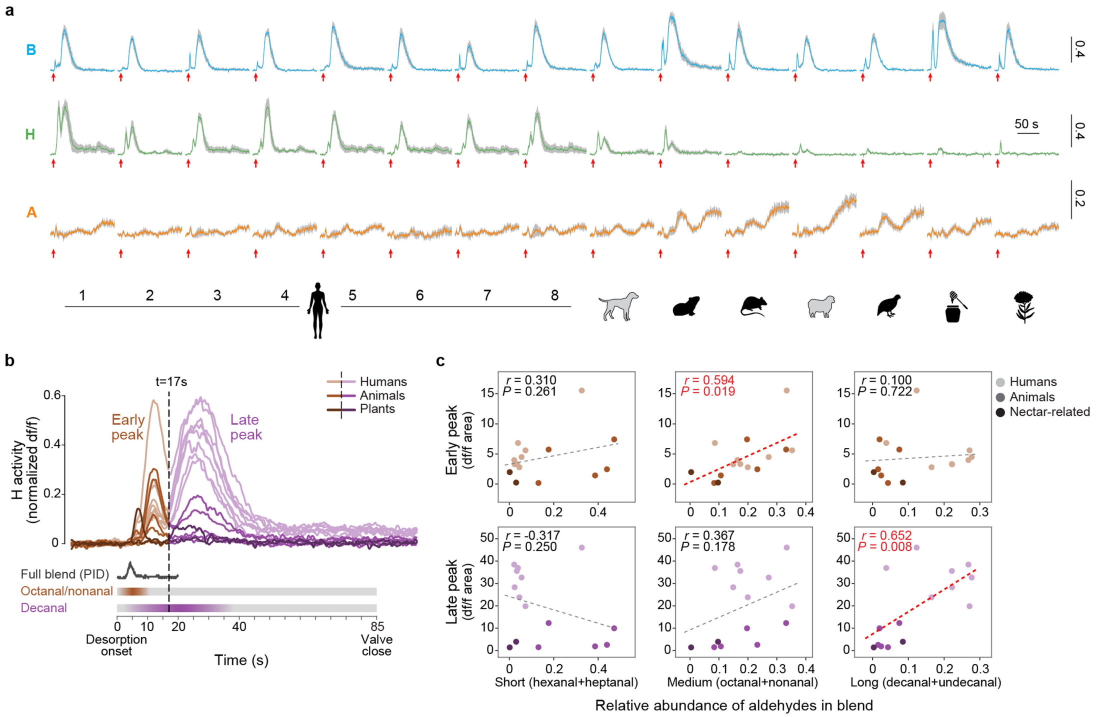


Extended Data Fig. 2 | See next page for caption.

