The Role of the Skin Microbiome and Host Genetics in Human Attractiveness to Mosquitoes

Alicia Showering

Thesis submitted in accordance with the requirements for the degree of
Doctor of Philosophy

of the

University of London

August 2023

Department of Disease Control

Faculty of Infectious and Tropical Diseases

LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

Funded by The Medical Research Council

Research group affiliations:

LSHTM Global Health Analytics

Institute of Medical and Biomedical Education, SGUL

LSHTM Logan Group

I, Alicia Showering, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: **Date: 28/12/2023**

I) Abstract

Mosquito-borne diseases like malaria and dengue are responsible for over 700,000 deaths each year. Some people produce specific body odours that make them more attractive than others to mosquitoes and consequently are at higher risk of contracting vector-borne diseases. The differential attractiveness of individuals to mosquitoes have been attributed to the differences in the skin microbiome, body odour and genetics. However, the relative contributions of the skin microbiome, body odour and human genetics to attractiveness to mosquitoes are poorly understood. To date, no studies have gone beyond exploration of the role of the skin microbiome in human attractiveness to mosquitoes.

In this thesis, the contribution of human genetics, the skin microbiome and body odour profile are investigated using twin cohorts from the UK and The Gambia to understand their effects on attractiveness to *Anopheles coluzzii* mosquitoes. The results revealed distinct skin microbiome compositions between individuals with varying levels of attractiveness. However, there were no discernible differences in their volatile odour profiles. My findings suggest that variation in the attractiveness of people to mosquitoes is related to the composition of the skin microbiota, knowledge that could improve odour-based vector control methods.

Additionally, the research extended to analysing the skin microbiome's impact on attractiveness to *Aedes aegypti* mosquitoes in another UK cohort. Differences in microbiome composition were confirmed between groups with high and low mosquito attractiveness. Attempts to transfer skin microbiomes from individuals less attractive to mosquitoes to those more attractive showed suggestive statistical evidence for a trend towards decreased mosquito attraction post-transfer.

II) Acknowledgements

I am immensely grateful to my advisory committee for their support and encouragement these past few years, despite the challenges posed by the global pandemic, field and lab disruptions, and most of my advisors relocating to different countries! A big thank you to Ernest and Julien for the patient explanations and prompt email responses, especially during the initial stages when I had little idea what I had got myself into. To Robert for his continued time, meticulous feedback and general support.

I would like to thank James and Rachel for allowing me to work independently from day one. They allowed me to pursue most of my ideas, apply for grants to attend training and conferences worldwide (when COVID restrictions eventually permitted), to gain teaching, project management and supervision experience. I owe the most enormous thanks to Chrissy and Bert, without whom this thesis would not have come to fruition. Thank you for taking me on for the final year. Your support and perspective have put this thesis in much better shape.

Thank you to the GenoScent collaborators, especially John Pickett, for his insightful discussions and Salvador for the stats calls. To all the participants who gave up their time to provide samples, putting your foot in an oven bag for 2 hours is not particularly pleasant! In the field, I would like to thank all the families who welcomed us into their homes. I also appreciate the MRC field team, especially Amadou, Babucarr, Abdul, and Mamadou at MRCG.

I am deeply grateful to all the ex-Logan group members, particularly Freddy, who has been a sounding board for the last couple of years when things got rough. Thanks to Scott, the best days in the lab were spent with you. I would also like to thank Laura R, Laura T, Ailie, Liz, Grace, Robert and Catherine for their chats and encouragement when it was needed.

I sincerely thank all the other lab groups that allowed me to use their labs and equipment, especially Susana and Martin. I will always be thankful to James A, Dan and Matt for their constant enthusiasm and invaluable support, especially when troubleshooting my numerous failed PCRs. Without you, I would not have finished in the laboratory, I owe you many drinks. Thanks to Shahida, Pat and Cheryl for the entomology guidance and advice (even on a Sunday night) it was much less lonely rearing mosquitoes knowing you were around!

Thanks to my fellow PhD students, ECRs, PHEG and malaria centre members at LSHTM. While my PhD journey was filled with challenges and a fair few frustrations, your unwavering support and encouragement have got me to this point. Many of you have become close friends.

Thanks to those that have read and suggested edits on parts of this thesis: Chrissy, Bert, Matt, James, Ernest, Julien, Mojca and Taff, but also to Emily, Hattie, Ade and Earl for checking for typos and helping me with the ordering. It reads a lot better, thanks to your suggestions (and patience).

