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The genetic basis of host preference of the European disease vector *Culex pipiens*

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Cover: Photograph of *Culex pipiens*, taken by Anders Lindstöm

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Abstract

Host seeking in mosquitoes is primarily driven by olfaction, with host odours comprising blends of odorants. Closely related mosquito species display marked differences in the expression and function of genes hypothesized to be involved in regulating host seeking and discrimination. The two biotypes of *Culex pipiens*, hereafter called Pipiens and Molestus, demonstrate differential host preference, however little is known about the molecular mechanisms regulating this behaviour. This thesis provides novel insights into the genetic basis of host preference of the two biotypes by identifying and characterising the behaviour of laboratory and wild European populations, and by studying the differences in expression and function of antennal chemosensory genes correlated with the observed behaviour. Behavioural analysis of laboratory populations of Pipiens and Molestus, using a Y-tube olfactometer, demonstrate a difference in host preference between the biotypes. To identify chemosensory genes correlated with this differential host preference, antennal transcriptome analysis of behaviourally phenotyped mosquitoes was used to identify differences in expression of chemosensory genes, including odorant receptor genes (Paper I). Differences in host preference were also observed in wild populations of the biotypes and their hybrids, with the host preference of the mosquitoes varying across a latitudinal gradient in Europe, along with differences in their relative populations (Paper II). Finally, the identified odorant receptors, from Paper I, were functionally characterised via heterologous expression in HEK293 cells in a ligand-induced fluorescence assay. Only one odorant receptor responded, with four compounds present solely in human odours, eliciting a dose-dependent response. A comparative analysis of the 3D-predictive protein structures of the Or205 of both biotypes revealed differences in gene sequence and protein structure, which could explain the observed differences in receptor function (Paper III). Together, the studies in this thesis expand our understanding of the genetic mechanisms regulating host preference in the *Cx. pipiens* biotypes.

Keywords: *Culex pipiens*, Molestus, host preference, Y-tube olfactometer, antennal transcriptome, odorant receptor, field collections, functional characterisation, HEK293 cells, predictive protein modelling

Den genetiska grunden för värdpreferensen hos den europeiska sjukdomsvektorn Culex pipiens

Abstract

Värdsökande hos myggor drivs främst av luktsinnet, där honmyggor attraheras till och särskiljer mellan värddjur beroende av blandningar av doftämnen som de avger. Närbesläktade myggarter kan uppvisa tydliga skillnader i uttryck och funktion av gener som korrelerar med värdsökande och -diskriminering. De två biotyperna av *Culex pipiens*, Pipiens och Molestus, uppvisar olika värdpreferenser, men lite är känt om de molekylära mekanismer som reglerar detta beteende. Målet med denna avhandling är att ge nya insikter i den genetiska grunden för värdpreferens hos de två biotyperna genom att initialt identifiera och karakterisera beteendet hos laboratorie- och vilda europeiska populationer, samt att studera skillnader i uttryck och funktion av kemosensoriska gener uttryckta i myggans antenn som korrelerar med myggornas preferens. Beteendeanalyser av laboratoriepopulationer av Pipiens och Molestus, med hjälp av en Y-rörsolfaktometer, bekräftar tidigare rapporter om en skillnad i värdpreferens mellan biotyperna. För att identifiera kemosensoriska gener kopplade till denna skillnad i värdpreferens användes transkriptomanalys av antenner från beteendefenotypade myggor för att identifiera skillnader i genuttryck, inklusive doftreceptorgener (Artikel I). Skillnader i värdpreferens observerades även i vilda populationer av biotyperna och deras hybrider, där myggornas värdpreferens varierade längs en latitudinell gradient i Europa, tillsammans med skillnader i deras relativa populationsstorlekar (Artikel II). Slutligen karakteriserades de identifierade doftreceptorerna från Artikel I funktionellt genom heterologt uttryck i HEK293-celler i en ligandinducerad fluorescensanalys. Endast en luktreceptor svarade, och fyra föreningar som enbart förekommer i mänskliga dofter framkallade ett dosberoende svar. En jämförande analys av de 3D-prediktiva proteinstrukturerna av Or205 hos båda biotyperna visade skillnader i gensekvens och proteinstruktur, vilket kan förklara de observerade skillnaderna i receptorfunktion (Artikel III). Tillsammans utvidgar studierna i denna avhandling vår förståelse av de genetiska mekanismer som reglerar värdpreferens hos *Cx. pipiens*-biotyperna.

Keywords: *Culex pipiens*, Molestus, värdpreferens, Y-rörsolfaktometer, antenntranskriptom, luktreceptor, fältinsamlingar, funktionell karakterisering, HEK293-celler, prediktiv proteinmodellering

Dedication

To everyone whose help and support has guided me on my academic journey.

"It's a magical world, Hobbs, ol' buddy… Let's go exploring!"
- Bill Watterson, It's a Magical World (Calvin and Hobbs #11)

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. **Rohan Menon**, Rickard Ignell and Sharon Rose Hill (2025). Differential expression of antennal chemosensory genes related to host preference of *Culex pipiens* biotypes. Parasites and Vectors, 18(1), 372. https://doi.org/10.1186/s13071-025-07028-y

II. **Rohan Menon**, Stefanos S. Andreadis, Constantianus J. M. Koenraadt, Niels O. Verhulst, Rickard Ignell and Sharon R. Hill (2026) Relative proportion and contrasting host preference of *Culex pipiens* biotypes across Europe. (submitted)

III. **Rohan Menon**, Jothi Kumar Yuvaraj, Martin N. Andersson, Rickard Ignell and Sharon R. Hill. Functional and predictive structural analysis of odorant receptors correlated with differential host preference of *Culex pipiens* biotypes (manuscript)

The published paper is available open access.

The contribution of Rohan Menon to the papers included in this thesis was as follows:

I. Conceptualized the idea with the co-authors. Designed the methodology along with co-authors. Performed the data collection and data analysis. Drafted the manuscript with input from all co-authors.

II. Conceptualized the idea with the co-authors. Designed the methodology along with co-authors. Performed the data collection and data analysis. Drafted the manuscript with input from all co-authors.

III. Conceptualized the idea with the co-authors. Designed the methodology along with co-authors. Performed the data collection and data analysis. Drafted the manuscript with input from all co-authors.

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Abbreviations

Or	Odorant receptor
Ir	Ionotropic receptor
Gr	Gustatory receptor
WNV	West Nile virus
OSN	Olfactory sensory neuron
VOC	Volatile organic compound
AL	Antennal lobe
LH	Lateral horn
MB	Mushroom body
OBP	Odorant binding protein
CSP	Chemosensory protein
ODE	Odorant degrading enzyme
TRP	Transient receptor potential
SNMP	Sensory neuron membrane protein
ppk	Pickpocket protein
PCA	Principal component analysis
GO	Gene ontology
Orco	Odorant receptor co-receptor
ZT	Zeitgeber time
CRISPR	Clustered regularly interspaced short palindromic repeats
CO_2	Carbon dioxide
dpe	Days post-eclosion
HEK	Human embryonic kidney
SSR	Single sensillum recording

1. Introduction

Despite the overwhelming prevalence of mosquitoes, both in geographic distribution and in species diversity, only a small fraction of mosquito species feed on human blood (Yee *et al*., 2022). Of these anthropophilic (human preferring) mosquitoes, less than 10% are either known or potential vectors of various diseases, including malaria, dengue, yellow fever and West Nile fever (Yee *et al*., 2022). As a whole, mosquito-borne diseases represent a major health risk to humans, affecting millions of people every year (Lim *et al*., 2025; WHO, 2022; 2023). Aside from the impact on human lives, many animals are also susceptible to mosquito-borne diseases, such as West Nile in birds and horses (Farajollahi *et al*., 2011; Ferraguti *et al*., 2024; Castillo-Olivares & Wood, 2004), Rift Valley fever and Japanese encephalitis in cattle (Tchouassi *et al*., 2016; Mansfield *et al*., 2017) and heartworm in dogs (Ledesma & Harrington, 2011). Great strides have been taken to mitigate the effects and spread of these disease vectors, through the use of insecticide-treated bed nets, more accurate surveillance strategies, as well as better medication, vaccines and repellents (Hemingway *et al*., 2016). However, the effects of anthropogenic climate change and human activity have enabled the spread of invasive mosquitoes into new habitats, including regions where they were never detected before, such as the recent discovery of mosquitoes in Iceland (BBC, 2025). The rapid encroachment of mosquitoes into new environments risks the spread of disease to newer regions, causing a greater strain on healthcare and livelihoods of people, and places a greater emphasis on the development of new strategies to control mosquito-borne disease transmission.

The factors affecting mosquito-borne disease transmission are numerous and interconnected, dependent on both internal (mosquito genetics, physiological state, viral or parasitic effects) and external factors (ecological conditions, vector-host interactions, population density and host genetics) (Kramer & Ciota, 2015). In the case of zoonotic disease transmission, *i.e.*, diseases transmitted from animals to humans, further complexity is added as the mosquitoes successfully feeding on both an infected animal and then vector the infection to humans (Kilpatrick *et al*., 2007). Understanding the host-seeking and feeding behaviours of mosquitoes may be useful in determining the transmission of vector-borne diseases (Kilpatrick *et al*., 2007). Prior studies have identified many species-specific variations in host-

seeking behaviours across mosquitoes (Takken & Verhulst, 2013) and have identified a need for an improved understanding of the genetics underlying these behaviours. While certain mosquito vectors have received greater attention due to their role in vectoring more lethal diseases, such as the dengue vector *Aedes aegypti* and the malaria vector *Anopheles gambiae*, little is known about the genetics of the members within the cryptic *Culex pipiens* species complex. Members of this complex are noted vectors for many zoonotic viruses, such as the West Nile virus (WNV), the Usutu virus, and the Sindbis virus. Within the species complex are four recognized species– *Culex quinquefasciatus*, *Culex australicus*, *Culex globocoxitus* and *Culex pipiens*, with *Culex pipiens pallens* being recognized as a subspecies (reviewed in Farajollahi *et al*., 2011; Aaedema *et al*., 2022). Two distinct forms, or biotypes, of *Cx. pipiens* have been identified, named *Culex pipiens* f. *pipiens* (hereafter called Pipiens) and *Culex pipiens* f. *molestus* (hereafter called Molestus), which are morphologically identical but vary genetically and behaviourally. In Europe, *Cx. pipiens* is the primary vector of WNV (Brugman *et al*., 2018), and the rising prevalence of WNV across Europe has been noted over the past few years (eCDC, 2024), placing a greater demand on understanding the mechanisms regulating vector-host interactions. While recent studies have been aimed at characterising the host seeking, and more specifically the host preference, of *Cx. pipiens* (Faraji & Gaugler, 2015; Noreuil & Fritz, 2021; Bell *et al*., 2024), the genetic mechanisms regulating this behaviour remain unclear.

This thesis addresses the gap in the knowledge concerning the genetic mechanisms regulating the host preference of *Cx. pipiens* biotypes. The studies presented within this thesis focus on the phenotypic differences demonstrated by the biotypes and correlate these behavioural differences with differential gene expression and function. By focusing on both laboratory colonies and field collected samples, the results presented in this thesis add to the growing body of knowledge about the various genetic mechanisms that regulate host preference in mosquitoes.

2. Background

2.1 The *Culex pipiens* biotypes

The two biotypes of *Cx. pipiens*, Pipiens and Molestus, though morphologically identical, display behavioural and physiological differences that aid in their identification (Vinogradova, 2000; Fonseca *et al*., 2004; Farajollahi *et al*., 2011; Harbach, 2012; Haba & McBride, 2022). Found throughout Europe and North America, Pipiens typically breed in large, open-air water sources above ground and form mating swarms at dusk (eurygamous) (Harbach, 2012). Similar to most blood-feeding mosquito species, Pipiens requires a blood meal for egg production (anautogenous), and they have been found to enter reproductive diapause, *i.e.*, a state of suspended animation, during winter (Vinogradova, 2000; Harbach, 2012). Pipiens is typically characterised as ornithophilic (bird preferring), though bloodmeal analyses of field-collected mosquitoes have identified a range of hosts, including humans and other mammalian hosts (Haba & McBride, 2022; Wehmeyer *et al*., 2024). The other biotype of *Cx. pipiens*, Molestus, is characterised by their ability to not just survive but thrive in smaller, underground habitats, as demonstrated by their first identification in the London underground tunnels (Vinogradova, 2000; Fonseca *et al*., 2004; Farajollahi *et al*., 2011, Harbach, 2012; Haba & McBride, 2022). Their adaptability to smaller, underground habitats is also seen in their mating habits, with Molestus forgoing the typical swarming behaviours in open areas by having males approaching resting females (stenogamous) (Tate & Vincent, 1936; Kim *et al*., 2018). More interestingly, females do not require a blood meal for the development of their first egg batch (autogenous), however, a bloodmeal is required for the development of further egg batches. Molestus has been characterised as being primarily anthropophilic, though, similar to Pipiens, Molestus have been found to feed on a range of possible hosts including other mammalian hosts and birds (Haba & McBride, 2022; Wehmeyer *et al*., 2024). Molestus has largely been found in the lower latitudes of Europe (Vinogradova, 2000; Farajollahi *et al*., 2011, Harbach, 2012; Haba & McBride, 2022) but, due to their ability to breed in more confined locations, have managed to establish small colonies in urban areas in northern Europe, largely driven by anthropogenic climate change and human migration (Haba & McBride 2022; Haba *et al*., 2025). Similar to

Pipiens, their numbers grow during the warmer months from June to September in Europe, however, likely due to their ability to breed in warmer underground habitats, they do not diapause during the winter. In Sweden, Pipiens is the more prevalent biotype of *Cx. pipiens* (Hesson *et al*., 2014), with Molestus first being identified in 2016 (Hesson *et al*., 2016), and largely restricted to urban areas.

Despite the physiological and behavioural differences between Pipiens and Molestus, hybrids between the two biotypes have been identified in regions where the two are sympatric (Haba & McBride, 2022; Wehmeyer *et al*., 2024; Haba *et al*., 2025). Genomic analyses of field-collected samples of Pipiens and Molestus identified a gradient of genomic similarity between the biotypes across latitudes in Europe (Haba *et al*., 2025). The biotypes show more genomic similarity in the south-east of Europe and become more differentiated as latitudes increased (Haba *et al*., 2025). Hybrids follow a similar pattern, with a higher incidence of hybrids being identified in the lower latitudes of Europe and becoming less prevalent in the north (Haba & McBride, 2022; Haba *et al*., 2025). Differences in the relative proportions of the biotypes have also been observed within countries where the biotypes and their hybrids are sympatric (Gomes *et al*., 2013; Osorio *et al*., 2014; Vogels *et al*., 2016b; Haba & McBride, 2022; Wehmeyer *et al*., 2024). The reasons for this separation of biotypes in sympatric areas remain unclear, but apart from the expected geographic and habitat differences that the biotypes exhibit, certain other reproductive barriers have been postulated, including differences in mating behaviours (Spielman, 1964; Bullini & Coluzzi, 1980; Urbanelli *et al*., 1981; Villani *et al*., 1986; Kim *et al*., 2018), cytoplasmic incompatibility caused by differing bacterial endosymbionts (Laven, 1959; Yen & Barr, 1971; Dumas *et al*., 2016) and even low rates of F1 hybrid egg viability as seen in certain laboratory populations (Kim *et al*., 2018). Despite these barriers, hybrids exist in large numbers in Europe and demonstrate feeding on both avian and human hosts (Haba & McBride, 2022; Wehmeyer *et al*., 2024).

A key characteristic of Pipiens, Molestus and their hybrids is their role as vectors for disease transmission in Europe and North America. In Europe, *Cx. pipiens* has been linked to the spread of viruses, such as WNV and Usutu virus, canine dirofilarial worms and avian malaria parasites (eCDC, 2024). For WNV in particular, *Cx. pipiens* has been shown to vector the virus between infected birds, and act as a bridge vector from birds to mammalian

hosts, such as humans (eCDC, 2024). The biotypes and the hybrids demonstrate differential vectorial capacities depending on temperatures (Vogels *et al*., 2016a), with Pipiens being less effective as a WNV vector as temperatures decrease, which has been postulated as a reason for the lack of WNV cases in northern Europe (Vogels *et al*., 2017). While the factors affecting the vector competence of the biotypes and their hybrids are complex (as discussed in Vogels *et al*., 2016a), understanding the factors affecting the host choice and host preference of the mosquitoes may provide useful information to help reduce viral transmission from birds to humans.

2.2 Host choice versus host preference of *Cx. pipiens*

Host choice refers to the ability of mosquitoes to detect and feed on potential blood-meal hosts present in the environment, while host preference refers to the process by which the mosquito preferentially selects a particular host over another when presented with equal access to both (Lyimo & Fergusson, 2009; Takken & Verhulst, 2013; Wolff & Riffel, 2018). Studies on field-collected Pipiens and Molestus across Europe and North America report significant variation in the host choice of the biotypes, depending on trap site ecology and country of sampling (studies consolidated in Wehmeyer *et al*., 2024; Paper II). For both Pipiens and Molestus, bloodmeal analyses largely identify avian hosts as the most abundant bloodmeal source, with significant feeding on humans and other mammalian hosts (Wehmeyer *et al*., 2024). These studies describe the host choice of the biotypes, with no prior field assays testing the host preferences of the wild populations. The ornithophilic and anthropophilic preferences of laboratory populations of Pipiens and Molestus, respectively, range from weak (Faraji & Gaugler, 2015; Paper I) to strong (Noreuil & Fritz 2021; Bell *et al*., 2024), depending on the geographic origin of the laboratory populations. Only recently has a larger focus been placed on the genes correlated with the differential host preference of the biotypes (Bell *et al*., 2024; Paper I) but many questions remain about the genetic mechanisms regulating this odour-mediated behaviour.

2.3 Odour detection in mosquitoes

2.3.1 Mosquito olfactory system

Mosquitoes possess three olfactory appendages, *i.e.*, the antennae, maxillary palps and the labellum (reviewed in Wheelwright *et al*., 2021). The morphology of these appendages is similar across mosquito species, with sex-specific differences present (Wheelwright *et al*., 2021). Each of the appendages are covered in various classes of small, hair-like sensilla, with each sensillum housing dendrites of the olfactory sensory neurons (OSNs) (McIver, 1987; Hansson, 2002; Suh *et al*., 2014, Wicher & Miazzi, 2021), surrounded by an aqueous sensillum lymph. Three accessory cell types, *i.e.*, the thecogen, trichogen and tormogen cells, surround the somata of the OSNs and play a role in the production of soluble chemosensory proteins, and the maintenance of the ionic concentrations within the sensillum lymph (Keil, 1999, Shanbhag *et al*., 2001, Larter *et al*., 2016; Prelic *et al*., 2022). Odorant detection begins when hydrophobic volatile organic compounds (VOCs) enter the appendages via diffusion though the sensillar pores or spokes (Leal, 2013) and are then transported through the sensillum lymph by the soluble chemosensory proteins to membrane-bound receptors, which selectively bind to certain VOCs (Wheelwright *et al*., 2021).