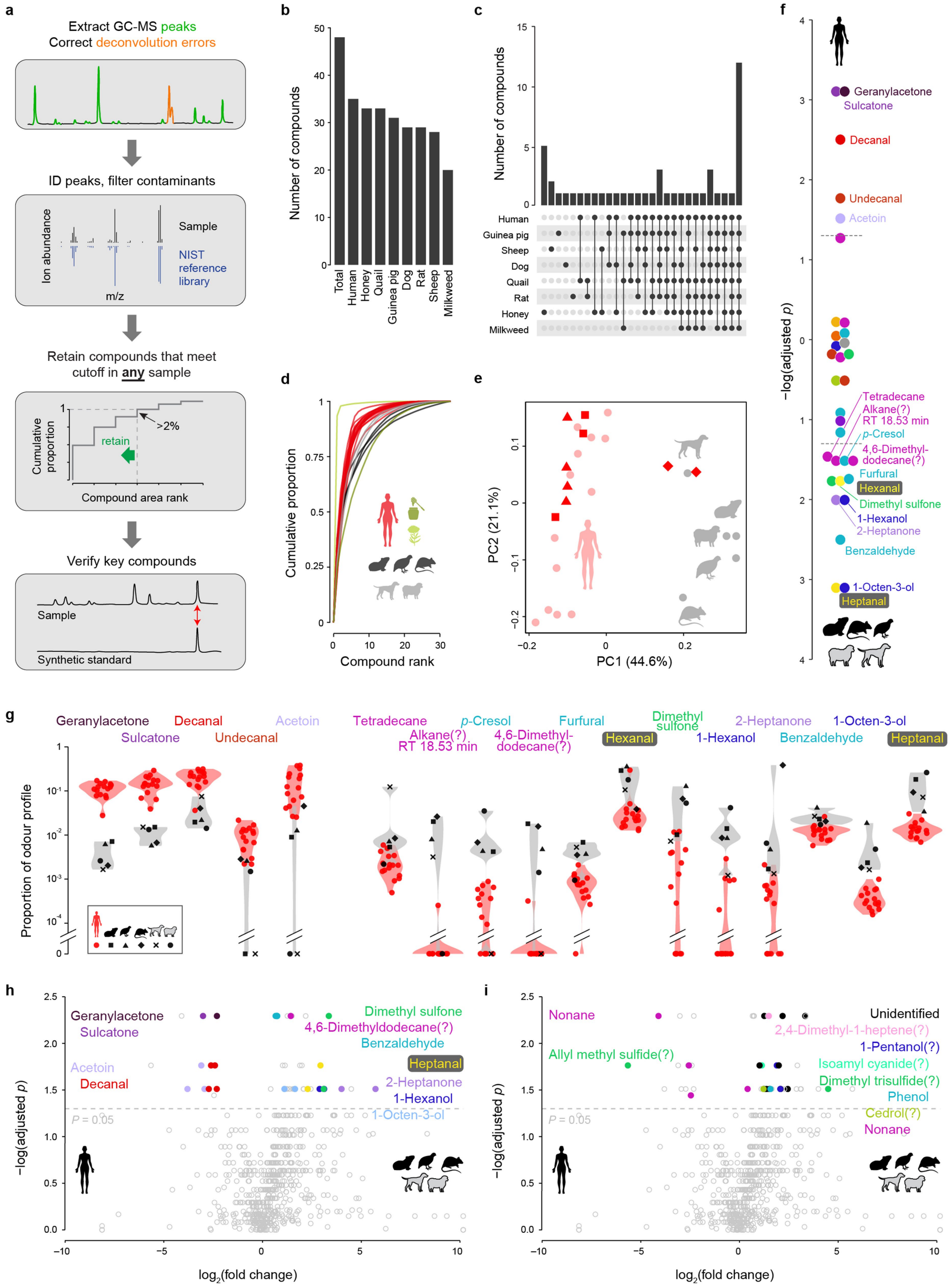
Extended Data Fig. 2 | Automated analysis of volumetric antennal lobe imaging data. a–**c**, Analysis pipeline schematic. After registration and unsupervised segmentation of all brains in a given data set (**a**), one brain was chosen as the reference and glomeruli from other brains were matched to those in the reference either manually (**b**) or using an automated pipeline (**c**). Colours in (**b**, **c**) show matched glomeruli (unmatched in white). Manual matching was performed for analyses focused on only B (cyan), H (green), and A (orange) glomeruli (Fig. 3, 5). Automated matching, which provides a more global picture of activity but is less reliable for the three focal glomeruli, was used for the complementary analysis presented here (**i**–**l**). In both cases, matching was based on spatial position and response to 14 reference odorants. **d**, Reference odorants were chosen from among 60 candidates based on their ability to account for a large part of the observed signal variance/neural activity (see Methods). The 10 top-ranked odorants belonged to 8 different chemical classes. **e**, Response of glomeruli from one mosquito to the final 14 reference odorants. Note that we delivered reference odorants at high concentration (neat, 10^{-1}, or 10^{-2} v/v dilutions) in order to activate as many glomeruli as possible. **f**, Evaluation of automated glomerulus matching. Glomeruli from 6 brains were matched as in (**c**). We then asked whether the matched glomeruli showed correlated responses to a new set of 13 test odorants. The plot shows the observed distribution of correlation coefficients across $n = 28$ sets of matched glomeruli (green) and 20 shuffled distributions where matches were reassigned at random (brown). Low correlations may be caused by mismatches or general lack of response by a given set of matched glomeruli to the test odorants. **g**, Same as (**f**) except showing the mean of the observed distribution (green line) and the distribution of means from 2000 shuffled datasets. **h**–**l**, Reanalyses of data presented in Fig. 3g–i relying in part or in whole on the automated pipeline. **h**, Human and animal odours were cleanly separated along the first three axes of an across-matrix PCA of unmatched signal clusters from all mosquitoes (see Methods). Symbols denote individual mosquitoes ($n = 5$); shades of red denote odour from different human subjects ($n = 8$). **i**–**l**, Human and animal odours were also cleanly separated in an analysis of signal clusters matched by the automated algorithm (**c**–**g**). Panel (**i**) shows signal clusters from the segmented antennal lobe of the reference mosquito, with key glomeruli highlighted. Panel (**j**) shows the mean normalized response to odour extracts (top) and select reference odorants (bottom) for those signal clusters (numbered across the bottom) that could be matched in the brains of at least 3 of 5 mosquito replicates. Panels (**k**, **l**) show a principal components analysis of data from (**j**). Note that the limited resolution of fast, volumetric imaging causes low level bleed through of signal from one glomerulus to proximal regions of adjacent glomeruli, especially along the z (depth) axis. This can make the delineation of adjacent glomeruli during unsupervised segmentation imperfect. Some glomeruli are split into two or more initial segments (e.g. two green H segments in (**i**) were initially split, but later merged by the automated algorithm due to correlated reference-odorant-responses, see Methods), while others show a shadow of the response pattern of their neighbors (e.g. clusters 11 and 12 shadow B in (**j**) and (**l**)). Nevertheless, the automated analysis supports the results of the manual analysis, showing that B, H, and A dominate host odour responses and contribute to the separation of human and animal odours. A fourth glomerulus (signal cluster #9) also contributes significantly and is highlighted in purple in (**i**–**l**). This glomerulus was obscured in the manual analysis because it is just posterior to B and has partially correlated responses to vertebrate odours. However, unlike B, it is strongly activated by the reference odorant 1-octen-3-ol (**j**), a known ligand of palp neurons[36] that project to this posterior-medial region of the antennal lobe[35] (Extended Data Fig. 1h–i).