I extend my appreciation to Mr Loynton and Professor Alan McCarthy for their belief in my potential. Mr Loynton ignited my passion for science in Year 8 with his enthusiasm, and Professor McCarthy gave me the confidence that pursuing a PhD was within my reach. Thanks to MRC LID for selecting me as an inexperienced undergraduate who believed they were ready to take on a multi-disciplinary project like this. I am thankful for MRCs support in funding my master's, PhD, training courses, competitions, and conferences, all of which would not have been possible without their financial assistance. A special thanks to Lara for being a star at MRC LID/registry and for many years of support.

Thanks to the innovation teams I have been part of, particularly YES20 (Emma, Stas, and Rhodri). I am also thankful to all at Bactobio, especially Carrie, Sibyl, Mark and Andre, for providing a placement that allowed me to dive into various projects and experience the world of science beyond academia.

Lastly, I want to thank my family and friends who have supported me throughout this journey. The biggest thank you to my housemates, both past and present. Particularly to Katey and Will, who always check up on me and have put up with me being miserable the last few months. To my friends in London (Emily, Hattie, Oscar, Becca, Ashley and Freddy), the north (Earl, Ade and Becca), Bristol (Amber, Belle, Elise, Brodie, and Alex) and Cambridge (Chris), who have all encouraged me and distracted me when necessary.

A final thanks to my parents, brother and Banksy, who have all kept me grounded in their own ways. I promise to finally get a job soon!

III) COVID-19 Impact Statement

The COVID-19 pandemic severely disrupted my primarily laboratory and field-based project from March 2020 to December 2020. My PhD project relied heavily on primary data collection and laboratory work in The Gambia. All sample collection was suspended in The Gambia on March 31, 2020, as the MRC unit focused on COVID-19 projects only. I had planned to travel back to The Gambia in April 2020 to support the field team in sample collection and to extract my first batch of Gambian microbiome swabs using the superior facilities at MRC Gambia. Unfortunately, the trip was cancelled due to the first lockdown. Although the field team was able to resume sample collection on June 15, 2020, it was slower than usual due to COVID-19 safety measures and disruptions when some of our team members contracted the virus. I was still unable to visit due to the Gambian borders remaining closed. To mitigate delays, we arranged for the transportation of materials necessary to continue collecting samples and how fragile glass samples could be transported back to LSHTM for processing. We transported the volatile, sock and microbiome samples back to LSHTM in autumn 2020.

Moreover, molecular laboratory work at LSHTM was halted when the labs closed on March 27th 2020. This work was not completed until January 2021 due to restrictions on laboratory access, which made it challenging to access equipment belonging to other groups and delays on laboratory consumables as companies shifted focus to producing COVID-related supplies. Furthermore, the mosquito colony required for processing the samples for behavioural work was destroyed before the closure of the LSHTM labs. We had to rear enough mosquitoes from stored eggs to perform behavioural assays on the samples from The Gambia. This delay resulted in the behavioural experiments on attractiveness starting in January 2021, seven months later than planned. Due to NHS COVID-19 delays, a more extensive operation was required to re-break and straighten my leg in August 2021, leading to an additional three-month delay in lab work.

Despite the disruption, I have completed all work that was originally planned for my PhD and additional skin microbiome transfer experiments by adapting to mitigate delays and self-teaching where possible. Transporting samples back to the UK, borrowing equipment from other groups and my perseverance in getting hold of consumables allowed the planned work to be conducted.

IV) Contents

V) List of tables

List Of Tables

VI) List of figures

List Of Images

the dispersion around the group means. The first component explains 10 % variance, and the second component 5 % variance, summing to a cumulative variance of 15 %. Additionally, density plots are placed above and to the right of the loading plot presenting the distribution of the scores for the first and second components for each group. These provide a view of the spread of the data for each component within the donor and recipient groups.