The response profile of the OSN to a particular VOC is defined by the sensitivity and/or selectivity of the chemosensory receptors expressed on the dendritic membrane. The binding of a VOC to a receptor triggers the generation of an action potential within the OSN, with the resulting signal being carried to the mosquito brain for initial processing (Sato & Touhara, 2008). If the signal originated in the antennae or maxillary palps, the signal is carried to the primary olfactory centre in the mosquito brain, the antennal lobe (AL), while the signals from the labellum are carried to the suboesophageal zone (Ignell *et al*., 2005; Ghaninia *et al*., 2007; Riabinina *et al*., 2016; Shankar & McMeniman, 2020). The AL is composed of glomeruli, formed by the synaptic contacts between OSNs, local interneurons (that relay signals between glomeruli) and projection neurons (that relay signals further to the higher brain centres) (Ignell *et al*., 2005; Vosshall & Stocker, 2007; Singh *et al*., 2023). At the AL level, the olfactory information conveyed by the peripheral OSNs are filtered based on the quality, quantity and temporal characteristics prior to relaying them to the higher brain centres (Ignell *et al*., 2010). It was previously assumed that OSNs expressing the same tuning

receptors converged onto the same glomerulus in the AL, as seen in *Drosophila melanogaster* (Vosshall *et al*., 2000; Couto *et al*., 2005), however recent studies challenged this one-receptor-one-neuron perception by identifying co-expression of multiple receptor genes from different receptor families in OSNs of *Ae. aegypti* and *Anopheles coluzzii* (Herre *et al*., 2022; Ye *et al*., 2022), and in *D. melanogaster* (reviewed in Rauscher & Wolff, 2023). Signals from the AL are carried by the projection neurons to either the lateral horn (LH) or the mushroom bodies (MB) of the protocerebrum for further processing. Little is known about the function of the LH and MBs in mosquitoes (Wolff & Riffel, 2018; Wolf *et al*., 2023), however in *Drosophila*, the LH has been shown to mediate innate responses to VOCs (Schultzhaus *et al*., 2017, Das Chakraborty & Sachse, 2021) whereas the MBs are involved in memory and learning (Heisenberg, 2003; Strutz *et al*., 2014). The processing and modulation of the olfactory and other sensory cues within the higher brain centres likely allow for the more complex responses displayed by the mosquito, as postulated in McMeniman *et al*. (2014), though how it occurs is currently unclear.

2.3.2 Molecular basis of olfaction – receptors and proteins

Odorant detection in insects is reliant on the function and expression of the various soluble chemosensory binding proteins and membrane-bound receptors found in the sensillum lymph and on the dendritic membrane of the OSNs, respectively. Two classes of soluble binding proteins, *i.e.*, the odorant binding proteins (OBPs) and the chemosensory proteins (CSPs), have been identified in insects (Vogt & Riddiford, 1981; Steinbrecht, 1996; Leal, 2013). Aside from chaperoning VOCs within the sensillum lymph, OBPs also regulate OSN sensitivity via gain control (Biessmann *et al*., 2010; Pelletier *et al*., 2010; Larter *et al*., 2016; Pelosi *et al*., 2018). Both OBPs and CSPs are expressed in high concentrations in the sensillum lymph of olfactory tissues, as well as in other tissues including the proboscis and legs (Pelletier & Leal, 2009; reviewed in Rihani *et al*., 2021), and display numerous functions within insects (Pelosi *et al*., 2018; Rihani *et al*., 2021). The structure of OBPs and CSPs are composed primarily of six α-helices, with a hydrophobic inner cavity where the VOCs typically bind (Sandler *et al*., 2000; Campanacci *et al*., 2003; Tegoni, *et al*., 2004). The mechanism regulating the binding and transport of the VOCs by these proteins is unclear, however pH-dependent conformational changes of the three-dimensional

protein structure has been postulated, which cause an increased binding of the VOCs at the sensillar pores and expulsion of the bound VOCs near the tuning receptors at the dendritic membrane (Leal *et al*., 2005; Zubkov *et al*., 2005; Manoharan *et al*., 2013, Pelosi *et al*., 2018). After receptor activation and signal transduction has occurred, signal termination occurs via the degradation of the VOCs by odorant degrading enzymes (ODEs). The rapid degradation of the ligands helps maintain OSN sensitivity and reliable coding of the temporal dynamics of the signal (reviewed in Chertemps & Maibeche, 2021). Though many questions remain regarding the regulation of ODEs in the context of odour detection, the overexpression of certain classes of ODEs such as cytochrome P450 have been linked to insecticide resistance in certain mosquito species (reviewed in David *et al*., 2013).

Once the VOCs have been transported across the sensillum lymph and are released by the soluble binding proteins, they bind to receptors expressed on the dendritic membrane. Three main receptor families have been identified, *i.e.*, odorant receptors (Ors), ionotropic receptors (Irs) and gustatory receptors (Grs) (reviewed in Guidobaldi *et al*., 2014; Wheelwright *et al*., 2021). Other transmembrane receptors and ion channels, namely the transient receptor potential channels (TRPs), sensory neuron membrane proteins (SNMPs) and pickpocket ion channels (ppk), have also been identified, though little is known about their role in mosquitoes. SNMPs play a role in pheromone detection in certain Diptera and Lepidoptera (Benton *et al*., 2007; Zhang *et al*., 2020; Cassau & Krieger, 2021). TRPs are involved in the mediation of multiple sensory modalities including chemo- and thermosensation in mosquitoes (Wang *et al*., 2009; Corfas & Vosshall, 2015; Li *et al*., 2019; Melo *et al*., 2021) and in other insects (Liu *et al*., 2007; Kang *et al*., 2010; Kwon *et al*., 2010; Fowler & Montell, 2013). The role of ppks in modulating mosquito oviposition behaviour have been described (Matthews *et al*., 2019), and ppks are also associated with modulating mating behaviours in *Ae. aegypti* (Wyer *et al*., 2023; Wyer *et al*., 2025).

2.3.3 Molecular basis of olfaction – odorant receptors

Insect Ors are comprised of seven transmembrane domains, composed of roughly 400 base pairs (Clyne *et al*., 1999; Vosshall *et al*., 1999, reviewed in Li *et al*., 2025). However, unlike mammalian Ors, the orientation of the domains is inverted, with an intracellular N-terminus and an extracellular C-terminus (reviewed in Li *et al*., 2025). Insect Ors are co-expressed with a co-

receptor named Orco (Vosshall & Hansson, 2011), forming ligand-gated non-selective cation channels that are activated by the binding of specific ligands to the tuning Or (Wang *et al*., 2024; Zhao *et al*., 2024). Expression of Orco is vital for dendritic localization of the Or/Orco complex (Larsson *et al*., 2004), with CRISPR-induced knockouts of Orco disrupting odour-mediated behaviours in insects (DeGennaro *et al*., 2013; Koutroumpa *et al*., 2016; Fandino *et al*., 2019; Sun *et al*., 2020; Zhang *et al*., 2024). Phylogenetic analyses of Ors and Orco across multiple insect species describe the conserved nature of the Orco sequence and protein structure (Butterwick *et al*., 2018; Li *et al*., 2025), with tuning Ors, on the other hand, evolving rapidly across insect species, likely via the birth-and-death model of gene evolution (Nei & Rooney, 2005). This model suggests that the rapid evolution and creation of Ors, which were initially derived from gustatory receptors, occurred through high rates of gene duplication, followed by purifying selection of the novel genes, either by deletion or pseudogene events (Sánchez-Garcia *et al*., 2009). The number of Ors vary across mosquito species, with around 80 Ors identified in *An. gambiae* (Neafsey *et al*., 2015), around 120 in *Ae. aegypti* (Matthews *et al*., 2018) and around 180 in *Cx. quinquefasciatus* (Arensburger *et al*., 2010).

Despite the variation in numbers of Ors across mosquito species, the tertiary structure of the tuning receptors and the Or/Orco complex is conserved, with recent *in vitro* structural analyses of Ors from *Ae. aegypti* and *An. gambiae* detailing a 1:3 Or:Orco stoichiometry of the complexes in both their ligand-bound and unbound states (Zhao *et al*., 2024). This study by Zhao *et al*. (2024), along with other structural studies on other insect Ors and Orco (Butterwick *et al*., 2018; Wang *et al*., 2024), describe a consistent division of the complex into two key regions – an intracellular "anchor" domain responsible for mediating interactions between the tuning Or and the nearby Orco subunits, and a transmembrane region, with a few extracellular loops linking the various helices of each subunit, where the majority of the protein complex is housed. Within the transmembrane region of the tuning Or, a hydrophobic pocket forms by the coalescence of the S2, S3, S4 and S6 helices where the ligand binds with the hydrophobic and aromatic residues, such as Tyr^{183} and Leu^{67} in the *Ae. aegypti* Or10 (Zhao *et al*., 2024). Mutations of these residues in the binding pocket to alanine causes a lower binding affinity of the tuning Or to the ligand (Zhao *et al*., 2024). Strong binding between the tuning Or and the ligand causes a shift in the

conformation of the Or/Orco complex, mediated by the interaction of the S7b helix of the tuning Or and the surrounding Orco subunits, which opens up the main ion channel and allows cation flow into the cell (Zhao *et al*., 2024). A similar mechanism of the Or/Orco complex is observed in another recent structural assay that focused on aphid Ors, in which the movement of the S5 and S6 helices in the anchor domain of the complex facilitated the conformational change caused by the S7b helices (Wang *et al*., 2024). Ion flow through the central ion channel then funnels into four lateral conduits, formed near the anchor domain (Zhao *et al*., 2024). Future *in vitro* studies on the structure of the Or/Orco complex aim to address how the complexes form and gate their responses within the OSNs, with *in vivo* expression of the Or/Orco complex aiming to better characterise the ligand-binding affinity of the tuning Or.

Tuning Ors respond to ligands of multiple chemical classes, including aldehydes, alcohols, ketones, aromatics, esters and terpenes (Carey *et al*., 2010; Wang *et al*., 2010; Omondi *et al*., 2019; Pullmann-Lindsley *et al*., 2024), and are classified as either broadly or narrowly tuned based on the number of ligands they respond to (Andersson *et al*., 2015). Certain Ors demonstrate enantio-selectivity, with differing responses based on the chiral forms of certain odorants. For example, differences in responses to (*R*)-(-)- and (*S*)-(+)-linalool were observed in cells expressing AgamOr29, with AgamOr29 being more sensitive to (*S*)-(+)-linalool than (*R*)-(-)-linalool (Huff & Pitts, 2019). The Or pathway has been shown to mediate host seeking and discrimination in mosquitoes (DeGennaro *et al*., 2013, Omondi *et al*., 2019; Zhao *et al*., 2022), with differences in the expression and function of certain receptors postulated to affect host preference (McBride *et al*., 2014).

2.3.4 Molecular basis of olfaction – ionotropic and gustatory receptors

Besides Ors, ionotropic and gustatory receptors play a key role in chemosensory detection. First identified in other insects (Benton *et al*., 2009), Irs are derived from ionotropic glutamate receptors and are involved in multimodal detection of stimuli, including odorants, tastants, humidity and temperature (Wicher & Miazzi, 2021; Laursen *et al*., 2023; Morita *et al*., 2025). Similar to Ors, Irs are highly expressed in the mosquito antenna and maxillary palps, and the tuning Irs are co-expressed alongside one of three Ir co-receptors, *i.e.*, Ir8a, Ir25a or Ir76b (Benton *et al*., 2009; Abuin *et al*., 2011;

2019). The predicted Ir/Irco structure is similar to Ors, with the four subunits Ir/Irco complex forming ligand-gated ion channels (Abuin *et al*., 2011; 2019). However, the stoichiometry of the Ir/Irco complex varies depending on the co-receptor being expressed (Abuin *et al*., 2011; 2019). In mosquitoes, the number of Irs vary with species, with 46, 95 and 69 Irs being identified in *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus*, respectively (reviewed in Raji & Potter, 2022). Irs bind to carboxylic acids and amines (Pitts *et al*., 2017; Raji *et al*., 2019), and in the context of odour-mediated behaviours, the Ir-pathway mediates host discrimination in mosquitoes (De Obaldia *et al*., 2022).

Phylogenetic analyses of Grs have identified a close link to Ors, with both receptor families comprising seven transmembrane helices and acting as ligand-bound receptors (Frank *et al*., 2024; Gomes *et al*., 2024). Unlike Ors, the role of Grs includes not just olfaction but also gustation (Gomes *et al*., 2025). Recently, the structure of Grs of other insects has been solved (Frank *et al*., 2024; Gomes *et al*., 2024), with a tetramer being formed from four identical sub-units (protomers) being arranged around a central pore (Frank *et al*., 2024; Gomes *et al*., 2024). Unlike the Or/Orco complex, the protomers comprise of the tuning Gr, with no co-receptor being identified. The ligand-binding and conformational changes associated with the activation and function of Grs is similar to that of Ors, except for the presence of more binding pockets (one pocket in each protomer of the Gr complex). In mosquitoes, 76, 81 and 60 Grs have been identified in *An. gambiae*, *Ae. aegypti*, and *Cx. quinquefasciatus*, respectively (Hill *et al*., 2002; Kent *et al*., 2008; Arensburger *et al*., 2010; Matthews *et al*., 2018). In the context of their role in olfaction, Grs play a key role in CO_2 detection in mosquitoes. In culicines and anophelines, Gr 1 – 3 and Gr 22 – 24, respectively, are expressed in the maxillary palps and play a key role in detecting CO_2 (Lu *et al*., 2007; McMeniman *et al*., 2014; Xu *et al*., 2020; Liu *et al*., 2020). This Gr pathway also plays a role in detecting acetone, a VOC present in exhaled breath of mammals (Ghaninia *et al*., 2019; Herre *et al*., 2022).

2.4 Odour-mediated host discrimination

2.4.1 Host odorants

Odours emitted from all sources, be it floral, blood-meal host or oviposition sources, are comprised of a multitude of different odorants (Bernier *et al*., 2008; Knudsen *et al*., 2006; Wondwosen *et al*., 2017; Nyasembe *et al*., 2018; Omondi *et al*., 2019; Spanoudis *et al*., 2022; Zhao *et al*., 2022). As with other insects, mosquitoes respond to only a fraction of the emitted odorants, restricted by the function of the various membrane-bound receptors (as detailed in Section 2.2). Despite the large number of potential VOCs emitted by various sources, certain VOCs are shared between the different sources, defined as chemical parsimony. The presence of parsimonious compounds, along with other salient compounds, create unique host odours that are detected by the mosquito and used for host discrimination (Syed, 2015; Ignell and Hill, 2020).

Perhaps the most important, and most ubiquitous, host odorant detected by host-seeking female mosquitoes is CO_2. Female mosquitoes are highly sensitive to changes in the relative concentration of CO_2, with species-specific differences in both the sensory and behavioural responses to CO_2 observed (Grant *et al*., 1995; Majeed *et al*., 2017). Levels of exhaled CO_2 from vertebrate hosts contain around 40,000 parts per million (Gillies, 1980; Clements, 1999), and for many mosquito species CO_2 acts as an activator, synergist and modulator of host-seeking behaviour by gating their responses to olfactory, thermal and visual stimuli (Dekker *et al*., 2005; McMeniman *et al*., 2014; van Breugel *et al*., 2015; Webster *et al*., 2015; Vinauger *et al*., 2019). For some species, such as *Ae. aegypti*, CO_2 alone can activate mosquito flight (Eiras & Jepson, 1991), and aids in orienting flight to the source, *i.e.*, acts as an attractant (Dekker *et al*., 2005; Dekker & Cardé, 2011). However, for *An. gambiae*, CO_2 alone does not act as an attractant and must be paired with other host odours to elicit attraction (Takken *et al*., 1997; Spitzen *et al*., 2008; Hinze *et al*., 2021). For Molestus and *Cx. quinquefasciatus*, CO_2 decreased or inhibited the behavioural responses, respectively, when paired with attractive host odours in laboratory settings (Spanoudis *et al*., 2022). The high sensitivity of *Cx. quinquefasciatus* to CO_2 was previously characterized (Syed & Leal, 2007; Majeed *et al*., 2017) and is postulated to have evolved as a result of the more opportunistic host

selection of *Cx. quinquefasciatus* compared to that of the anthropophilic *Ae. aegypti* and *An. coluzzii* (Majeed *et al*., 2017).

Host odours are comprised of blends of VOCs from various chemical families, including aldehydes, ketones, carboxylic acids and even non-organic compounds, such as ammonia (Omondi *et al*., 2019; Spanoudis *et al*., 2022; Zhao *et al*., 2022, De Obaldia *et al*., 2022; reviewed in Dormont *et al*., 2021; Hinze *et al*., 2022). Of many hundreds of emitted VOCs present in host odours (reviewed in Dormont *et al*., 2021), only a small subset of the VOCs is detected by the mosquito, dependent on the tuning repertoire of the expressed chemosensory receptors (reviewed in Hinze *et al*., 2022). Zhao *et al*. (2022) highlight that the odour profiles of multiple host species include several overlapping VOCs in common and those that our distinct, with the mosquito responding to the qualitative and quantitative differences in the host odours profiles. For example, sulcatone, geranylacetone, and long-chained aldehydes, such as nonanal and decanal, are more abundant in human odours compared to other non-human hosts (McBride *et al*., 2014; Zhao *et al*., 2022; reviewed in Dormont *et al*., 2013), while shorter chained aldehydes, such as heptanal and octanal, are more prevalent in chicken odours (Bernier *et al*., 2008; Spanoudis *et al*., 2022). These VOCs are formed as byproducts from the breakdown of other biomolecules, with some differences between species – the degradation of sebum in humans produces sulcatone and the longer-chained aldehydes (reviewed in Zung & McBride, 2025) while degradation of uropygial gland secretions (which functions similarly to sebum, in feather maintenance) in birds produces the short-chained aldehydes (Bernier *et al*., 2008). Differences in the microbiota on host species also affects VOC emissions (Verhulst *et al*., 2011; 2018). Intra-specific variation in the host odour profiles has also been noted, with mosquitoes demonstrating differential attraction to hosts based on the abundance of certain compounds (Zhao *et al*., 2022; De Obaldia *et al*., 2022).