Extended Data Fig. 3 | Further characterization of the thermal-desorption odour-delivery system. a, Puff shape for hexanal, measured with a photoionization detector (PID) at the location of mosquito antennae in imaging set-up (*n* = 3 puffs). Time = 0 indicates the onset of focusing-trap desorption. It takes ~3 s for the desorbed odour to reach the mosquito. **b**, Puff shape for hexanal (orange), methyl laurate (green), and their mixture (blue), showing that the temporal dynamics of odour release are similar for odorants with markedly different volatility (*n* = 3 puffs each). **c**. Puff shape for human odour delivered via thermal desorption and detected using a PID. Note that the PID may not detect all blend components given their low individual concentrations. As demonstrated in (**d**, **e**), a few components are released over a longer period of time. **d**, GC–MS traces showing the composition of replicate puffs of human odour collected for a period of 10, 20, 40, or 85 s following the onset of trap desorption. Inset shows focusing-trap temperature across each interval. **e**, Fraction of major aldehydes that were released within the given intervals (calculated from (**d**)). Consistent with PID measurements (**c**), most major components were released within the first 10 s, including octanal and nonanal, but decanal took longer to fully desorb. Abundance of undecanal is too low for precise quantification, but it also appears to experience delayed release. **f**, Schematic of process for pooling ('stacking') odour samples and matching their concentrations before use in imaging. We stacked multiple collection tubes from the same individual human subject (or different individuals of the same animal species) to generate a single raw aliquot (brown). We then quantified 1/25th of each raw aliquot via GC–MS in order to inform the subsequent generation of concentration-matched aliquots (red) with the same total odour content (Fig. 2g). **g**, Concentration of five replicate puffs of hexanal delivered from each of four sample tubes (different colours) demonstrating repeatability of the delivered stimulus.