- Figure 51. Bacterial genera with the greatest contribution to differences in microbiome composition between unattractive and attractive groups of relative attractiveness in the UK cohort on component 1 of the sPLS-DA. The loading plot represents the bacterial genera contributing the most to differences between the attractiveness group on component 1 (10 ASVs) of the sPLS-DA. Bars represent the loading weights or correlation coefficients of each bacterial genus to the components of the sPLS-DA. The direction of the bars (left or right) relates to the direction of the loadings in Figure 50. Dark blue and green bars indicate a higher abundance in the unattractive and attractive groups, respectively.
- Figure 52. Bacterial genera with the greatest contribution to differences in microbiome composition between unattractive and attractive groups of relative attractiveness in the UK cohort on component 2 of the sPLS-DA. The loading plot represents the bacterial genera contributing the most to differences between the attractiveness group on component 2 (50 ASVs) of the sPLS-DA. Bars represent the loading weights or correlation coefficients of each bacterial genus to the components of the sPLS-DA. The direction of the bars (left or right) relates to the direction of the loadings in Figure 50. Dark blue and green bars indicate a higher abundance in the unattractive and attractive groups, respectively.
- Figure 53. ROC curve for UK sPLS-DA for microbiome composition and category of attractiveness (unattractive vs attractive relative attractiveness) to mosquito on A) The first component and B) All four components selected with tuning.
- Figure 54. DESeq2 differential abundance of ASVs between unattractive and attractive groups. Volcano plot of ASVs (black dots) showing differential abundance between the relative attractiveness groups. DESeq2 was used to calculate log 2-fold changes, i.e. if bacterial ASVs are more or less abundant in the unattractive compared to the attractive group. The x-axis represents log 2-fold change abundance in the unattractive compared to attractive groups, with the biggest changes furthest from the centre. Y axis indicates the negative log-10 transform of the nominal p-value, i.e. increasing significance away from the origin. The red line represents P = 0.05 for exploratory purposes. There are 57 ASVs above the red line without adjustment for multiple testing.
- Figure 55. ASVs that were significantly differential abundant at an FDR cut-off of 0.05 between unattractive and attractive relative attractiveness groups in the UK cohort.
- Figure 56. Boxplot comparing similarities in the Shannon diversity per pair between MZ (red), DZ (blue) and unrelated (grey) pairs in the UK cohort.
- Figure 57. Summarises the taxonomy at the genus level as a taxa barplot of relative abundances for A) positive controls, B) negative controls and C) samples. There are clear differences in taxonomy between the samples and controls. Samples are a subset of the 20 most abundant taxa at the genera level,

NA is the less abundant taxa combined.

- Figure 58. Comparison analysis of the top ten most prevalent bacterial genera between unattractive (dark purple) and attractive (dark orange) participants in the Gambian cohort. The x-axis categorises the bacterial genera, ordered from least to most abundant from left to right. The y-axis illustrates the absolute abundance of each genus on a logarithmic (base 10) scale. Boxplot whiskers include the full range of genus abundances, represented as sequence variant (ASV) counts and displayed as log base 10 absolute abundance. Statistical differences in median abundances between the donor and recipient groups for each genus were assessed using a Wilcoxon test, with adjustments for multiple testing via the Benjamini-Hochberg method. The statistical significance is visualised by asterisks above each boxplot. The absence of an asterisk suggests no significant difference, while a single (*) and double (**) asterisk denote significant (adjusted p-value < 0.05) and highly significant (adjusted p-value < 0.005) differences, respectively.
- Figure 59. Comparison analysis of the top ten most prevalent bacterial genera relative abundance between unattractive (dark purple) and attractive (dark orange) participants in the Gambian cohort. The x-axis categorises the bacterial genera, ordered from least to most abundant from left to right. The y-axis illustrates the relative abundance of each genus on a logarithmic (base 10) scale. Boxplot whiskers include the full range of genus abundances, represented as sequence variant (ASV) counts and displayed as log base 10 relative abundance. Statistical differences in median abundances between the donor and recipient groups for each genus were assessed using a Wilcoxon test, with adjustments for multiple testing via the Benjamini-Hochberg method. The statistical significance is visualised by asterisks above each boxplot. The absence of an asterisk suggests no significant difference, while a single (*) and double (**) asterisk denote significant (adjusted p-value < 0.05) and highly significant (adjusted p-value < 0.005) differences, respectively.
- Figure 60. Comparison analysis of Shannon diversity between unattractive and attractive groups prior to transfers. The x-axis categories fall into two groups: unattractive (dark purple) and attractive (dark orange) in the Gambian cohort. The y-axis represents the Shannon diversity index. The boxplot displays the data distribution for each group, demonstrating the median and interquartile range and total range of the data. The individual data points overlaid as scatter represent the individual samples.
- Figure 61. Sample plot visualises differences between the groups from a sparse partial least squares discriminant analysis (sPLS-DA) implemented in the MixOmics package (Rohart et al. 2017). It compares skin microbial composition (beta diversity based on relative amounts of ASVs) between the unattractive (dark purple) and attractive (dark orange) in the Gambian cohort. Individuals are presented as small circles (unattractive) or small triangles (attractive). Microbiome data were pre-processed by filtering, CLR transformed and scaled (centred and standardised). The centroids, depicted as black stars, represent the average microbiome composition in the two-dimensional space on the first and second components of the analysis for each group. The enclosing ellipses (large circles) represent these groups' 95 % confidence intervals, visually estimating

the dispersion around the group means. The first component explains 6 % variance, and the second component 4 % variance, summing to a cumulative variance of 10 %. Additionally, density plots are placed above and to the right of the loading plot presenting the distribution of the scores for the first and second components for each group. These provide a view of the spread of the data for each component within the donor and recipient groups.