2.4.2 Odour-mediated host discrimination

Odour-mediated host discrimination in mosquitoes is based on qualitative and quantitative differences in host odour profiles (Zhao *et al*., 2019; De Obaldia *et al*., 2022; Paper I; reviewed in Hinze *et al*., 2022). The presence of certain taxa-specific VOCs in host odours has been shown to play a role in mediating host discrimination in mosquitoes, such as 3-octanone in chicken odour (Spanoudis *et al*., 2022; Paper I) and (*R*)-1-octen-3-ol in

mammalian odours (Majeed *et al*., 2016; Paper I). The relative ratios of VOCs that overlap in the odour profile of hosts also affects host discrimination (reviewed in Hinze *et al*., 2022). For example, levels of lactic acid in human odours are around 10-100 times higher when compared to other mammalian odours and has been a key odorant mediating host discrimination in anthropophilic mosquitoes (Dekker *et al*. 2002). Addition of human levels of lactic acid to cattle odours causes an increase in attractiveness for *Ae. aegypti* to similar levels as human odours (Dekker *et al*., 2002). A similar increase in attraction to previously unattractive host odours was observed when human levels of (*R*)-1-octen-3-ol were added to chicken odours, causing an increase in attraction to levels comparable to human odours for *Ae. aegypti* and *An. coluzzii* (Majeed *et al*., 2016). Meanwhile, the addition of human odour-levels of (*R*)-1-octen-3-ol to attractive chicken odours led to decreased attraction for *Cx. quinquefasciatus* (Majeed *et al*., 2016). The abundance of carboxylic acids in human odours has also been shown to affect intra-specific host discrimination in anthropophilic mosquitoes (De Obaldia *et al*., 2022). This context-dependent detection of host odorants highlights the importance of olfaction in regulating host preference, and places greater emphasis on understanding the molecular mechanisms involved in the regulation of this behaviour.

2.4.3 Molecular pathways involved in host discrimination

In mosquitoes, the Or and Ir pathways play a role in mediating host seeking (Or pathways) and host discrimination (Or and Ir pathways) (DeGennaro *et al*., 2016; Sun *et al*., 2020; McBride *et al*., 2014; Omondi *et al*., 2019; De Obaldia *et al*., 2022, Paper II). The function of the co-receptors in mediating host discrimination is vital, as mutant *Ae. aegypti* lacking Orco or Irco receptors are unable to discriminate between human and non-human hosts, and affects intra-specific host discrimination, respectively (DeGennaro *et al*., 2013; De Obaldia *et al*., 2022). Moreover, mutation of Orco in *An. coluzzii* Orco reduces host seeking (Sun *et al*., 2020). Differences in the expression and/or function of the tuning Ors are also postulated to affect host discrimination (McBride *et al*., 2014; Rinker *et al*., 2013). Differences in the expression and sensitivity of alleles of *Or4*, which binds to sulcatone have been observed in anthropophilic and zoophilic sub-populations of *Ae. aegypti* (McBride *et al*., 2014). Other studies focusing on the genomics and transcriptomics of closely related mosquito species demonstrate that

differential host preferences correlate with differences in gene sequence and/or expression of tuning *Ors* (Rinker *et al*., 2013; Neafsey *et al*., 2015; Main *et al*., 2016; Athrey *et al*., 2017). For example, Rinker *et al*. (2013) identified several *Ors* that were differentially expressed between the anthropophilic *An. gambiae* and the zoophilic *An*. *quadriannulatus* and proposed that the divergent host preferences relate to differences in the tuning profiles of a subset of the identified Ors. While the genetic mechanisms guiding this example of differential host discrimination are not fully characterised, it strengthens the idea that differences in the expression levels and gene structure of tuning Ors play a key role in regulating host discrimination.

3. Aims and objectives

The overall aim of this thesis was to understand the mechanisms driving host preference differences between Pipiens, Molestus and their hybrids by integrating behavioural assays, population-level ecological analyses across Europe, and functional characterisation of odorant receptors.

The first objective was to characterise the differential host preference demonstrated by laboratory populations of Pipiens and Molestus, and to correlate the differences in antennal gene expression with the observed behaviour (Paper I).

The second objective was to study the different host preference of wild populations of Pipiens, Molestus and their hybrids across a latitudinal gradient in Europe, and to determine if differences in the relative populations of the biotypes and their hybrids were correlated with a difference in host preference (Paper II).

The final objective was to functionally characterise the odorant receptors identified in Paper I using the HEK293 cell system, and to correlate differences in Or structure and function with the differential host preference of the biotypes (Paper III).

4. Methodology

4.1 Mosquito rearing (Paper I)

At SLU, laboratory colonies of Pipiens and Molestus were established in October 2021 from eggs and larvae provided by Prof. Sander Koenraadt (Wageningen University). These eggs originated from colonies established at Wageningen University in 2016, obtained from field collected mosquitoes (Vogels *et al*., 2016b). Mosquitoes were reared at 27 ± 2 °C, 65 ± 2% relative humidity with a 12 h light: 12 h dark photoperiod, with the light: dark cycle chosen to mimic the natural light conditions in Europe at times of the year with the highest incidence of West Nile virus transmission (August–September) (eCDC, 2024). Eggs and larvae of each biotype were placed in clear, food-grade plastic trays (23.5 cm ×18 cm × 7.5 cm) filled with 1 L of tap water, with approximately 150 – 200 larvae per tray. The quality of the plastic used in the trays was selected to ensure optimal rearing conditions of the larvae. During larval growth, L1 larvae were fed ½ tablet of fish food (Tetramin®, Melle, Germany) daily. After the larvae reached L2, the number of larvae per tray was reduced to < 150 (to minimize competitive stress and to achieve healthy growth), and each new tray of larvae was provided with 1 tablet of fish food daily until pupation. Pupae were collected in water-filled 30 mL plastic cups and placed in Bugdorm 4E1515 cages (17.5 cm × 17.5 cm × 17.5 cm, Megaview Science Co., Taichung, Taiwan) with *ad libitum* access to 10% sucrose in glass vials with filter paper wicks for the newly emerging adults.

For colony maintenance of adult Molestus, eggs produced from the anautogenous egg batch, *i.e.*, eggs laid without a bloodmeal, by 3-4 days post-eclosion (dpe) females were collected in a 30 mL clear plastic cup filled with tap water, and the eggs were placed in a fresh rearing tray. For the colony maintenance of Pipiens, which requires a bloodmeal to induce a gonotrophic cycle, 4 dpe females were bloodfed overnight with defibrinated cow blood (Håtunalab, Bro, Sweden) via a Hemotek membrane feeding system (Hemotek Ltd, Blackburn, UK). Around 2-3 days post blood feeding, the freshly deposited eggs were collected in a 30 mL clear plastic cup filled with tap water and placed in a fresh rearing tray.

For the behavioural experiments (detailed in section 4.2), female mosquitoes at peak host-seeking conditions were tested. Pilot experiments conducted to determine the peak host-seeking age of the mosquitoes found that 8 dpe Molestus and 4 dpe Pipiens exhibited peak host-seeking. Mosquitoes used in the behavioural experiments were not blood fed and were provided with *ad libitum* access to sucrose only, and 24 h prior to testing the adults were starved with access to water alone. All behavioural experiments were conducted during peak host-seeking time for *Cx. pipiens*, which is around the beginning of scotophase (Zeitgeber time 15 ± 2 h) (Yee & Foster, 1992).

4.2 Behavioural analysis (Paper I)

A Y-tube olfactometer was used to assess the differential host preference of Pipiens and Molestus (Box 1). The Y-olfactometer (120 cm × 10 cm), illuminated from above with red light at 40 lx, was placed in a room mimicking rearing conditions (27 ± 2 °C, 70 ± 5% relative humidity). Air was passed through a charcoal filter and humidified before entering the olfactometer at 0.3 m s^{-1}. Synthetic host odour blends, based on the VOCs that are associated with chickens and humans, and that are detected by the mosquitoes, were made as previously described (Omondi *et al*., 2019; Spanoudis *et al*., 2022) (Table 1), with the stock concentrations of the blends diluted in pentane (≥ 95%, Carlo Erba Reagents, Emmendingen, Germany). The blends and solvent control were released via diffusion using wick dispensers (Karlsson *et al*., 2013) to control for a consistent release of all components of the odour blends throughout the behavioural assay. The wick dispensers were placed in glass wash bottles (250 mL; Lenz Laborglas, Wertheim, Germany), and the blends or control were delivered into the upwind end of either arm of the olfactometer via Teflon™ tubing during the behavioural assays.

Groups of five female mosquitoes of either biotype were placed in cylindrical release cages (10 cm length × 10 cm i.d.) for 2 h to acclimatize to the room conditions prior to testing. The release cages were then attached to the downwind end of the olfactometer, and the mosquitoes were allowed 5 min to acclimatize before the behavioural assay was conducted. Upon opening the door of the release cages, the mosquitoes were given 5 min to make a choice between either of the two arms of the olfactometer, with

mosquitoes that remained in the cages or the downwind arm of the olfactometer being considered non-responding (and therefore not demonstrating a clear behavioural phenotype) and thereby excluded from future analysis. A preference index, calculated by (T – C)/ (T + C) where T is the number of mosquitoes responding to the odour of interest and C is the number responding to the solvent control or the other odour tested, was used to determine the host preference of the biotypes.

Three behavioural assays were conducted in Paper I to determine the host preference of Pipiens and Molestus. Pilot experiments were conducted to identify the dose-dependent responses of the biotypes to (a) either odour blend versus a pentane control, and (b) between the odour blends, *i.e.*, chicken odour versus human odour, with the goal of identifying a dose of the odour blends that displayed a clear differential host preference between the two biotypes. The final assay (c), which was conducted using the dose identified in (b), was performed to determine whether the mosquitoes demonstrate a consistency in host preference over time. Pipiens and Molestus that demonstrated a clear preference to chicken odour and human odours, respectively, were collected into Bugdorm cages with *ad libitum* access to water, and the assay was repeated after 24 h. Mosquitoes that made a consistent choice, *i.e.*, Pipiens that chose the chicken odour and Molestus that chose the human odour in both days of testing, were collected and used for subsequent transcriptomic analyses. Three replicates of each biotype that made a consistent choice, including mosquitoes of different cohorts, were conducted to obtain 50 individuals for each replicate.

Box 1 – Methods to assess host preference in laboratory assays

Assessing host preference in laboratory assays requires the assessment of the mosquito behaviour when presented with (at least) two odour sources. The purpose of this box is to briefly introduce the various assays described in prior research and highlight the advantages of each experimental set-up.

1. *Y-tube* olfactometers allow for testing two odour sources simultaneously and have been used to study the host preference of *An. gambiae* (Lefèvre *et al*., 2009) and *Cx. pipiens* (Paper I). Multiple types of odour sources (live hosts, host odour samples collected on nylon sleeves, or synthetic host odour blends) can be used in the assays. Single or groups of mosquitoes can be tested simultaneously, allowing for rapid testing. Most Y-tubes are connected to purified air filters and CO_2 cylinders, allowing for greater control of the experimental conditions during testing.

2. *Dual port olfactometers* are similar to Y-tubes, and can also be test two odours simultaneously, as demonstrated by DeGennaro *et al* (2013) when assessing host preferences in *Ae. aegypti* Orco mutants. The olfactometers can be either still air or connected to purified air flow with odours added to either port, with single or groups of mosquitoes being released into a large cage and then allowed to make a choice

3. *Landing assays* are used to test the close-range attractiveness of host odours and are composed of a test arena with single or multiple host odour sources. Single or groups of mosquitoes can be tested simultaneously. A significant advantage of this system is that they are inexpensive, allowing for landing assays to be used both in laboratory (Faraji & Gaugler, 2015; Noreuil & Fritz, 2021; Bell *et al*., 2024) as well as in semi-field conditions (Dekker & Takken, 1998), with the larger testing assay allowing for a greater analysis of the mosquito behaviour.

4.3 Tissue dissection and transcriptomic analysis (Paper I)

The antennae of cold-anesthetized adult females were collected immediately (< 2 h) after the behavioural assays were completed and, to prevent RNA degradation, were stored in RNAlater® solution (Thermo Fisher Scientific, Stockholm, Sweden) at -20 °C until RNA extraction. Three biological replicates of 50 pairs of antennae per biotype were generated. During RNA extraction, RNAlater® was removed, and the antennae were disrupted and homogenized using a power pestle with a disposable RNA-se free plastic pestle. Total RNA extraction and DNase digestion was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and the extracted RNA was stored at -80 °C. RNA quality and quantity was analysed via fluorescence using the TapeStation system 1200

(Agilent Technologies, Stockholm, Sweden), and total RNA samples were shipped on dry ice to Eurofins Genomics (Ebersberg, Germany) for sequencing, with sample libraries constructed using the INVIEW Transcriptome Ultra Low workflow. This generated paired-end reads of 2 × 150 bp coverage with a depth of 20 Mb. Sequences were trimmed and cleaned using CLC Genomics Workbench (http:// www.clcbio.com, version 23.0.5; Qiagen, Vedbæk, DK). and mapped to the *Cx. quinquefasciatus* reference genome from VectorBase (*Culex quinquefasciatus* JHB2020, VectorBase rel. 66, 28-NOV-2023).

To compare differential antennal transcript abundance between the two biotypes, the trimmed mean of the M value (TMM) adjusted counts per million (TPM) for each replicate was calculated. Transcripts with a TPM > 0.6 were considered significantly expressed ($P < 0.05$). A gene ontology analysis was performed on the significantly expressed transcripts, as well as the genes that were differentially expressed between the two biotypes (FDR $P \leq 0.05$, fold change ≥ 1.5) to identify differences in gene expression between the two biotypes. Heat maps, comparing the Log_{10} average TPM for the library of each biotype alongside the fold change, were generated to analyse differences in gene expression between the two biotypes.

4.4 Field collections of *Cx. pipiens* (Paper II)

To test whether the host preference of Pipiens, Molestus and their hybrids changes as latitudes increase across Europe, host-seeking *Cx. pipiens* were collected in Wageningen, the Netherlands (51.98° N, 5.66° E), in Zürich, Switzerland (47.38° N, 8.54° E) and in Thessaloniki, Greece (40.67° N, 22.79° E). Six trapping sites, located in peri-urban areas of each city were selected, with each site located at least 100 m apart. Sites were selected, with traps placed under standing foliage (> 1 m). At each site, two BioGents Sentinel type 2 traps (Biogents AG, Regensburg, Germany) were set 1 – 2 m apart at ground level. Both traps were connected via Teflon® tubing to a single 10 L CO_2 cylinder (99% purity), set to a flow rate of 200 mL min^{-1}. The CO_2 was used as an attractant at an intermediary level between humans and chickens (Mboera & Takken, 1997). In each trap pair, one trap was baited with the synthetic chicken odour blends, while the other trap was baited with the synthetic human odour blend (from Paper I), released by diffusion from wick dispensers. The stock synthetic host odour blends were

prepared as previously described and diluted in heptane (99 % purity, Merck, Steinheim, Germany) to obtain a release rate of 4 μL min^{-1} of the diluted blend. Control traps were set up in a similar manner, with heptane alone being used as lures in both traps.

Trapping was conducted over 12 days per country between the months of July to September 2023, following a Latin-square design, with treatment sites (*i.e.*, a site containing two traps, one baited with the synthetic chicken odours and the second baited with synthetic human odour), and a control site (baited with CO_2 alone), randomly assigned on the first day and then rotated between sites to minimise location bias over the following days. Traps were connected to a 12 V battery and were set to operate from 2 h before sunset to 2 h after sunrise, after which the traps were shut down, and the captured mosquitoes were collected. Collected mosquitoes were stored at -20 °C immediately after collection and were morphologically identified under a microscope using available identification keys (Becker *et al*., 2020). Only female *Cx. pipiens* mosquitoes were counted and stored at -20 °C in 1.5 mL tubes for further molecular identification of biotypes and hybrids.

4.5 Biotype identification of field samples (Paper II)

DNA of the collected *Cx. pipiens* mosquitoes was extracted using the ThermoFisher Phire Tissue Direct PCR kit, as per the manufacturer's instructions, and stored at -20 °C for further use. Samples were identified as either Pipiens, Molestus or hybrids using a modified version of the real-time PCR assay described previously in Vogels *et al*. (2016b), using a BioRad CFX96 real-time thermocycler (BioRad, Hercules, USA), with the following thermocycler conditions: 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 62 °C for 1 min. The data was analysed using the BioRad CFX manager software (BioRad). Samples with C_q values below 45, which showed exponential amplification, were treated as positives and used for further analysis. Samples that displayed responses to both Pipiens and Molestus probes were classified as hybrids. Negative controls and positive biotypes controls, from laboratory colonies of Pipiens and Molestus, were included in each PCR run.

4.6 Molecular cloning and generation of HEK293 cells (Paper III)

The differentially expressed antennal Or transcripts that correlated with the differential host preference of Pipiens and Molestus in Paper I were subjected to functional characterisation (Box 2). The amino acid sequences of these 14 Or sequences were codon optimized for better expression in the HEK293 cells (Roberts *et al*., 2021; 2022), cloned into the pcDNA5/TO expression vector (Thermo Fisher Scientific, Carlsbad, CA, USA), and then transformed into HB101 competent cells (Promega, Madison, WI, USA) as previously described (Corcoran *et al*., 2014; Andersson *et al*., 2016; Yuvaraj *et al*., 2021).

Colonies of transformed HB101 cells were screened via colony PCR using vector specific primers, with positive colonies being subcultured overnight to extract the plasmid DNA. Plasmid DNA was sequenced using Sanger Sequencing (Eurofins Genomics, Copenhagen, Denmark) to confirm successful Or insertion. Plasmids with successful Or insertion were then purified, linearized and transfected into the previously established HEK293 cell line that expresses the *Ae. aegypti* odorant co-receptor (AaegOrco), (whose protein sequence is > 90% similar to the *Cx. pipiens* Orco sequence and likely maintains functionality despite minor differences in sequence and structure) and an exogenous tetracycline-inducible repressor (Trex) (Corcoran *et al*., 2014). Successfully transfected cells were selected for using hygromycin, and cell lines were passaged three times and then frozen at -80 °C prior to testing.