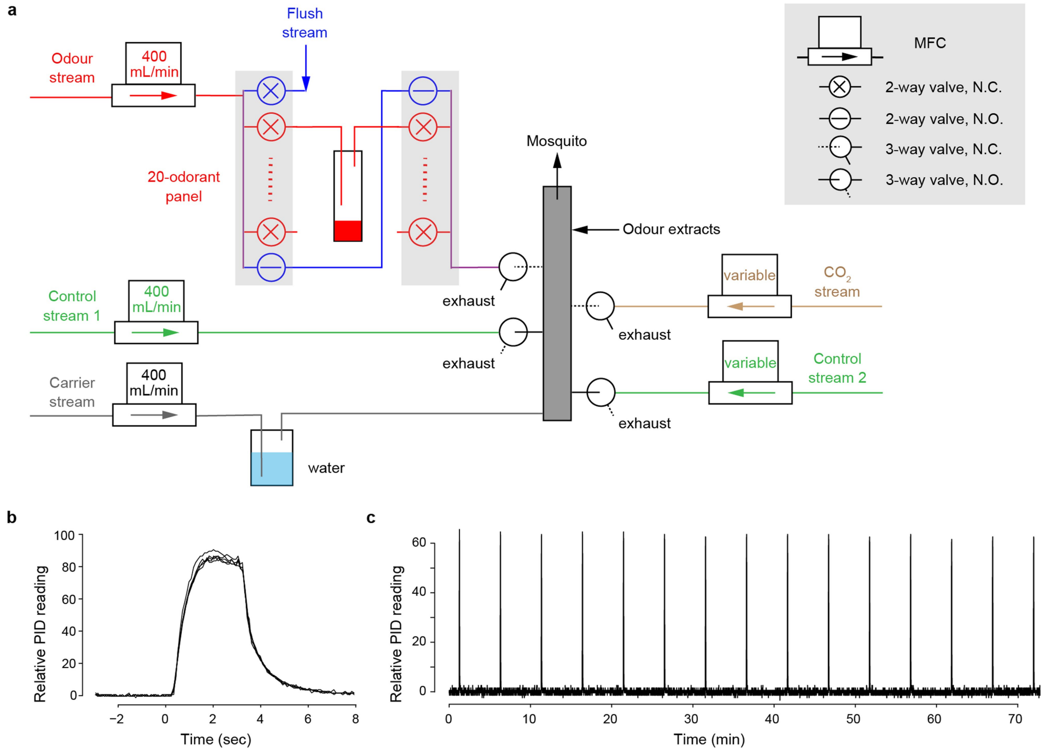


Extended Data Fig. 4 | Temporal features of glomerular response to complex odour extracts. a, Response of three target glomeruli to 1X concentrations of the given stimuli (same data summarized in Fig. 3h, i). Coloured lines and grey shading show mean ± SEM response (*n* = 5 mosquitoes). Red arrows under each trace mark desorption (heating) onset. Y-axis scale bars indicate normalized df/f. The biphasic responses seen for B and H and the delayed responses seen for A are likely a technical artifact of thermal desorption odour delivery, resulting from delayed release of a few key compounds (see **b**–**c**). **b**, Overlay of H responses from (**a**), recoloured to distinguish the early (brown) and late (purple) peaks. Schematics below show the human-odour puff shape estimated with a PID (Extended Data Fig. 3c) and the inferred timing of release of three major aldehyde components (Extended Data Fig. 3d, e). **c**, Correlations between the area under the peaks in (**b**) and the relative abundance of major aldehydes in the respective stimuli. Dashed lines show linear regressions. The early H peaks are significantly correlated with the abundance of medium-chain aldehydes (which are fully released within the first 10 s), while late H peaks are correlated with the abundance of long-chain aldehydes (which take 20–40 s to fully desorb). Taken together, the biphasic response of the H glomerulus is therefore likely caused by the different release dynamics of medium- and long-chain aldehydes. The late peaks seen in B and A traces may also be caused by delayed release of strong activators. However, other temporal features of these responses, including the prolonged tonic nature of the A response, are biological, since we saw them both here and when single synthetic odorants were delivered in a more traditional way (Extended Data Fig. 7). To account for temporal artifacts, we always report glomerular responses as area under the full df/f curve (rather than peak df/f). We do not expect this to significantly impact our findings as the responses of olfactory sensory neurons to different compounds and their mixtures are mostly additive in insects[21].