Box 2 – Functional characterisation of odorant receptors

Functional characterisation of insect chemosensory receptors is essential for assessing the tuning repertoire of the insect receptor or neuronal response either *in vitro* or *in vivo*. The purpose of this box is to briefly introduce the various assays described in prior research and highlight the advantages of each experimental set-up.

1. *Human embryonic kidney* (*HEK293*) *cells* are an immortalized mammalian cell line (Graham *et al*., 1977) used to characterise insect Ors in calcium-imaging assays (Corcoran *et al*., 2014; Yuvaraj *et al*., 2021; Roberts *et al*., 2021; Biswas *et al*., 2024; Paper III). HEK293 cells can reliably express inducible recombinant proteins (*i.e.*, the tuning Or and Orco), with fluorescence assays characterising receptor functionality on stimulation with a ligand. The cells can be stored in deep freeze as needed without much inter- or intra-assay variation (Corcoran *et al*., 2014), and large panels of odorants can be rapidly tested with multiple replicates on a single 96-well plate. Drawbacks to this system include that certain receptors may not function well in non-insect cells, likely due to differences in the second messenger systems in the HEK cells and the insect cells.

2. *Spodoptera frugiperida 9 (Sf9*) cells are a moth-derived cell line used to study insect receptor function in calcium-imaging assays (Anderson *et al*., 2009, Claudianos *et al*., 2014, Jordan *et al*., 2009, Kiely *et al*., 2007). A proportion of *Sf9* cells endogenously express Orco, so only the tuning Or sequence is transiently transfected into the cell line and, similar to *HEK293* assays, measures ligand-induced changes in calcium fluorescence. A drawback lies in the fact that the cells have to be transiently transformed for each assay, which may lead to significant inter- and intra assay variation in Or expression.

3. Assays using *Xenopus laevis oocytes* involve the injection of cRNA encoding the Or of interest and Orco for expression on the membrane of the oocytes. Differential electrical potential, in response to ligand application, is recorded using two-electrode voltage clamp recordings to analyse receptor channel function (Wang *et al*., 2010). Unlike the assays mentioned above, this assay provides robust data on receptor channel activity, since the oocytes do not express other transmembrane proteins. However, compared to the previous two systems, assays are slower and expensive to maintain, requiring a stable supply of fresh oocytes, which leads to increased inter- and intra-assay variability.

4. *Drosophila "empty" neuron system* allows for functional characterisation of candidate receptors in a functional insect sensillum, thereby producing a robust analysis of the neuronal response *in vivo.* The system uses *Drosophila melanogaster* mutants that lack an endogenous Or and uses an endogenous promoter, *e.g.,* Or22a/b, to ectopically express an endogenous Or (Carey *et al*., 2010). Fast in-depth screens of ligands via single sensillum recordings (SSR), measure neuronal function upon stimulation with an odorant ligand. The SSR setup can also be combined with gas chromatography to identify natural odour ligands in complex extracts (Omondi *et al*., 2019).

5. The use of *CRISPR-Cas9 mediated genetic manipulation* allows for the knockdown or knockout of Ors within the mosquito antennae. This process benefits from testing the tuning Or of interest in the insect of study, allowing for the clearest understanding of the receptor function at the behaviour and physiological level (Hinze *et al*., 2023). However, the CRISPR-Cas9 system is still under development and may not be available for all mosquito species. Laboratory colonies of mutant lines are also harder to generate and maintain.

4.7 Protein extraction and western blot analysis (Paper III)

To quantify Or protein expression in the transfected HEK293 cells, cells were cultured in two flasks for 24 h. Prior to extraction (-16 h), one flask of cells were induced to transcribe the heterologous Or with doxycycline, while the other was sham induced with a similar volume of buffer, as a negative control. A western blot was performed using 20 µg of total protein from each of the sham-induced and induced cells using standard protocols for mixed molecular weights proteins.

4.8 Functional characterisation of the *Cx. pipiens* Ors (Paper III)

The HEK293 cells that co-expressed the Ors and AaegOrco were tested using a calcium fluorescence assay with ligand evoked responses, measured using a CLARIOstar Omega plate reader (BMG Labtech, Ortenberg, Germany), as per previously described protocols (Corcoran *et al*., 2014; Andersson *et al*., 2016; Yuvaraj *et al*., 2021). Briefly, cells were plated in a 96-well plated two days prior to testing and incubated at 37 °C overnight. To induce Or and Orco expression in the cells, half of the wells containing the cells were induced with doxycycline prior to testing (-16 h), with the remaining cells used as negative controls. A calcium-sensitive fluorophore Fluo-4AM (Life technologies) was loaded into all wells 1 h prior to testing, and the plates were incubated in the dark at room temperature for 30 min (to the cells in the wells taken in the fluorophore and display the ligand-induced fluorescence in the assay). The cells were then washed using assay buffer (to remove any excess fluorophore) and incubated for an additional 30 min in the assay buffer prior to testing (to control for baseline fluorophore readings in the assay), and after testing the cells were discarded.

The panel of odorants used in the assay were identical to that used for the behavioural phenotyping of the two biotypes in Paper I (Table 1), since the expression of the Ors in the antennal tissue of the mosquitoes displaying the differential host preference was likely due to the detection of these odorants in the synthetic host odour blends in the Y-tube assay in Paper I. The compounds were diluted to 100 mM in DMSO as a stock solution, and screening doses of 30 µM (the lowest dose that produces a clear response) were aliquoted before each assay. An Orco agonist, VUAA1, and DMSO

(0.5% diluted in assay buffer) were included in each assay as positive and negative controls, respectively. Prior to screening, the background fluorescence was measured for non-induced and induced cells. On the addition of the compounds to the cells, the ligand-induced response was measured as a percentage increase in fluorescence from background levels 10 s after stimulation, with a ≥ 2% increase in fluorescence in induced cells compared to the non-induced cells being considered a significant ligand-induced response. Compounds that triggered a significant response in the 30 μM screening were then included in a dose-response assay, with a similar set up as described above. The dose-response assay consisted of a two-fold serial dilution, commencing at 200 μM for 12 wells. Three technical replicates of each compound were tested on a single plate in the screening assays, with three biological replicates of cells tested for each compound for both the screening and dose-response assays.

4.9 Protein modelling and ligand docking (Paper III)

For the CppOr that responded to the ligands in the HEK293 cell assay above (CppOr205) and its homolog in Molestus (CpmOr205), predictive protein models were generated using AlphaFold 3 (Abramson *et al*., 2024). Structural models were obtained from the PDB database for the four ligands eliciting responses in the assay, i.e., (*R*)-1-octen-3-ol, linalool, 2-nonanol and 3-octanol. Ligand docking simulations were performed using Autodock Vina (Eberhardt *et al*., 2021) in Chimera (ver 1.19) (Pettersen *et al*., 2004). Five possible protein-ligand docking models for each ligand-receptor simulation were computed and the highest scoring models were used for analysis. Or/AaegOrco models were also generated in AlphaFold, and the receptors were visualized using PyMol (Pymol Molecular Graphics team, 2020). Predictive Or models for receptors that have been previously functionally characterised with any of the four ligands, *i.e.*, the Or8 lineage (Bohbot & Dickens, 2009; Carey *et al*., 2010; Wang *et al*., 2010; Hill *et al*., 2015; Xu *et al*., 2015; Pullmann-Lindsley *et al*., 2024) and CquiOr5 (Zeng *et al*., 2019), were also created using the same process described above, and ligand docking simulations were also run to observe similarities in the predicted ligand binding pockets of all the receptors.

4.10 Statistics (Papers I – III)

Statistical analyses for papers I – III were performed using R (version 4.3.1) (R core team, 2021), unless otherwise stated. The following packages in R were used - "readxl", "emmeans", "car", "lme4", and "multicomp".

For Paper I, the behavioural responses to the synthetic chicken and human odour were analysed using a beta binomial model followed by Tukey's multiple comparisons post-hoc test. The multiday assays of the biotypes were analysed using a one-way analysis of variance (ANOVA) to determine the difference in response of the mosquitoes over the two days of testing. False discovery rate *P*-values in the transcriptomic analyses, to determine the expression of the antennal chemosensory genes, were determined using CLC Genomics Workbench (http://www. clcbio. com, version 23.0.5; Qiagen, Vedbæk, DK) using the method described by Benjamini and Hochberg (1995).

For Paper II, the biotypes of the field-collected samples were determined by qPCR and were subsequently analysed using a linear mixed model followed by Tukey's multiple comparisons post hoc test. The model included the interaction of location of trapping, lure odour, and biotype ID of the samples. An ANOVA was subsequently run on the model to compare the variance of the samples in each location by biotype. To analyse the effect of the synthetic host odours on the number of collected *Cx. pipiens*, a linear model was created, with Tukey's post-hoc analysis. To analyse the host preference of the biotypes across countries, a preference index (PI) was calculated using the formula – $PI = (H - C)/ (H + C)$, where H is the number of mosquitoes of each biotype caught in the traps baited with synthetic human odour and C is the number of mosquitoes of each biotype caught in the traps baited with synthetic chicken odour. A linear model was created to compare the preference indices for each biotype across all locations, followed by Tukey's post hoc analysis.

For Paper III, a general linear mixed model (GLMM) was calculated to analyse changes in fluorescence of the induced HEK cells, with "induction" as the fixed factor and "plate" as the random factor, assuming a normal distribution. The mean responses for the ligand-induced fluorescence were calculated for the screening assays, and a nonlinear curve fit regression was used to calculate and compare the half-maximal effective concentrations (EC_{50}) for the dose-response assays using GraphPad Prism 10.5.0 (GraphPad Software Inc., La Jolla, CA, USA).

Table 1. Compounds present in the synthetic host odour blends. The blends were used in Papers I and II, and the individual compounds were used in the odour panel tested in Paper III. Stock solutions of the odour blends (2 mL) were prepared using pentane as a solvent. All compounds were obtained from Merck as liquids, with purities ranging from 95 – 99%, except for phenol and α-terpineol, which were obtained as solids, with the amount listed in µg. The human odour blend was prepared as per Omondi *et al*. (2019), and the chicken odour blend was prepared as per Spanoudis *et al*. (2022).

	Compound	CAS no.	Volume (µL mL^{-1} solvent)
Human odour blend	**1-Hexanol**	111-27-3	5.7
	3-Octanol	589-98-0	18
	(*R*)-1-octen-3-ol	3687-48-7	1
	2-Nonanol	628-99-9	1.1
	Octanal	124-13-0	7.7
	Nonanal	124-19-6	76
	Decanal	112-31-2	115
	Butyl acetate	123-86-4	4.8
	Sulcatone	110-93-0	51
	Phenol	108-95-2	10.7
	Benzaldehyde	100-52-7	4.4
	Acetophenone	98-86-2	0.4
	Limonene	5989-27-5	150
	Linalool	78-70-6	5.7
	α-Terpineol	98-55-5	30.7
Chicken odour blend	**Heptanal**	111-71-7	1
	Octanal	124-13-0	5
	Nonanal	124-19-6	50
	Decanal	112-31-2	10
	Sulcatone	110-93-0	7
	3-Octanone	106-68-3	5
	Benzaldehyde	100-52-7	2

5. Summary and discussion of results

5.1 Differential host preference behaviour and antennal gene expression in Pipiens and Molestus (Paper I)

Host preference in blood-feeding mosquitoes is described as the process by which a female mosquito selects a suitable host over others when presented with equal access (Lyimo & Fergusson, 2009; Takken & Verhulst, 2013; Hinze *et al*., 2022). Pipiens and Molestus demonstrate differences in host preference, with Pipiens preferring birds (ornithophilic) and Molestus preferring humans (anthropophilic) (Faraji & Gaugler, 2015; Noreuil & Fritz, 2021; Bell *et al*., 2024; Paper I). This preference, as observed in the various laboratory assays, ranges from strong (Noreuil & Fritz, 2021; Bell *et al*., 2024) to weak (Faraji & Gaugler, 2015; Paper I), depending on the geographic origin of the lab strains tested. This host preference contrasts the host choice of the mosquitoes, *i.e.*, the ability to detect and feed on an available host (Lyimo & Fergusson, 2009; Takken & Verhulst, 2013; Hinze *et al*., 2022), as demonstrated by blood-meal analyses performed on field caught mosquitoes across Europe and North America (Haba & McBride, 2022; Wehmeyer *et al*., 2024), with Pipiens and Molestus demonstrating a variation in host choice. The genetic mechanism(s) guiding this differential host preference in Pipiens and Molestus is unclear, with only a recent study comparing the genomes of the two biotypes against that of *Cx. quinquefasciatus*, identifying potential genes involved in regulating host preference (Bell *et al*., 2024). The aim of this study (Paper I) was to (a) demonstrate that laboratory colonies of Pipiens and Molestus, originating from the Netherlands, display a clear and consistent difference in host preference when tested in a laboratory conditions, and (b) to correlate the expression of antennal chemosensory genes with the differential host preference, thereby identifying a potential genetic basis of the host preference of these mosquitoes.

In a Y-tube assay, Pipiens and Molestus demonstrated a dose-dependent response to the synthetic chicken and human odour blends, respectively. When assessed pairwise, Pipiens demonstrated a preference to the chicken odour blend at lower concentrations, with preference shifting to the human odour blend as the concentration of these blends increased. This trend was not observed in Molestus, which maintained a consistent preference to the

human blend. When tested at a concentration that elicited the highest number of responses for both biotypes, the mosquitoes maintained a similar ratio of host preference over the two days of testing (Figure 1), with Pipiens and Molestus significantly preferring the synthetic chicken and human odour blends, respectively. The host preference of the biotypes observed in this study is similar to that found in previous studies and strengthens the argument that the strength of the host preference is dependent on geographic origin of the laboratory colonies (Faraji & Gaugler, 2015; Noreuil & Fritz, 2021; Bell *et al*., 2024). When compared to the highly anthropophilic *Ae. aegypti* and *An. gambiae* (McBride *et al*., 2014; Zhao *et al*., 2022; Takken & Verhulst, 2013), or the highly zoophilic *Anopheles quadriannulatus* (Dekker *et al*., 2001; Takken & Verhulst, 2013), the host preferences of Pipiens and Molestus is lower, implying that the biotypes used in this study are opportunistic, though biased to birds and humans, respectively. The observed variance in host preference observed in laboratory studies may explain the demonstrated variation in host choice across Europe (Wehmeyer *et al*., 2024). The results of this study, however, emphasise that Pipiens and Molestus are attracted to, and are able to discriminate between, host odours, likely due to the differential expression of antennal chemosensory genes.

The antennal transcriptome created from the harvested antennae of the behaviourally phenotyped mosquitoes identified differences in expression profiles between biotypes (Figure 2). A gene ontology (GO) analysis of the reliably expressed genes found similar gene families to be expressed in the antennae of the two biotypes. A further analysis on the differentially expressed genes using GO slim terms for molecular function, identified subsets of genes related to olfaction, *e.g.,* odorant binding (Figure 3). Of the chemosensory genes that were reliably and differentially expressed between the two biotypes, 9 Ors, 3 Irs, 12 OBPs were identified, along with 3 CSPs and one SNMP (Figure 4).

For the Ors, three Ors were more highly expressed in Molestus, with three of the remaining six Ors being solely expressed in Pipiens. None of the Ors identified in this study have been functionally characterised in *Cx. pipiens,* nor in the closely related *Cx. quinquefasciatus*. However, orthologs of the Or2 gene, which was more highly expressed in Pipiens, have been studied in other species of mosquitoes and have been shown to bind to indole, 4-methyl phenol and benzaldehyde (Omondi *et al*., 2019; Ruel *et al*., 2019), all of which are commonly found in human odours (Omondi *et al*., 2019; Zhao *et*

al., 2022), and play a role in mediating other behaviours (Khan *et al*., 2022). The Or pathway plays a role in mediating host seeking and discrimination in mosquitoes (DeGennaro *et al*., 2013; Omondi *et al*., 2019). Taking this into account, I hypothesize that the Ors identified in this study likely play a role in regulating discrimination between humans and birds, similar to that proposed for the anthropophilic and zoophilic *Ae. aegypti* (McBride *et al*., 2014). For the Irs, Ir75l and Ir100b were more highly expressed in Molestus, while the co-receptor Ir76b was more highly expressed in Pipiens (Figure 4). The Ir pathway has been shown to mediate host discrimination (De Obaldia *et al*., 2022), so similar to the Ors, the Irs identified in this study may also be involved in mediating host discrimination in Pipiens and Molestus. The role of the other chemosensory proteins in olfaction has been described in prior studies (detailed in section 2.3), and I hypothesize that they likely play a role in regulating the antennal sensitivity of the biotypes to host VOCs.

In conclusion, the results from this study support previous laboratory observations of the host preference of Pipiens and Molestus and demonstrate that the preference is innate and not individualistic. By correlating the differential host preference with differences in the expression of certain antennal chemosensory genes, the study provides possible molecular mechanisms involved in the regulating the host preferences of the biotypes.

5.2 Variation in relative proportion and host preference of Pipiens, Molestus and their hybrids across Europe (Paper II)

The biotypes of *Cx. pipiens* and their hybrids are commonly found throughout Europe (Farajollahi *et al*., 2011; Yurchenko *et al*., 2020; Haba & McBride, 2022) (Figure 5). Pipiens is the most prevalent and has been observed in a variety of ecological habitats, *i.e.*, urban, peri-urban and rural habitats (Yurchenko *et al*., 2020; Haba & McBride, 2022; Wehmeyer *et al*., 2024). Molestus has typically been more prevalent in south-eastern Europe, with smaller populations arising in urban and peri-urban habitats in northern Europe (Yurchenko *et al*., 2020; Haba & McBride, 2022). The incidence of Molestus in these new regions has been linked to increased travel and trade, with hybrids between the biotypes also forming in sympatric regions (Vogels *et al*., 2016b; Hesson *et al*., 2016; Haba & McBride, 2022; Blom *et al*., 2024; Wehmeyer *et al*., 2024). Field collections of these mosquitoes have identified

a variation in the feeding patterns, *i.e.,* host choice, of *Cx. pipiens* across Europe, with both Pipiens and Molestus largely feeding on avian hosts, followed by human and other mammalian hosts (Wehmeyer *et al*., 2024).