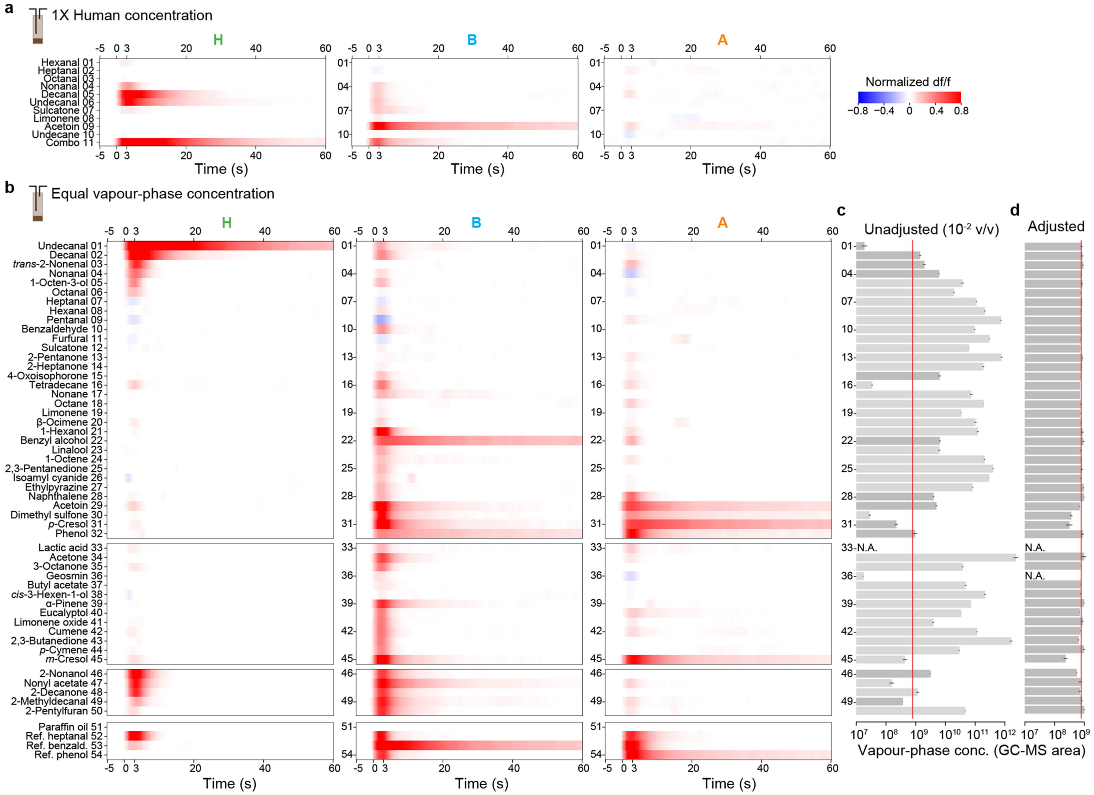


Extended Data Fig. 5 | See next page for caption.

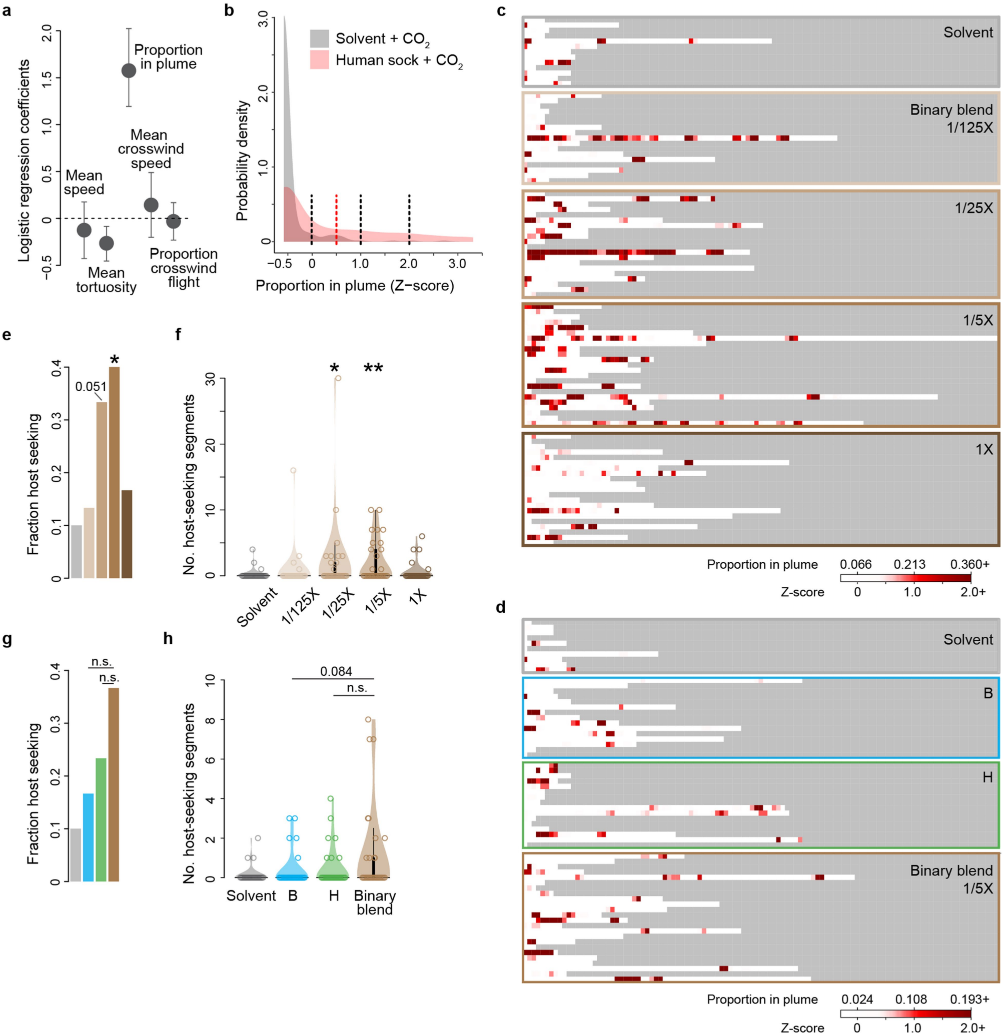
Extended Data Fig. 5 | Quantitative analysis of human and animal odours. **a**, Analysis pipeline for GC-MS data. **b**, Total number of compounds found in each odour extract. **c**, Number of compounds found exclusively in the given combination of odour extracts. **d**, Cumulative distribution of odorants in each odour profile. **e**, Unscaled principal components analysis of human and animal odour profiles similar to Fig. 4b but including 2–4 replicate odour extractions for three of the human subjects. The subjects with replicate data are denoted by triangles, squares, and diamonds, respectively; all other subjects are represented by light red circles. **f**, *P*-values from Kolmogorov-Smirnov tests for a difference in the relative abundance of each odorant between humans and animals (with Benjamini-Hochberg multiple test correction). Values extend up or down from zero for human- or animal-biased odorants, respectively. Dashed lines mark $P = 0.05$. **g**, Violin plots showing on a log scale the relative abundance of odorants that passed the significance threshold in (**f**). **h**,**i**, Alternative analysis of human and animal odours using the program xcms, which matches the component ions of compounds across samples (rather than the compounds themselves, see Methods). Volcano plots show differences in abundance of all identified chromatogram components, with colour highlighting those corresponding to compounds that were (**h**) or were not (**i**) also significant in the original analysis in (**f**). *P*-values were calculated using Kolmogorov-Smirnov tests, corrected for multiple testing using the Benjamini-Hochberg procedure.