To assess the relative populations of Pipiens, Molestus and their hybrids across a latitudinal gradient of Europe, in the context of their host preference, trapping assays were conducted in three countries of varying latitude – The Netherlands, Switzerland and Greece. Unlike prior field studies from regions in similar latitudes and ecological habitats (references within Haba & McBride, 2022 and Wehmeyer *et al*., 2024), which typically use a single trap baited with or without CO_2, the trapping assay in this study was conducted using a two-choice assay solely in peri-urban sites (to allow for equal access to human and bird hosts). The trapping assay comprised of six pairs of traps, each placed 1 - 2 m apart and baited with CO_2. Within the trap pair, each trap was baited with either the synthetic chicken or human odour blends used in the laboratory assay (Paper I). This two-trap set up allowed for the testing of the host preference of wild mosquitoes in each country in a similar manner as the laboratory assays in Paper I, by comparing the number of mosquitoes caught in the chicken odour baited trap against those caught in the human odour baited trap.

The use of the blends also helped greatly increase the number of mosquitoes caught in each country when compared to control sites (baited with CO_2 alone) (Paper II) and those in prior field collections (references within Haba & McBride, 2022 and Wehmeyer *et al*., 2024). The study (Paper II) found that the relative population of Pipiens (the most prevalent biotype), Molestus and the hybrids varied across Europe, with the relative proportions of Pipiens increasing as latitudes increased (Paper II). The proportions of Molestus and the hybrids were higher in Greece than in the lower latitudes (Paper II) and were in line with results from regions in similar latitudes (references within Haba & McBride, 2022 and Wehmeyer *et al*., 2024).

The results of the host preference analysis varied based on country (Figure 6). The observed host preference of Pipiens (ornithophilic) and Molestus (anthropophilic) in Greece was in line with previously reported host choice studies from regions in similar latitudes for Pipiens but not for Molestus (Wehmeyer *et al*., 2024). In addition, the observed host preference in Greece was in line with the previously reported laboratory studies on their host preference (Faraji & Gaugler, 2015; Noreuil & Fritz, 2021; Bell *et al*., 2024; Paper I). The host preference shifted as the latitude increased, with

Pipiens demonstrating a weak anthropophilic preference in the Netherlands and Switzerland, compared to the preference observed in Greece. This shift in preference for Pipiens was in line with previously available data from similar latitudes (Wehmeyer *et al*., 2024). Similarly, Molestus, demonstrated a more ornithophilic preference in the Netherlands and Switzerland than that observed in Greece (Paper II). In contrast to the biotypes, the hybrids remained opportunistic regardless of location of trapping, in line with available host choice data (Wehmeyer *et al*., 2024).

Though the selective pressures regulating the observed differences in host preference between the different populations of the biotypes across latitudes are unknown, there is likely a genetic basis, as seen in other mosquito species (Rinker *et al*., 2013; McBride *et al*., 2014; Main *et al*., 2016). The results also highlight that in regions of high gene flow and hybridization (as described in Haba *et al*., 2025), the hybrids demonstrate a more intermediate phenotype than their parent biotypes, which further strengthens the argument for a genetic basis of host preference put forth in Paper I. *Culex pipiens* is a noted vector of arboviruses such as WNV (Farajollahi *et al*., 2011; Vogels *et al*., 2017; Yurchenko *et al*., 2020), but the host preference of the hybrids have yet to be assessed in laboratory settings, which may provide greater insight into the vectorial capacity of these mosquitoes.

5.3 Functional characterisation and predictive structural analysis of *Cx. pipiens* Ors correlated with differential host preference (Paper III)

Two ways by which the strength of the innate response of OSNs to a host VOC may be altered are either through changes in Or expression (Section 5.1; Paper I) or changes in the structure of the Or complex (elaborated on in section 2.2.3). Paper I demonstrated that the differential expression of nine antennal Ors in Pipiens and Molestus correlated with the observed differences in host preference. The aim of Paper III was to functionally characterise these nine Ors, using the odorants from the synthetic host odour blends used in Paper I.

The nine identified Ors (Section 5.1; Paper I) were functionally characterised using an inducible HEK293 cell line co-expressing AaegOrco (Corcoran *et al*., 2014). Of the Ors tested, only one Or, Pipiens Or205 (CppOr205), demonstrated a dose-dependent response to four VOCs – (*R*)-

1-octen-3-ol, linalool, 2-nonanol and 3-octanol (Figure 7), all of which are exclusively found in the synthetic human odour blend. Of these four compounds, both (*R*)-1-octen-3-ol and linalool have been demonstrated to play an important role in mediating attraction and discrimination in anthropophilic mosquitoes, when detected in a context-dependent manner (Majeed *et al*., 2016; Omondi *et al*., 2019; reviewed in Hinze *et al*., 2022; Paper I). Altering the level of (*R*)-1-octen-3-ol in attractive host odours causes a reduction in attraction in *Cx. quinquefasciatus* (Majeed *et al*., 2016). Similarly, if tested alone, (*R*)-1-octen-3-ol and linalool elicit a repellence in *Cx. quinquefasciatus* (Xu *et al*., 2015; Zeng *et al*., 2019). The pathways mediating the attraction or repellence in response to (*R*)-1-octen-3-ol detection involve the expression of Or8 on OSNs in the maxillary palps (Majeed *et al*., 2016) or the antennae (Xu *et al*., 2015), respectively, with each neural pathway likely involved in mediating these distinct behaviours. Considering that CppOr205 was expressed in the antennae, I hypothesize that by detecting (*R*)-1-octen-3-ol and linalool present in the human odours, CppOr205 is likely also involved in an aversive response pathway. The Or205 homolog in Molestus (CpmOr205) did not respond to the highest dose of these VOCs in the HEK cell assay, with a sequence alignment between the two Ors identifying a single amino acid difference between the two receptors (Figure 8).

Using predictive modelling software (AlphaFold 3), the difference in structure between the Or205 of both biotypes was located between the S7a and S7b helices (Figure 8), a region that has been implicated in the interaction between subunits in the anchor domain of the Or-Orco heterotetramer, and not in the predicted binding pocket of the tuning Or (Zhao *et al*., 2024). This region, when present alongside the Orco subunits, form a lateral pore conduit, and the observed changes in this region of the structure of CpmOr205 may restrict ion flow into the cell, affecting receptor function (Figure 9). Ligand binding analyses using the predictive structures of CppOr205 and CpmOr205 was conducted to compare the binding affinities of the receptors to the four ligands of CppOr205. The predictive binding structures of other Ors that have been previously reported to bind (*R*)-1-octen-3-ol and 3-octanol, *i.e*., the Or8 lineage (Bohbot & Dickens, 2009; Carey *et al*., 2010; Wang *et al*., 2010; Hill *et al*., 2015; Xu *et al*., 2015; Pullmann-Lindsley *et al*., 2024) and linalool and 3-octanol, *i.e.*, CquiOr5 (Zeng *et al*., 2019), were also included in the ligand binding analyses to

identify possible similarities in binding pockets. The analyses observed that the convergence in function of these receptors correlates with a conservation of binding pocket structure and residue polarity (Figure 10). Recent studies on the structure and binding affinities of other insect Ors (del Mármol *et al*., 2021; Wang *et al*., 2024; Zhao *et al*., 2024) elaborate on the role of the tertiary structure of the Ors in mediating strong binding affinities, though the factors affecting the regions outside the binding pockets, i.e., the lateral pore and anchor domain, require further attention.

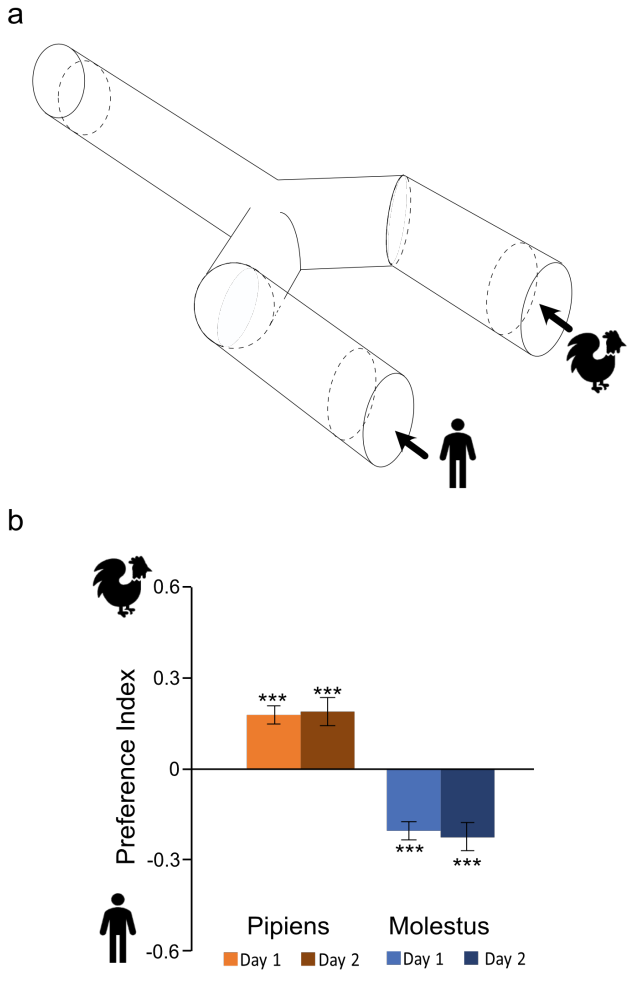

Figure 1. Pipiens and Molestus demonstrate a clear and consistent host preference to synthetic chicken and human odour blends, respectively. (a) Diagram of the Y-tube olfactometer used to assess host preference of the biotypes. (b) Consistency in host preference for each biotype was assessed over 2 days, by the response to synthetic odour blends (dose = 10^{-5}). Error bars represent the standard error of proportions, with asterisks denoting statistical difference from 0 ($P < 0.0001$) for each day.

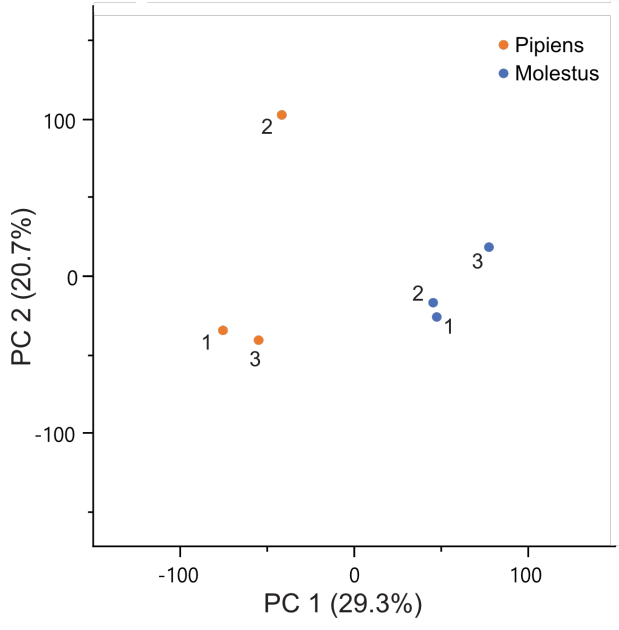

Figure 2. Gene expression profiles differ between antennal libraries of host-seeking Pipiens and Molestus. Principal component analysis (PCA) highlighting the difference in expression profiles of the antennal libraries of Pipiens (orange) and Molestus (blue) obtained from RNA-sequencing.

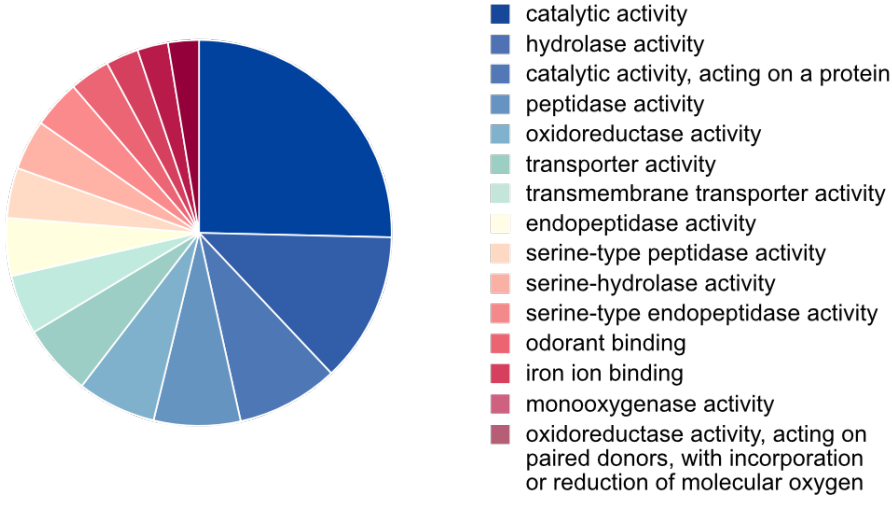

Figure 3. Gene ontology analysis of differentially expressed antennal transcripts of Pipiens and Molestus. Odorant binding genes represent 5 % of the differentially expressed genes identified in the GO analysis. Genes were characterised based on their molecular function, using GO slim categorization.

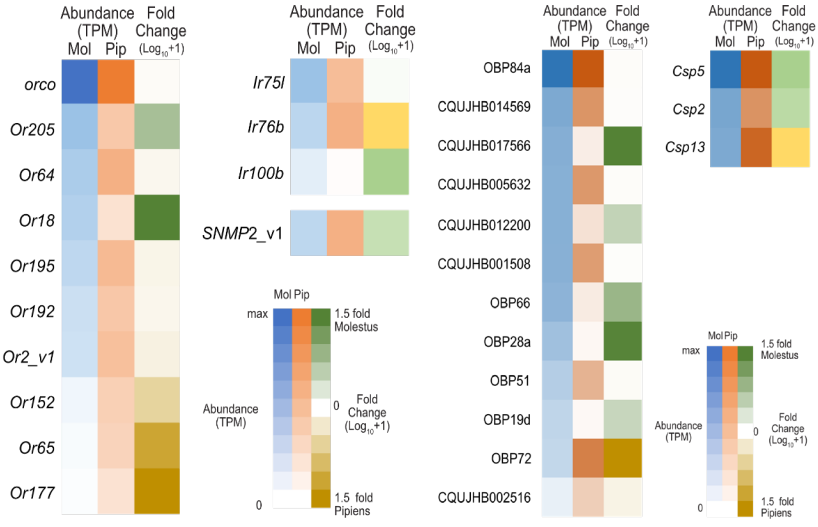

Figure 4. Comparison of differential antennal chemosensory gene expression in Pipiens and Molestus correlated with divergent host preferences. Transcript abundance values represented in -Log_{10}+1 scale for Pipiens (P) and Molestus (M) in orange and blue, respectively, with fold change (FC) representing a comparison between the two biotypes. Genes were labelled as per the reference genome (*Culex quinquefasciatus* JHB2020, VectorBase rel. 66, 2-NOV-2023), and if they were not annotated, the VectorBase gene IDs are stated. Left - Transcript abundance of odorant receptors (Ors), ionotropic receptors (Irs), and sensory neuron membrane proteins (SNMPs). Right - Transcript abundance for odorant binding proteins (OBPs) and chemosensory proteins (Csps).

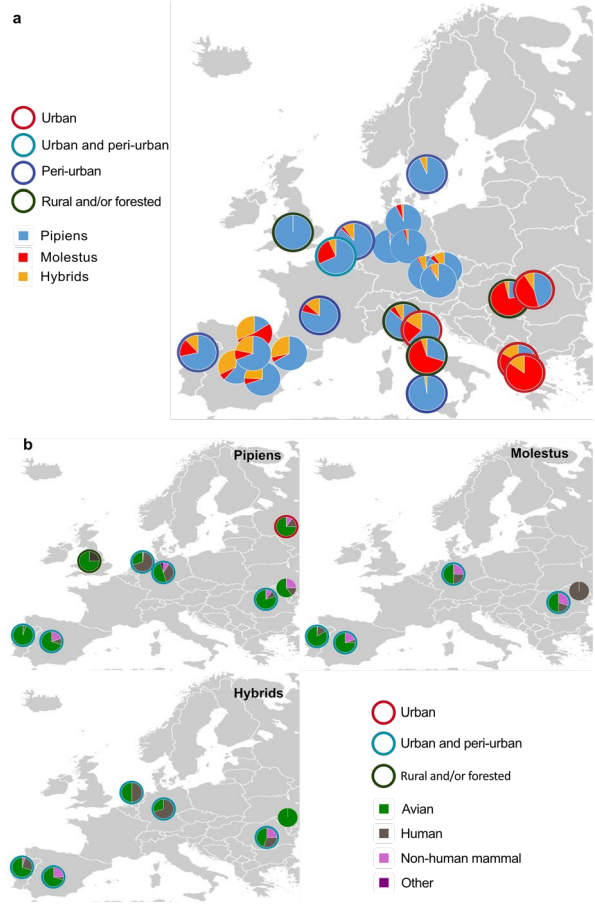

Figure 5. Relative proportion and blood-feeding patterns of *Culex pipiens* biotypes and hybrids across Europe. (a) Relative proportion of Pipiens, Molestus and their hybrids across Europe based on data aggregated in Haba & McBride, 2022. Coloured rings around the pie charts indicate the ecology of trapping sites for each study. (b) Blood-meal analysis identifying the host choice of Pipiens (top left), Molestus (top right) and the hybrids (bottom left) from data aggregated in Wehmeyer *et al*., 2024. Coloured rings around charts indicate the ecological context of the trapping sites, and colours within each pie chart represent identified host species.

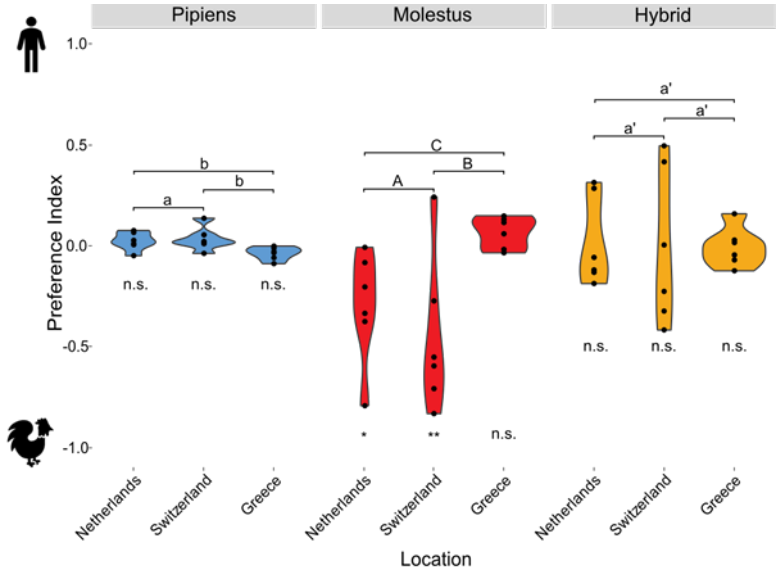

Figure 6. Host preference of *Culex pipiens* biotypes and hybrids, across three locations in Europe, assessed using a two-choice trapping assay baited with synthetic chicken and human odours. Preference index ranges from -1 (chicken preferring) to 1 (human preferring). Asterisks indicate levels of significance for Pipiens (blue), Molestus (red) and hybrids (yellow) within each country, and letter indicate level of significance for comparisons between countries (a = not significant, b = $P < 0.05$, c = $P < 0.01$), calculated using a linear model with Tukey's post hoc test.