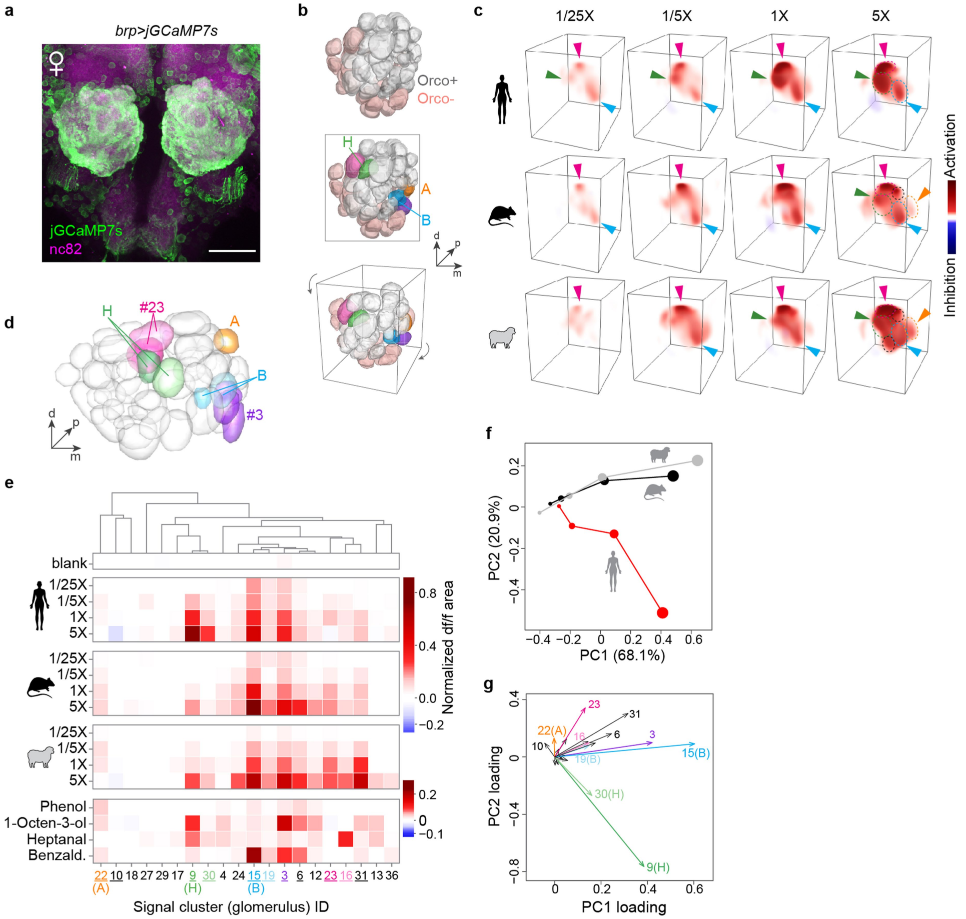
Extended Data Fig. 6 | Design and characterization of the single-odorant delivery system. a, Design schematic. Filtered air is split into 5 streams, each regulated by a mass flow controller (MFC). The humidified carrier stream (grey) flows continuously through the mixing manifold (grey box) to the mosquito. Normally, the two control streams (green) are also flowing through 3-way valves to the manifold. Synthetic odorants and CO_2 are delivered through the odour stream (red) and CO_2 stream (brown), respectively. The odour stream has 20 odour channels (red) plus a bypass (blue). To puff the odorant in a given vial, the bypass closes, 2-way valves flanking the odour vial open, and the headspace of the odour vial is carried by the odour stream to a 3-way valve that diverts the stream from exhaust to the mixing manifold with a delay. Meanwhile, control stream 1 is diverted to exhaust to maintain a constant flow rate. When delivering CO_2, the CO_2 stream (fed by a carbogen tank) is similarly diverted to the mixing manifold and offset by control stream 2. The high-flow flush (blue, 2000 ml/min) opens between odour puffs to remove residual odorant from the system. Output of the thermal-desorption system used to deliver complex odours also joins the final mixing manifold. MFC, mass-flow controller; N.C., normally closed; N.O., normally open. See Methods for more detail. **b**, Shape of odour puffs delivered by the system, featuring fast rise/decay and stable peak height. Five replicate 3-second puffs of 2-heptanone (10^{-2} in paraffin oil) were aligned to the command onset (time = 0). **c**, Long-term stability of odour puffs delivered by the system. A 3-second puff of 2-heptanone (10^{-2}) was delivered every 5 min for 75 min.