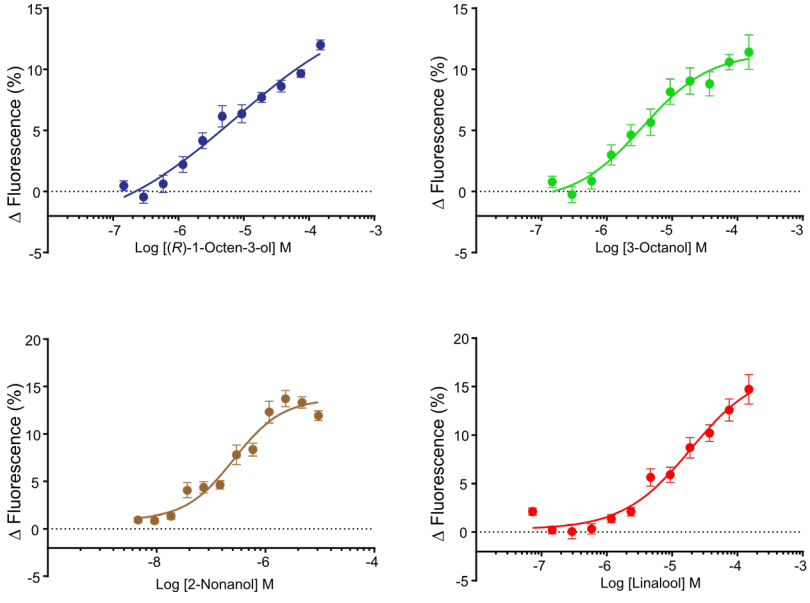

Figure 7. Dose-dependent responses of Trex/HEK293 cells expressing the *Culex pipiens* biotype pipiens Or205 to the four active volatile organic compounds identified from the screening assays. Changes in calcium fluorescence were measured for the cells when tested with (*R*)-1-octen-3-ol (blue, top left), 3-octanol (green, top right), 2-nonanol (brown, bottom left) and linalool (red, bottom right) in the HEK cell assay (n = 3, N = 3). Error bars represent standard error of the mean.

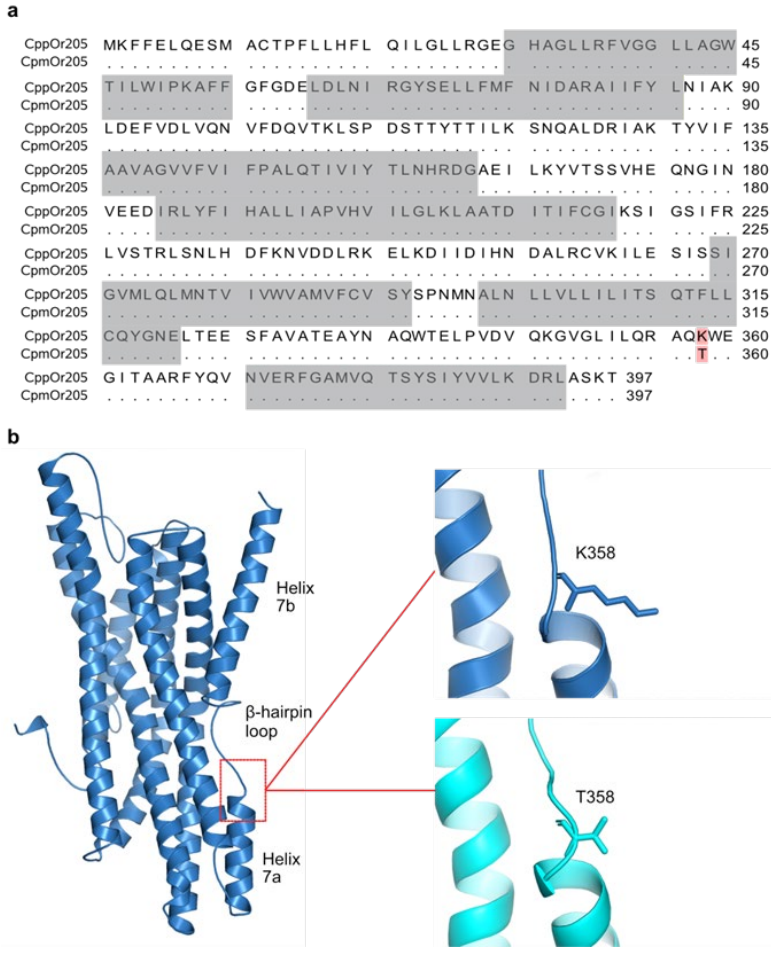

Figure 8. *Culex pipiens* biotypes Pipiens and Molestus Or205 display a high degree of sequence and structure conservation. (a) Amino acid sequence alignment for Pipiens Or205 (CppOr205) and Molestus Or205 (CpmOr205), with the single differing amino acid highlighted in red. Grey boxes indicate predicted transmembrane domains. (b) Predicted protein model for Or205, focusing on the β-hairpin loop (red box) between the 7a and 7b helices, in which the differing amino acid is present, for CppOr205 (top) and CpmOr205 (bottom). Predicted protein models were created using AlphaFold (Abramson *et al*., 2024) and visualized using PyMol (Schrödinger & DeLano, 2020).

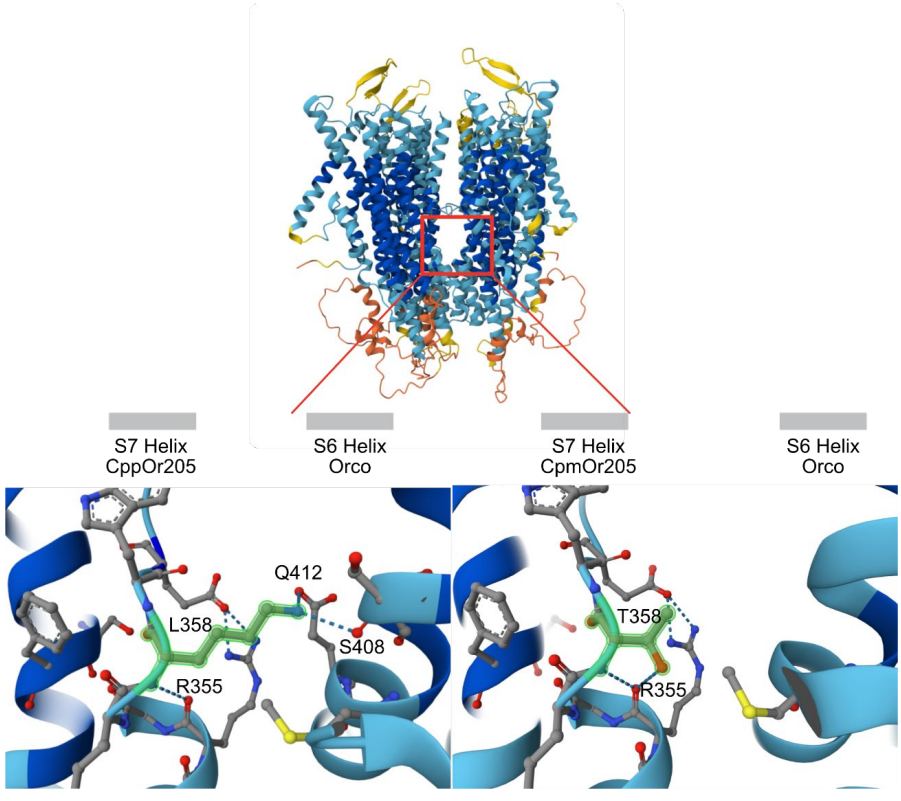

Figure 9. Differences in predicted Or/Orco subunit interaction due to differential Or structures of *Culex pipiens* biotypes Pipiens and Molestus in a less well conserved region. Predicted structure of the Or205/Orco structure for Pipiens and Molestus, created in AlphaFold 3 (Abramson *et al*., 2024). Red box highlights the region between the S7a and S7b helices of Or205 and the S6 helix of neighbouring Orco subunit, with zoomed in insert demonstrating this differential interaction for CppOr205 (left) and CpmOr205 (right) likely occurring due to the differing amino acid at position 358 for the receptor (highlighted in green).

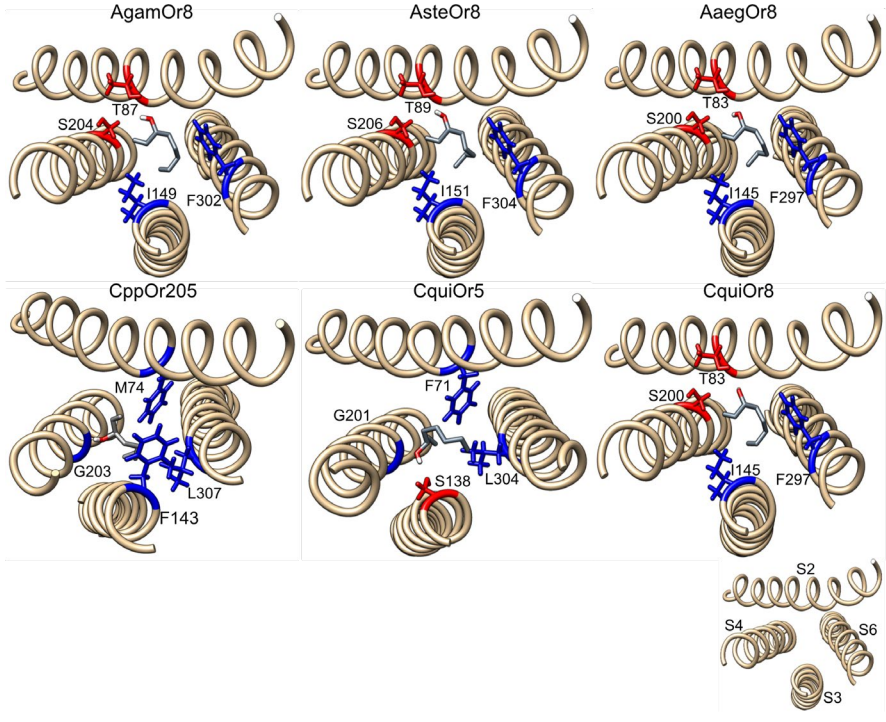

Figure 10. Comparison of predicted ligand binding pockets of Ors that bind to (*R*)-1-octen-3-ol across mosquito species. The amino acids identified in the binding analyses are coloured based on polarity, with nonpolar residues in blue and polar uncharged residues in red. (*R*)-1-octen-3-ol is shown in silver, with the hydroxyl group highlighted in red. Predictive Or structures of the evolutionarily conserved Or8 receptors (top row and bottom right), CppOr205, and CquiOr5, were created using AlphaFold 3, with ligand docking analyses done in Chimera with Autodock Vina. For AaegOr8, an alternate kozak sequence was used based on RNA-seq data on Vectorbase. Inset shows legend, labelling the different helices of the receptor.

6. Conclusion and future perspectives

As the number of WNV cases in Europe increases (eCDC, 2024) and spreads to new regions (UEVP, 2026), a greater understanding of *Cx. pipiens*, and the factors regulating its vectorial capacity, is required. *Culex pipiens* remains a largely understudied mosquito species compared to other notable mosquito vectors, with little prior knowledge available about the factors regulating the differential host seeking behaviours displayed by the biotypes.

Using a combination of behavioural and genetic analyses, the studies in this thesis propose a genetic mechanism that regulates the host preference of the biotypes. The results highlight that, similar to other mosquito species, the differential expression and/or function of key odorant receptors in the antennae of the biotypes regulates their host preference, and postulates that this genetic mechanism affects, in part, the vectorial capacity of the mosquitoes. The studies outlined in this thesis provide novel information that not only expands the scientific knowledge about the species (both behaviourally and genetically) but also opens new avenues of research into the molecular mechanisms guiding their host preference behaviours, which may be useful in future epidemiological studies on *Cx. pipiens*.

Papers I and II assessed the differential behaviour of laboratory and wild populations of the biotypes and correlated this behaviour, of the laboratory populations, with the differential antennal gene expression. This combined behavioural and transcriptomic analysis allows us to identify certain Ors that may play a role in regulating the host preference in the biotypes, but there remain a few questions. Future studies may focus on comparing the differences in antennal gene expression between different populations of the biotypes from different regions across Europe, providing further information about the genetic mechanisms regulating the host preference. A genomic analysis of these differing populations, utilizing the recently published genome of *Cx. pipiens* (Hesson *et al*., 2025), may also shed further light on differences between the populations, and identify which chemosensory genes, if any, are under high selective pressures.

A limitation of Paper I is the lack of knowledge regarding the host preference of the hybrids. Generating and maintaining laboratory populations of the hybrids is difficult, thereby hampering any laboratory assays. Future analysis on the issues surrounding the mating behaviours of

the biotypes in laboratory conditions may help develop better rearing techniques for the easy and timely generation of hybrids, allowing for more behavioural experiments.

Paper III demonstrated that out of the differentially expressed Ors in Paper I, only one Or responded to the host ligands, and a comparison of the Or sequence and structure from both biotypes identified differences that are likely responsible for their differing functions. This analysis of Or205 further expands on the proposed genetic mechanism postulated, with future studies focusing on other possible genes. Since the other Ors tested in the assay did not respond to any of the host odours, questions remain regarding what caused the differential expression of the Ors in the behavioural analysis of Paper I. Further analysis is required to deorphanize the receptors and to analyse their role in any other odour-mediated behaviours of the biotypes.

Another possible avenue of exploration would focus on the structural differences of the Ors, as highlighted in Paper III. Currently, only two mosquito Ors have been solved *in vitro* (Zhao *et al*., 2024), so validating the models proposed in Paper III may help further the understanding of the mechanisms guiding Or function. Solving these structures may also explain the role of the structures present in the anchor domain, and how the interactions between the Or and Orco subunit impact protein function and expression within the cell. The development of better CRISPR-Cas9 driven tools to alter the protein sequence and structure may also provide more information about Or function and expression.

Taking the results of Papers I – III together, these predictive structural models comparing CppOr205 and CpmOr205 provide a possible genetic mechanism involved in regulating the differential host preference observed in Paper I. These pathways likely play a role in the more opportunistic preference observed in the hybrid populations sampled in Paper II, though further analysis is required.

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Popular science summary

Mosquitoes are among the most abundant and deadliest insects, responsible for transmitting diseases that result in numerous fatalities each year. Due to anthropogenic climate change and human activity, mosquitoes have spread into new regions and now pose greater health risks not only to humans but also to other animals such as birds and cattle. While some of the major disease-vectoring mosquito species are well studied, there remains a significant gap in knowledge regarding other disease-vectoring species.

The focus of this thesis is one such species, the northern house mosquito *Culex pipiens*. It is found throughout Europe and North America and is a known vector of viruses such as West Nile virus (WNV), which affects both humans and birds. There are two biotypes of *Cx. pipiens*, called Pipiens and Molestus, which are look identical but display marked differences in behaviour and genetics. One important difference between the two biotypes is host preference: Pipiens prefers birds, whereas Molestus prefers humans. The genes that control this behaviour are unknown, but identifying and studying them may help develop tools to reduce the spread of WNV. Since mosquitoes rely on their sense of smell to locate food sources, the genes involved in their olfactory pathways are likely responsible for regulating host preference. The aim of this thesis was to study the host preference of Pipiens and Molestus and to identify the genes involved in regulating this behaviour.

The first paper examined laboratory populations of Pipiens and Molestus and showed that their attraction to chickens and humans, respectively, was not very strong. A closer analysis of the different olfactory genes expressed in the mosquitoes identified certain odorant receptors (Ors) that may be responsible for regulating the different behaviours. The second paper investigated wild populations of Pipiens, Molestus, and their hybrids in Europe and found that host preference varied across countries. Finally, the third paper examined the Ors identified in Paper I more closely, as little is known about their function in Pipiens and Molestus. One Or in particular, called Or205, differed between Pipiens and Molestus, both in function and gene sequence. I hypothesize that differences in the Or205 gene sequence and structure are, in part, responsible for the differences in host preference behaviour between Pipiens and Molestus.

Overall, this thesis links the behaviour and genetics of Pipiens and Molestus, and provides novel information about this species that may be used to further study the transmission of WNV in Europe.

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Populärvetenskaplig sammanfattning

Myggor är bland de mest talrika och dödligaste insekterna och ansvarar för spridning av sjukdomar som orsakar många dödsfall varje år. På grund av antropogen klimatförändring och mänsklig aktivitet har myggor spridit sig till nya områden och utgör nu större hälsorisker, inte bara för människor utan även för andra djur såsom fåglar och boskap. Även om vissa av de viktigaste myggarterna som sprider sjukdomar är väl studerade, finns det fortfarande ett betydande kunskapsgap när det gäller andra sjukdomsvektorerande arter.

Fokus för denna avhandling är en sådan art, den norra husmyggan *Culex pipiens*. Den förekommer i hela Europa och Nordamerika och är en känd vektor för virus såsom West Nile-virus (WNV), som påverkar både människor och fåglar. Det finns två biotyper av Cx. pipiens, kallade Pipiens och Molestus, som ser identiska ut men uppvisar markanta skillnader i beteende och genetik. En viktig skillnad mellan de två biotyperna är värdpreferens: Pipiens föredrar fåglar, medan Molestus föredrar människor. De gener som styr detta beteende är okända, men att identifiera och studera dem kan hjälpa till att utveckla verktyg för att minska spridningen av WNV. Eftersom myggor förlitar sig på sitt luktsinne för att hitta födokällor, är det troligt att de gener som är involverade i deras olfaktoriska banor ansvarar för reglering av värdpreferens. Målet med denna avhandling var att studera värdpreferensen hos Pipiens och Molestus och att identifiera de gener som är involverade i regleringen av detta beteende.

Den första artikeln undersökte laboratoriepopulationer av Pipiens och Molestus och visade att deras attraktion till kycklingar respektive människor inte var särskilt stark. En närmare analys av de olika olfaktoriska gener som uttrycktes i myggorna identifierade vissa luktreceptorer (Ors) som kan vara ansvariga för att reglera de olika beteendena. Den andra artikeln undersökte vilda populationer av Pipiens, Molestus och deras hybrider i Europa och visade att värdpreferensen varierade mellan olika länder. Slutligen undersökte den tredje artikeln de Ors som identifierades i Artikel I mer ingående, eftersom lite är känt om deras funktion hos Pipiens och Molestus. En Or i synnerhet, kallad Or205, skilde sig mellan Pipiens och Molestus, både vad gäller funktion och gensekvens. Jag hypotheserar att skillnader i Or205-genens sekvens och struktur delvis är ansvariga för skillnaderna i värdpreferensbeteende mellan Pipiens och Molestus.

Sammanfattningsvis kopplar denna avhandling samman beteende och genetik hos Pipiens och Molestus och bidrar med ny kunskap om denna art, vilket kan användas för att vidare studera spridningen av WNV i Europa.

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Parasites & Vectors

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Differential expression of antennal chemosensory genes related to host preference of *Culex pipiens* biotypes

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Abstract

Background The northern house mosquito, *Culex pipiens,* is a noted arboviral disease vector commonly found throughout Europe and North America. Two morphologically identical biotypes of this species, *Culex pipiens pipiens* and *Culex pipiens molestus*, display differential host preference to birds and humans, respectively; however, little is known about the genetic mechanisms regulating this behavior.

Methods Using a Y-tube olfactometer, the host preference of the host-seeking female mosquitoes of both biotypes was tested by providing a choice between synthetic chicken and human odor blends, across 2 days of testing. Antennal transcriptomes, from the mosquitoes that demonstrated a clear and consistent preference to either of the odor blends, were created to observe differences in antennal chemosensory gene expression.

Results In the host preference experiments, *Cx. pipiens pipiens* and *Cx. pipiens molestus* demonstrated a weak, but significant, preference to the synthetic chicken and human odor blends, respectively, when tested across multiple days. The transcriptome created from the antennae of mosquitoes that made a consistent choice over 2 days of testing identified 9 odorant receptors, 3 ionotropic receptors, and 12 odorant binding proteins, and other chemosensory genes, that were differentially expressed between the two biotypes, which correlate with the observed differential host preference.

Conclusions This study identified a set of chemosensory genes that are putatively correlated with the differential host preference of the two biotypes. Future research is required to increase the understanding of the function of the identified chemosensory receptors, and how they can be used as genetic markers of host preference of wild mosquitoes.

Keywords *Culex pipiens*, Biotypes, Host preference, Antennal transcriptome, Chemosensory genes, Odorant receptors

Background

The northern house mosquito, *Culex pipiens,* is a vector for arboviruses, e.g., West Nile and Usutu viruses, and is commonly found throughout North America and Europe [1–3]. Two biotypes of the species have been identified: *Culex pipiens* f. *pipiens* (hereafter called Pipiens) and *Culex pipiens* f. *molestus* (hereafter called Molestus), which are morphologically indistinguishable, but differ in terms of ecology, mating, gonotrophic cycle, oviposition site preference, and more notably host preference, with Pipiens and Molestus predominantly feeding on birds

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(ornithophilic) and humans (anthropophillic), respectively [2]. Although the mechanism regulating the differential host preference is currently unknown, an increased understanding of the genes, and variants thereof, associated with host preference may identify novel targets for vector control, and may be used to create better predictive tools for determining factors regulating vectorial capacity.

Host preference in blood-feeding mosquitoes is defined as the process by which mosquitoes preferentially select one host over others when presented with equal access, while host choice can be defined as the process of detection and feeding on any available host present in the environment [4, 5]. While the factors regulating host preference of the highly anthropophillic vectors of dengue and malaria, *Aedes aegypti and Anopheles gambiae*, respectively, have been well-characterized [5–9], the host preference of *Cx. pipiens* has received less attention [10, 11]. Blood meal analysis, indicating host choice of field-captured Pipiens and Molestus, demonstrates feeding patterns on birds, as well as human and nonhuman mammals, across Europe, which is likely affected by the availability of hosts and trapping location [2, 12–14]. Laboratory studies, however, support a mostly ornithophilic preference of Pipiens [10–12], while Molestus demonstrates a stronger anthropophillic preference [2, 10–12, 15]. These studies, similar to those conducted on other mosquitoes, emphasize that mosquitoes predominantly use olfaction for host discrimination and selection.

Host odors comprise blends of volatile organic compounds (VOCs), which regulate host discrimination and selection [4, 5, 16]. Attraction of blood-seeking mosquitoes to a host is regulated by species-specific differences in detection of odor composition and ratios, with certain classes of chemical compounds, including aldehydes and ketones, being shared across host odors [4, 7, 9]. Mosquitoes may use these shared compound classes for intra- and interspecific host discrimination [4, 7, 9]. For example, differences in the composition of short- and long-chained aldehydes, as well as sulcatone, are thought to regulate interspecific host preference in *Ae. aegypti* [7, 9]. In addition, taxon-specific VOCs, such as (*R*)-1-octen-3-ol, have been demonstrated to regulate both intra- and interspecific host discrimination [7, 9, 17].

Volatile organic compounds associated with vertebrate hosts are detected by the peripheral olfactory system of the mosquito, which comprise the antennae, maxillary palps, and labellum [4, 18]. Odorants enter via pores in the hair-like sensilla on these olfactory organs, and are transported through the aqueous sensillum lymph by soluble proteins, including odorant binding proteins (OBPs) and chemosensory proteins (Csps), to the chemosensory receptors on the olfactory sensory neuron dendrites [19–21]. The chemosensory receptors include odorant receptors (Ors), ionotropic receptors (Irs), and gustatory receptors (Grs), with other membrane-bound proteins, such as sensory neuron membrane proteins (SNMPs), also being involved in signal transduction [4, 19, 20]. Both Ors and Irs are heteromeric proteins constituted of a coreceptor, orco, as well as Ir8a, Ir25a, and Ir76b, respectively [19–21]. The Or pathway is sufficient for eliciting host seeking, while both the Or and Ir pathways play a role in host discrimination [22–24]. While the majority of Grs are involved in taste, a subset of Grs is associated with the detection of CO_2 required for activation and attraction of host-seeking mosquitoes [4, 25–27]. Differential expression of chemosensory genes in the peripheral olfactory system, predominantly *Ors*, and functional characterization of sequence variants, in anthropophillic and zoophilic mosquito subspecies and species, furthermore demonstrate a correlation with host preference [7, 8, 28]. While a genome-wide association study identified select Ors correlating with host preference in the two *Cx. pipiens* biotypes [12], there is a lack of studies linking the host preference phenotype with differential chemosensory gene expression in the peripheral olfactory system of Pipiens and Molestus.

The aim of this study was to identify differential expression of chemosensory genes correlating with host preference of Pipiens and Molestus. For this purpose, a two-choice assay was used to assess a consistent host preference of the two biotypes to either synthetic chicken or human odor blends [15, 24]. An antennal transcriptome was obtained from mosquitoes that displayed a consistent preference, to identify differentially expressed genes. The identification of differentially expressed chemosensory genes may provide an insight into the molecular mechanisms regulating the host preference of *Cx. pipiens* biotypes.

Methods

Mosquito rearing

Eggs of Pipiens and Molestus were provided in October 2021 by Prof. Sander Koenraadt (Wageningen University, Netherlands), and were reared from colonies established at Wageningen University in 2016, from field collected mosquitoes [29]. Larvae and adult Pipiens and Molestus were reared at 27 ± 2 °C, 65 ± 2% relative humidity with a 12 h light: 12 h dark photoperiod, with the light: dark cycle chosen to mimic the natural light conditions in Europe at times of the year with the highest incidence of West Nile virus transmission (August–September) [30]. Moreover, these rearing conditions have been used in previous experiments aimed at assessing the odor-mediated response of Molestus [15]. Eggs of each biotype were placed in plastic trays (23.5 cm × 18 cm × 7.5 cm)

filled with 1 L tap water and fed with Tetramin® fish food (Tetramin, Blacksburg, Germany), with approximately 150–200 larvae per tray. Pupae were collected in water-filled 30 mL plastic cups and placed in Bugdorm-4E1515 cages (17.5 cm × 17.5 cm × 17.5 cm, Megaview Science Co., Taichung, Taiwan) with ad libitum access to 10% sucrose in glass vials with filter paper wicks. Molestus were maintained on a sucrose diet alone, whereas female Pipiens females were provided defibrinated cow blood (Håtunalab, Bro, Sweden) via a Hemotek membrane feeding system (Hemotek Ltd, Blackburn, UK). Molestus adults were allowed to complete their first gonotrophic cycle (4–5 days post-emergence (dpe)), and were tested at peak host-seeking conditions (8 dpe) in accordance with Spanoudis et al. [15]. Pipiens adults used for experiments were not given access to a blood meal and were tested at 4 dpe, concordant with the activity period of Molestus. All mosquitoes were starved with access to water 24 h prior to experimentation and tested at peak host-seeking time (Zeitgeber time 15 ± 2 h) [15].

Behavioral analysis

A Y-tube olfactometer [15, 24] (120 cm × 10 cm), illuminated from above with red light at 40 lx, was used to assess the host seeking of the two biotypes. Air was passed through a charcoal filter and humidified before entering the olfactometer at 0.3 m s^{-1}, with room conditions mimicking rearing conditions (27 ± 2 °C, 70 ± 5% relative humidity). Synthetic host odor blends for human and chicken were made as previously described [15, 24] (Supplementary Table S1). The stock concentration of the odor blends was diluted in pentane (≥ 95%, Carlo Erba Reagents, Emmendingen, Germany) and released by diffusion from wick dispensers to control for a consistent release of all components of the odor blend throughout the behavioral assay [15, 24]. The wick dispensers were placed in glass wash bottles (250 mL; Lenz Laborglas, Wertheim, Germany) and the odors or solvent control were delivered into the upwind end of either arm of the olfactometer via Teflon™ tubing.

Groups of five mosquitoes were placed in cylindrical release cages (10 cm × 10 cm) for 2 h to acclimatize to room conditions. The release cages were then placed downwind of the olfactometer, and the mosquitoes were allowed 5 min to acclimatize, before the odor blend(s) and/or solvent control were introduced into the upwind ends of either arm of the olfactometer. The door of the release cages was opened, and the mosquitoes were given 5 min to make a choice between the two arms. Mosquitoes that did not leave the release cage or that remained within the downwind tube prior to the arms were considered nonresponding, and were excluded from further analyses. A preference index, calculated by $(T - C)/(T + C)$, where T is the number of mosquitoes responding to the test odor, i.e., chicken odor for Pipiens and human odor for Molestus, and C is the number of mosquitoes responding to the other odor tested, was used to determine the host preference of the mosquitoes.

To test differences in host preference of the two biotypes, three assays were conducted using the Y-tube olfactometer. Initially, two pilot experiments were conducted to (a) identify the dose-dependent response to the host odor blends versus a pentane control, and (b) to identify the dose-dependent preference to either odor blend. The purpose of the latter experiment was to identify a dose at which the two biotypes displayed a clear differential host preference. The main experiment (c) was designed to assess whether the biotypes demonstrate a consistency in host preference over time. For this, mosquitoes were provided with a choice between the chicken and human odor blend, using a dose (10^{-5}) that elicited a clear differential host response in (b). Pipiens and Molestus that demonstrated a preference to either chicken or human odor, respectively, were collected into Bugdorm cages with ad libitum access to water, and then the assay was repeated 24 h later. Mosquitoes that made a consistent choice over the 2 days were used for subsequent antennal transcriptomic analyses. Three replicates of individuals that displayed a consistency in preference, including mosquitoes of different cohorts for both biotypes, were conducted to obtain 50 individuals for each replicate, which were then subjected to tissue dissection.

Tissue dissection and RNA extraction

The antennae of cold-anesthetized adult females were collected using sterilized forceps, immediately (< 2 h) after the behavioral assays were completed, and rapidly transferred into RNAlater® (Thermo Fisher Scientific, Stockholm, Sweden), stored at room temperature overnight, and then stored at −20 °C until RNA extraction. Four biological replicates of 50 pairs of antennae per biotype were generated. For RNA extraction, RNAlater was removed and the antennal tissue was disrupted and homogenized using a power pestle with a disposable RNAse-free plastic pestle. Total RNA extraction and DNAse digestion were performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and then stored at −80 °C. Prior to sequencing, the RNA quality and quantity were analyzed using a TapeStation system 1200 (Agilent Technologies, Stockholm, Sweden).

Sequencing and RNA-seq analysis

The eight total antennal RNA samples were shipped on dry ice to Eurofins Genomics (Ebersberg, Germany). Of the eight replicates sent for sequencing, six replicates

met the total RNA-seq requirements from Eurofins (three each from Molestus and Pipiens). Sample libraries were constructed using the INVIEW Transcriptome Ultra Low workflow (Eurofins Genomics), which generated paired-end reads of 2 × 150 bp coverage with a depth of 20 Mb. Raw read data was cleaned and trimmed to remove adaptors, and sequences with a Phred score ≤ 20 were discarded using CLC Genomics Workbench (http://www.clcbio.com, version 23.0.5; Qiagen, Vedbæk, DK). Cleaned sequences were mapped to the *Cx. quinquefasciatus* reference genome from VectorBase (*Culex quinquefasciatus* JHB2020, VectorBase rel. 66, 28-NOV-2023) (Supplementary File S1). Owing to discrepancies between the new (JHB 2020) and previous reference genome (Johannesburg) [31], the annotations for the chemosensory gene families in the previous genome were correlated with that in the most recent genome (Supplementary Table S2).

Differential gene expression analysis

All RNA-seq analyses were performed using CLC Genomics WorkBench. To visualize differential antennal transcript abundance between the two biotypes, the trimmed mean of the M value (TMM) adjusted counts per million, i.e., TPM, for each replicate were calculated. The threshold of 0.6 TPM was chosen following the rationale that this is a reasonable approximation of thresholds used with other normalization methods (i.e., 1 RPKM; 1 FPKM). A gene ontology (GO) analysis was performed on transcripts to confirm the expected expression of functional gene ontologies in the antennae of Pipiens and Molestus, and to observe differences in gene expression between the biotypes associated with host preference, similar to other antennal transcriptomic studies [32, 33]. The GO analyses were performed on transcripts showing significant expression in the transcript libraries, as well as on the genes that were differentially expressed between the two biotypes (FDR $P \leq 0.05$, fold change ≥ 1.5), using the Vectorbase reference genome annotations stated above. Heat maps were generated by comparing the Log_{10} average TPM for the library of each biotype alongside the FC to compare expression between the biotypes.

Statistical analyses

The behavioral response to the synthetic chicken and human odor blends was analyzed using a beta binomial model followed by Tukey's multiple comparisons post-hoc test with R software (version 4.3.1) using the packages "readxl," "emmeans," and "car." A beta binomial model was chosen to account for the group of mosquitoes being flown per replicate, using the following formulae:

$$\begin{aligned} \text{Yij} &= \text{BetaBin}(\mu\text{i}) \\ \text{logit}(\mu\text{i}) &= \alpha\text{i} \end{aligned}$$

,in which i denotes the blend, with $i = 1$ being the blend the moquitoes choose, and $i = 2$ being the other blend, j denotes the jth replicate in treatment i, μ_i denotes the mean of treatment i, α_i denotes the logit-transformed mean, and Y_{ij} is the success probability. The multiday assays of the biotypes were analyzed using a one-way analysis of variance (ANOVA) to determine the difference in response of mosquitoes over the 2 days of testing. The FDR P-values were determined in CLC Genomics Workbench [34].

Results

Behavior

When assayed in pilot Y-tube olfactometer assays (Fig. 1a), female host-seeking Pipiens and Molestus demonstrated a differential dose-dependent behavioral response when presented with a choice between either the synthetic chicken or human odor blends and a solvent control (Supplementary Fig. S1a and b), as well as between the two blends (Supplementary Fig. S1c). Pipiens and Molestus responded to lower doses of the synthetic chicken and human odor blends, when compared with the other biotype, respectively (N = 7–8, $n = 50$), when choosing between either of the odor blends versus the solvent control (Supplementary Fig. S1a and b). For the two-choice experiments, 982 Pipiens and 886 Molestus were tested, with 773 (79%) Pipiens and 759 (86%) Molestus responding to any of the two odor blends. In a choice between the two odor blends (Supplementary Fig. S1c), Pipiens showed a preference to the chicken odor blend at lower doses, which shifted to the human odor blend at the highest dose tested ($F = 3.07$, $df = 31$, $P = 0.04$). In contrast, Molestus showed a dose-dependent preference to the human odor blend. To assess the consistency in preference over time, the two-choice behavioral assay was repeated using the dose eliciting a clear differential host preference in both biotypes. Both biotypes maintained a similar ratio of host preference over 2 days, with Pipiens and Molestus significantly preferring the synthetic chicken ($z = 4.832$, $P < 0.001$) and human ($z = -5.992$, $P < 0.001$) odor blends, respectively (Fig. 1b). Females that demonstrated consistent host odor preference were subsequently used for tissue collection.

RNA sequencing

Expression profiling of antennal total RNA from the six libraries (three each from Molestus and Pipiens), constructed from paired-end reads of 2 × 150 bp coverage with a depth of 20 Mb, showed a similar average level of reliably expressed genes of 10,067 and 10,166

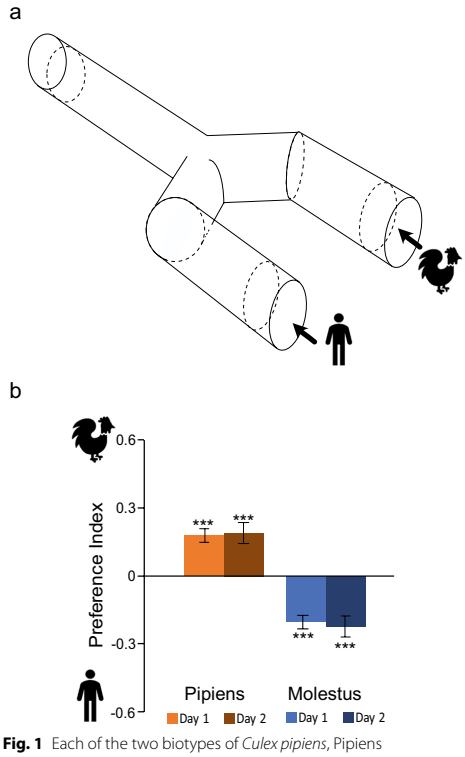


Fig. 1 Each of the two biotypes of *Culex pipiens*, Pipiens and Molestus, demonstrate a low but consistent preference to synthetic chicken and human odor blends, respectively. **a** Diagram of the Y-tube olfactometer used to assess host preference of the biotypes. **b** Consistency in host preference for each biotype was assessed over 2 days, by the response to synthetic odor blends (dose = 10^{-5}). Error bars represent the standard error of proportions, with asterisks denoting statistical difference from 0 ($P < 0.0001$) for each day

(transcripts per kilobase million (TPM) > 0.6) in Molestus and Pipiens, respectively (Supplementary File S2). A core eukaryotic gene (CEG) analysis identified 353 and 352 out of the 361 CEG genes [35] to be reliably expressed (TPM > 0.6) in Pipiens and Molestus, respectively (Supplementary File S2), demonstrating sufficient sequencing depth and coverage of the sample libraries. A principal component analysis among the antennal transcriptomes of the six libraries demonstrated differential expression of antennal genes between the two biotypes along principal component 1, accounting for 29.3% of the variation (Fig. 2a).