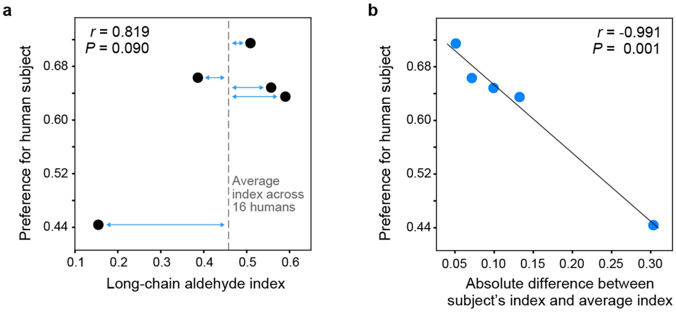
Extended Data Fig. 7 | Response of three target glomeruli to single odorants. **a**, Mean response to major components of human odour delivered at their respective concentrations in a 1X human sample. Combo is a mix of all the individual components except acetoin. **b**, Mean response to individual odorants delivered at equal vapour-phase concentration (but see a few exceptions in **d**). Odorants are grouped according to those identified in our natural extracts (#1–32), biologically relevant compounds discussed in the literature (#33–45), compounds structurally similar to decanal/undecanal (#46–50), and high concentration reference odorants (#52–54). In both (**a**) and (**b**), responses from individual mosquitoes ($n = 4$–5) were normalized to the response of glomerulus H to decanal. Note that glomerular responses to single components are often prolonged, lasting well beyond the 3-second stimulus. This is consistent with recent single-sensillum recordings that found a prolonged response by olfactory sensory neurons to certain odorants, including aldehydes[54]. **c**–**d**, Vapour-phase concentration (estimated via GC-MS peak area, arbitrary units) of single-odorant puffs coming off the headspace of a 10^{-2} v/v liquid dilution (**c**) or an adjusted dilution calibrated individually for each odorant to generate a uniform target vapour-phase concentration (red lines) (**d**). Bars and black lines indicate mean ± SEM ($n = 2$–11 replicate puffs per odorant). Odorants ordered as in (**b**). Light-grey bars in (**c**) indicate high or low volatility odorants for which the 10^{-2} data are an extrapolation from a different initial, pre-calibration dilution (anywhere from neat to 10^{-6} v/v), which was necessary to match the dynamic range of the GC-MS. Note that dimethyl sulfone, *p*-cresol, and *m*-cresol were too insoluble/nonvolatile to achieve the target concentration, and geosmin was purposely delivered at a lower concentration (undetectable via our GC-MS set-up, headspace of a 10^{-3} liquid v/v dilution) to avoid contamination of our delivery system (as it readily adsorbs to surfaces). Lactic acid cannot be quantified via standard approaches and was placed in the odour vial undiluted.



Extended Data Fig. 8 | Supporting analyses of the wind-tunnel experiments. a–**d**, Data supporting the automated analysis of host seeking presented in Fig. 6. **a**, We first assessed the utility of various flight parameters for the identification of host-seeking behaviour. We divided the flight trajectories of female mosquitoes exposed to human-worn socks *versus* odourless solvent into 10-second segments and then used a multivariable logistic regression to test the predictive power of each variable in discriminating between the two types of segments. Values of all variables were standardized as z-scores, making the plotted regression coefficients directly comparable. Dots and lines indicate mean ± SEM. The proportion of each 10-second segment that a mosquito spent in the odour plume had by far the most predictive power. **b**, Distribution of proportion-in-plume z-scores for human-worn sock (red) and solvent (grey) segments. A simple threshold of 0.5 (dotted red line) was used to identify 'host seeking' in Fig. 6h–i, l–m, but other thresholds (dotted black lines) produced similar results. **c**, **d**, Flight trajectories for individual mosquitoes visualized as the proportion of each consecutive 10-second segment spent in the plume. We tested $n = 30$ mosquitoes for each treatment, but trajectories are only shown for those that entered the filmed volume (Fig. 6f, j). **e**–**h**, Complementary analysis of wind-tunnel data that identifies host-seeking segments based on k-means clustering with all 5 flight variables (see Methods) rather than a simple proportion-in-plume threshold.



Extended Data Fig. 9 | Antennal lobe response to human and animal odours during pan-neuronal imaging. a, Antibody staining of mosquito antennal lobe in an animal expressing jGCaMP7s under the control of the *brp* pan-neuronal driver. All glomeruli are strongly labelled with jGCaMP7s. Scale bar 50 µm. **b**, AL reconstruction from confocal imaging highlighting Orco$^+$ and Orco$^-$ glomeruli (top), five focal glomeruli discussed below (middle), and the viewing angle used in (**c**) (bottom). **c**, 3D renderings of the response of a single representative female mosquito to human, rat, and sheep odour. Dashed circles outline glomeruli that responded strongly at 5X. Arrowheads highlight key glomeruli, including an non-Orco glomerulus (magenta) adjacent to H that responded strongly to both human and animal odour in most replicate mosquitoes. **d**–**g**, Automated analysis of pan-neuronal imaging data, showing segmented antennal lobe of the reference mosquito (**d**), mean normalized response for all signal clusters that could be matched in the brains of at least 3 of 4 replicate mosquitoes (**e**), and principal components analysis of mean responses (**f**, **g**). As explained in Extended Data Fig. 2, the limited resolution of fast, volumetric imaging causes low-level bleed-through of signal from one glomerulus to adjacent regions of neighboring glomeruli, especially along the z (depth) axis. This can make the delineation of adjacent glomeruli during unsupervised segmentation imperfect, especially when all neurons express GCaMP as they do here. Some glomeruli are split into two or more initial segments (*e.g.* two green H segments in (**d**)), while others show a shadow of the response pattern of their neighbours. For example, signal cluster #30 shadows H, #19 shadows B, and #16 shadows #23 (magenta). To help readers more easily distinguish shadow signals from independent glomeruli, we have used dark and light shades of the same colour to highlight what we believe are source and shadow clusters, respectively, in (**e**) and (**g**). The overall pattern is consistent with the result from Orco$^+$ imaging (Extended Data Fig. 2) in that four key Orco$^+$ glomeruli contribute to host responses: B, H, A, and the 1-octen-3-ol-sensitive glomerulus posterior to B (signal cluster #3, purple). Importantly, H is still the only glomerulus that is selective for human odour. However, we now see two to three broadly host-sensitive signals coming from Orco$^-$ regions of the AL. Of these, the magenta glomerulus adjacent to H was the most consistent upon manual data inspection. It can be seen clearly in (**c**), but is less prominent in the automated analysis (signal cluster #23) due to the challenges of matching glomeruli across brains in this non-model species. Based on position, this glomerulus may be one of those that express the Ir8a co-receptor[51,52].



Extended Data Fig. 10 | Suggestive correlations between preference for individual humans and their aldehyde profiles. a, Relationship between the extent to which a given human subject was 'preferred' (over animals in live-host preference assays) and the long-chain aldehyde content of the subject's body odour. Preference estimates come from a reanalysis of the data in Fig. 1c, including human subject as a fixed effect. The long-chain aldehyde index is the ratio of long-chain aldehydes to total aliphatic aldehydes in a subject's body odour. Dashed line indicates the average index across the $n = 16$ humans analysed in Fig. 4a. **b**, Same as (**a**), except x-axis now represents the difference between a subject's long-chain aldehyde index and the average human index (blue arrows in **a**). Line shows linear regression.

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Mosquitoes locate and discriminate among potential hosts primarily based on olfactory cues. This thesis investigated odour-mediated host seeking and discrimination in two disease vector mosquitoes, *Anopheles gambiae* and *Aedes aegypti,* from chemistry to neural coding, behaviour and modulation.

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