Gene ontology analysis

The gene ontology (GO) slim terms of genes, related to their molecular function, present at background levels (Fig. 2b and c) and of the genes that were reliably expressed in the antennae (Fig. 2d and e) (TPM > 0.6) of Pipiens and Molestus identified few differences. The GO slim terms of the most abundant genes in all comparisons were ion binding (GO:0043167), RNA binding (GO:0003723), and ATP-dependent activity (GO:0140657). The only significant difference in GO slim terms between the two biotypes was the number of genes in the molecular function category hydrolase activity, acting on carbon–nitrogen (but not peptide bonds) (GO:0016810) in the Pipiens library, which represented 3% of the total number of identified genes (data not shown). The most frequent GO terms of differentially expressed genes between the two biotypes (genes with an absolute fold change ≥ 1.5 and a threshold false discovery rate *P*-value of ≤ 0.05) were catalytic activity (GO:0003824), hydrolase activity (GO:0016787), and catalytic activity, acting on a protein (GO:0140096) (Fig. 2f). Odorant binding (GO:0005549) represented 5% of the differentially expressed genes between the two biotypes, and these genes were selected for further expression analysis.

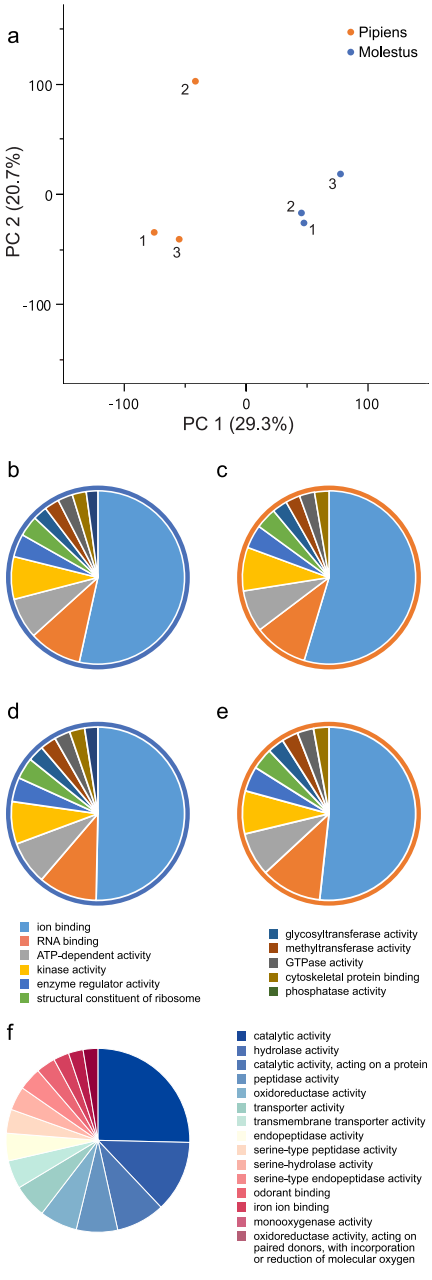
Differential expression of chemosensory genes

Odorant receptors

Of the 156 annotated *Or*s obtained from the reference genome (*Culex quinquefasciatus* JHB2020, VectorBase rel. 66, 28-NOV-2023), 104 and 105 *Or*s were reliably expressed in Pipiens and Molestus, respectively, with the odorant coreceptor *Orco* (CQUJHB017442) being highly expressed in both biotypes (Supplementary File S3). Among the reliably expressed *Or*s, nine were differentially expressed (TPM > 0.6 and fold change > 1.5 or < −1.5), with three: *Or18*, *Or64*, and *Or205* demonstrating higher transcript abundance in Molestus than Pipiens (Fig. 3a). Of the remaining six *Or*s, three, *Or2*, *Or192*, and *Or195*, had higher transcript abundance in Pipiens than Molestus, while *Or65*, *Or152*, and *Or177* were exclusively expressed in Pipiens (Fig. 3a).

Ionotropic receptors and other transmembrane chemosensory proteins

Of the 160 annotated *Ir*s obtained from the reference genome, 34 and 40 *Ir*s were reliably expressed in Pipiens and Molestus, respectively, with the *Ir* coreceptors, *Ir8a* (CQUJHB009988), *Ir25a*, and *Ir76b*, being highly expressed in both biotypes (Supplementary File S3). *Ir76b* was the only coreceptor that showed higher abundance in Pipiens than Molestus (Fig. 3a). Out of the variable



◀ **Fig. 2** Gene expression and function differ between antennal libraries of host-seeking Pipiens and Molestus. **a** Principal component analysis of the six sample libraries, collected from antennal tissue of the Pipiens and Molestus females that displayed consistent host preference to synthetic chicken and human odor blends, respectively, revealed a clear separation of biotypes along principal component (PC) 1. **b–e** Gene ontology (GO) analysis, using GO slim terms of the genes identified in the transcriptome analysis, demonstrated differences in function of genes in the antennae compared with the background (**b** and **c**) and of genes showing reliable expression (**d** and **e**) in the antennae of Molestus and Pipiens, respectively. Charts with blue borders (**b** and **d**) refer to Molestus, while charts with orange borders refer to Pipiens (**c** and **e**). **f** Gene ontology terms, which were identified when comparing differentially expressed genes between biotypes. The legends (**b–f**) indicate terms representing ≥ 5% of the total expressed transcripts for all comparisons

tuning *Ir*s, two, *Ir75l* and *Ir100b*, had a higher abundance in Molestus than Pipiens (Fig. 3a). Of the 53 annotated *Gr*s, six and ten were reliably expressed in Pipiens and Molestus, respectively, with no *Gr*s being differentially expressed between the two biotypes (Supplementary File S3). Both annotated *SNMP*s were reliably expressed in Pipiens and Molestus (Supplementary File S3), with *SNMP2,* transcript variant X1 (CQUJHB014288) being more abundant in Molestus than in Pipiens (Fig. 3a).

Soluble odorant binding proteins

Of the 88 annotated *OBPs*, 36 and 44 were reliably expressed in Pipiens and Molestus, respectively (Supplementary File S3). A total of 12 *OBP*s were differentially expressed between the biotypes, with three, *OBP66*, CQUJHB012200 and CQUJHB017566, having a higher abundance in Molestus than Pipiens, and *OBP19d* and *OBP28a* being exclusively expressed in Molestus (Fig. 3b). The remaining seven *OBP*s exhibited a higher abundance in Pipiens than in Molestus (Fig. 3b). Of the 22 annotated *Csp*s, 13 and 7 were reliably expressed in Pipiens and Molestus, respectively, with *Csp2* and *Csp5* being more abundant in Molestus than in Pipiens, whereas *Csp13* was more abundant in Pipiens than in Molestus (Fig. 3b).

Discussion

The two biotypes of *Cx. pipiens* demonstrated a preference for either humans or birds [this study, 10, 12], albeit more variable compared with highly anthropophillic and zoophilic mosquito species [5–9]. An antennal transcriptome created from the *Cx. pipiens* biotypes, demonstrating a consistent host preference, identified differentially regulated chemosensory genes, encoding Ors, Irs and OBPs that confer sensitivity and selectivity to host VOCs, and mediate host seeking and discrimination in mosquitoes [this study, 7, 8]. These genes are targets for future

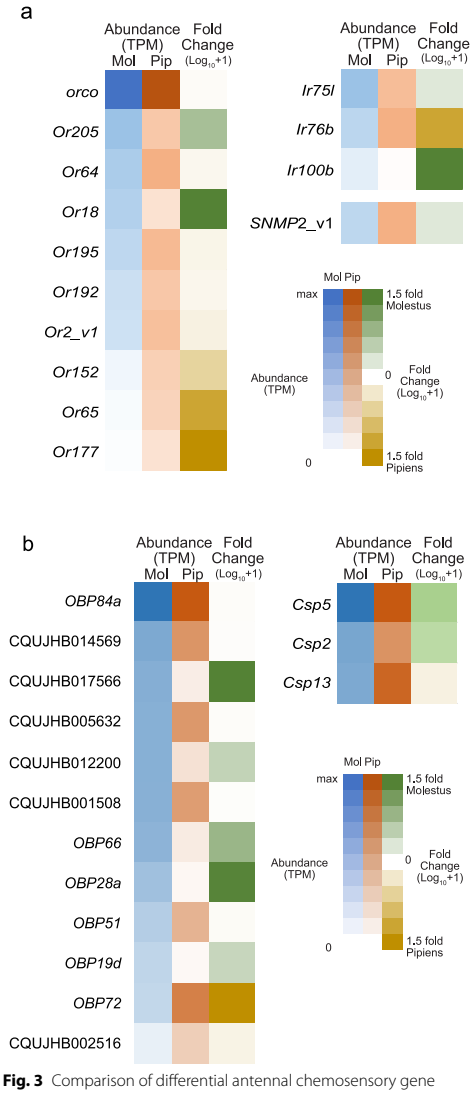


Fig. 3 Comparison of differential antennal chemosensory gene expression in Pipiens and Molestus correlated with divergent host preferences. Transcript abundance values represented in $Log_{10}+1$ scale for Pipiens (P) and Molestus (M) in orange and blue, respectively, with fold change (FC) representing a comparison between the two biotypes. Genes were labelled as per the reference genome (*Culex quinquefasciatus* JHB2020, VectorBase rel. 66, 2-NOV-2023), and if they were not annotated, the VectorBase gene IDs are stated. **a** Transcript abundance of odorant receptors (*Ors*), ionotropic receptors (*Irs*), and sensory neuron membrane proteins (*SNMPs*). **b** Transcript abundance for odorant binding proteins (*OBPs*) and chemosensory proteins (*Csps*)

functional characterization aimed at understanding the molecular mechanisms regulating host selection and discrimination of *Cx. pipiens*, which ultimately regulates vectorial capacity.

Pipiens and Molestus demonstrated an ornithophilic and anthropophilic preference, based on their response to the synthetic chicken and human odor blends, respectively, similar to that found in previous studies [10–12]. In a two-choice assay, both biotypes demonstrated a low, however consistent, preference to either host odor, with lower anthropophillic preference of Molestus than previously described [11]. When compared with the highly anthropophillic *Ae. aegypti* and *An. gambiae* [5–7, 36], and the highly zoophilic *Anopheles quadriannulatus* [5, 37], the host preference of Pipiens and Molestus was lower, implying that both biotypes are opportunistic although biased to either humans or birds, which is reflected in blood meal analysis of field caught mosquitoes throughout Europe [13]. Whether the consistently low preference to the synthetic odor blends of the biotypes reflects the low ability of *Culex* mosquitoes to learn odors, compared with strongly anthropophilic species, correlated with differential dopaminergic innervation of the primary olfactory center, the antennal lobe [38], remains to be confirmed. Available data, however, emphasize that Pipiens and Molestus are attracted to, and discriminate between, host odors [this study, 10, 12, 15], which is likely linked to differential expression and sequence variants of chemosensory genes in the primary olfactory organ, the antenna [7, 8]. From an evolutionary perspective, and for closely related taxa, such changes may affect the tuning of OSNs, resulting in a change in preference at a low cost [39, 40].

The Or and Ir pathways mediate host seeking (Ors) and discrimination (Ors and Irs) in mosquitoes [22–24], with other membrane-bound and soluble chemosensory proteins regulating selectivity and sensitivity of the olfactory system [19–21]. In host seeking *Cx. pipiens*, which demonstrated a consistent host preference, three Ors each were more abundant in the antennal transcriptome of Pipiens and Molestus, while three Ors were exclusively expressed in Pipiens, providing a potential molecular mechanism regulating discrimination between humans and birds, similar to that proposed for the anthropophillic and zoophilic subspecies of *Ae. Aegypti* [7]. While the majority of these Ors have not been functionally characterized, Or2 has been shown to bind to indole, 4-methyl phenol, and benzaldehyde, compounds found in human body emanations, but also released by a wide variety of organisms, mainly bacteria, and proposed to mediate host- and oviposition site-seeking [3, 9, 24, 41, 42]. Out of the potential members among the Irs regulating host discrimination, the coreceptor Ir76b and two tuning

Irs, were differentially expressed in the two biotypes. Of these, Ir76b and Ir75l mediate responses to carboxylic acids and/or amines, both classes of which are present in human and avian odors [43–46]. *Ir76b* mutant *Ae. aegypti* display a decreased sensitivity and attraction to human odor, while retaining the ability to discriminate between humans, emphasizing a key role of the Or pathway in host discrimination [46]. While the role of OBPs and Csps in regulating mosquito behavior is currently unclear, their role in odorant transport, receptor interaction, and gain control [22], and the differential expression of predominantly OBPs, suggests that these soluble proteins may regulate sensitivity to (select) host VOCs. In summary, the observed differential expression of chemosensory genes provides targets for further functional characterization aimed at understanding the molecular mechanism(s) regulating host preference in *Cx. pipiens* biotypes.

Conclusions

This study supports the host preference of Pipiens and Molestus, and demonstrated that this preference is innate and not individualistic under laboratory conditions. The transcriptome analyses of expressed antennal genes, including chemosensory genes, in phenotyped female mosquitoes, identified possible molecular mechanisms regulating host preference in the two biotypes. Future research will determine the function of these genes and how they regulate host preference in *Cx. pipiens*, as well as their implication for speciation.

Abbreviations

VOCs	Volatile organic compounds
OBPs	Odorant binding proteins
Csps	Chemosensory proteins
Ors	Odorant receptors
Irs	Ionotropic receptors
Grs	Gustatory receptors
SNMPs	Sensory neuron membrane proteins
TPM	Transcripts per million
GO	Gene ontology

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-025-07028-y.

Supplementary material 1. Supplementary Table S1. Compounds used for the formulation of the synthetic human and chicken odor blends. Both odor blends used pentane as a solvent. All compounds were obtained from Merck, with purity ranging from 90-99%. Phenol and α-terpineol were obtained as solids, with the amount listed in µg. Supplementary Table S2. List of differentially expressed chemosensory genes identified in the transcriptome analysis, with the corresponding VectorBase gene IDs for the reference genome (*Culex quinquefasciatus* JHB2020, VectorBase rel. 66, 28-NOV-2023) and the corresponding gene IDs from the previous reference genome (*Culex quinquefasciatus* Johannesburg, VectorBase rel. 66)

Supplementary material 2. Supplementary Figure S1. Differential responses of Pipiens and Molestus to the synthetic odor blends and solvent control in a Y-tube olfactometer. Error bars show standard error of proportions, and the preference index (PI) was calculated as PI = (*T* − *C*)/(*T* + *C*) where T is the number of mosquitoes responding to the odor blend and C is the number of mosquitoes responding to the solvent control. Mosquito responses to the synthetic human odor blend (**a**) and chicken odor (**b**) against a solvent control (pentane) showed higher sensitivity to the human and chicken odor blends for Molestus and Pipiens, respectively. **c** Dose-dependent responses of Pipiens and Molestus when presented with a choice between the synthetic odors. N= 7 − 8, *n* = 50 for each assay

Supplementary material 3. Supplementary File S1. Total number of reads, mapped reads (%), transcripts and reliably expressed transcripts (>0.6 TPM) of antennal transcriptomes, obtained from behaviorally phenotyped Pipiens and Molestus females

Supplementary material 4. Supplementary File S2. Gene expression data from antennal transcripts of Pipiens and Molestus showing differential host preference. Sheets include all antennal transcripts (Sheet – Antennal genes), genes used for CEG analysis (Sheet – CEG genes), as well as all chemosensory gene families (Sheets – *Ors*, *Irs*, *Grs*, *SNMPs*, *OBPs*, *Csps*). Gene names and VectorBase gene IDs are provided as per the *Culex quinquefasciatus* reference genome (*Culex quinquefasciatus* JHB2020, VectorBase rel. 66, 28-NOV-2023). All expression values are expressed in transcripts per million (TPM) and are color coded for Pipiens (orange) and Molestus (blue). Fold change and false discovery rate (FDR) *P*-values are provided. For the chemosensory genes, heat maps showing expression differences between the two biotypes are provided

Supplementary material 5. Supplementary File S3. Gene expression data from the differentially expressed chemosensory genes of Pipiens and Molestus that showed differential host preference. Gene names, as well as VectorBase gene annotations and IDs, are provided for each gene family. All expression values are expressed in transcripts per million (TPM) and are color coded for Pipiens (orange) or Molestus (blue). Fold change and false discovery rate (FDR) *P*-values are provided. For the chemosensory genes, heat maps showing expression differences between the two biotypes are provided

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Author contributions

R.M.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing—original draft, review and editing. R.I.: conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, resources, supervision, validation, visualization, writing—review and editing. S.R.H.: conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, resources, supervision, validation, visualization, writing—review and editing.

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Data availability

All raw sequence data obtained by RNA-seq analysis were deposited in the National Center for Biotechnological Information (NCBI) database under BioProject accession number PRJNA1197149. RNA-seq reads for Molestus 1-3 and Pipiens 1-3 samples are found under the BioSample accession numbers SAMN45774340, SAMN45774341, SAMN45774342, SAMN45774343, SAMN45774344, and SAMN45774345, respectively, in the NCBI database.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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The two biotypes of *Culex pipiens* display differences in host preference. By combining behavioural assays, population-level ecological assays and the functional characterization of odorant receptors correlated with the observed behaviour, the studies in this thesis propose a genetic mechanism that regulates this differential host preference.

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