- Figure 62. Bacterial genera with the greatest contribution to differences in microbiome composition between unattractive and attractive groups of relative attractiveness in the Gambian cohort on component 1 of the sPLS-DA. The loading plot represents the bacterial genera contributing the most to differences between the attractiveness group on component 1 (50 ASVs) of the sPLS-DA. Bars represent the loading weights or correlation coefficients of each genus to the components of the sPLS-DA. The direction of the bars (left or right) relates to the direction of the loadings in Figure 61. Dark purple and dark orange bars indicate a higher abundance in the unattractive and attractive groups, respectively.
- Figure 63. Bacterial genera with the greatest contribution to differences in microbiome composition between unattractive and attractive groups of relative attractiveness in the Gambian cohort on component 2 of the sPLS-DA. The loading plot represents the bacterial genera contributing the most to differences between the attractiveness group on component 2 (10 ASVs) of the sPLS-DA. Bars represent the loading weights or correlation coefficients of each bacterial genus to the components of the sPLS-DA. The direction of the bars (left or right) relates to the direction of the loadings in Figure 61. Dark purple and dark orange bars indicate a higher abundance in the unattractive and attractive groups, respectively.
- Figure 64. ROC curve for sPLS-DA for microbiome composition of Gambia cohort and category of attractiveness (unattractive vs attractive absolute attractiveness) to mosquitoes on A) the first component and B) both components (selected by cross-validation).
- Figure 65. DESeq2 differential abundance of ASVs between unattractive and attractive groups. Volcano plot of ASVs (black dots) showing differential abundance between the relative attractiveness groups. DESeq2 was used to calculate log 2-fold changes, i.e. if bacterial ASVs are more or less abundant in the unattractive compared to the attractive group. The x-axis represents log 2-fold change abundance in the unattractive compared to attractive groups, with the biggest changes furthest from the centre. Y axis indicates the negative log-10 transform of the nominal p-value, i.e. increasing significance away from the origin. The red line represents P = 0.05 for exploratory purposes. There are 121 ASVs above the red line without adjustment for multiple testing.
- Figure 66. Boxplot comparing the Shannon diversity per pair between MZ (red), DZ (blue) and unrelated (grey) twins in the Gambia cohort.
- Figure 67. The library size for the samples (blue points) and the negative controls (red points) varied.
- Figure 68. Comparison of 20 samples randomly selected A) Pre-decontam and B) Post-decontam. Shows the top 20 genera relative abundances. Grey is used for the other taxa not in the 20 most abundant.

and scaled (centred and standardised). The centroids, depicted as black stars, represent the average microbiome composition in the two-dimensional space on the first and second components of the analysis for each group. The enclosing ellipses (large circles) represent the 95 % confidence intervals for these groups, providing a visual estimate of the dispersion around the group means. The first component explains 7 % variance, and the second component is 7 % variance, summing to a cumulative variance of 14 %. Additionally, density plots are placed above and to the right of the loading plot presenting the distribution of the scores for the first and second components for each group. These provide a view of the spread of the data for each component within the donor and recipient groups.

- Figure 116. Visualisation of the top 20 most abundant genera over time (colours) for each recipient. The figure combines 15 grouped bar plots, one per recipient. The plots are organised in rows, grouped by the donor the recipient received the transfer from. Each plot represents the relative abundance of the genera displayed on the y-axis at six-time points on the x-axis: the Donor before the transfer, Recipient before the transfer, immediately post-transfer (Recipient After_0), 6 hours post-transfer, 24 hours post-transfer and 72 hours post-transfer.
- Figure 117. Sample plot visualises differences between the groups from a sparse partial least squares discriminant analysis (sPLS-DA) implemented in the MixOmics package (Rohart et al. 2017). It compares skin microbial composition (beta diversity) between the donors before transfer (green), recipients before transfer (red) and recipients immediately after transfer (light grey). Individuals are presented as small circles (donors), small triangles (recipients after transfer) or small squares (recipients before transfer). Microbiome data were pre-processed by filtering, CLR transformed and scaled (centred and standardised). The centroids, depicted as black stars, represent the average microbiome composition in the two-dimensional space on the first and second components of the analysis for each group. The enclosing ellipses (large circles) represent the 95 % confidence intervals for these groups, providing a visual estimate of the dispersion around the group means. The first component explains 5 % variance, and the second component 4 % variance, summing to a cumulative variance of 9 %.
- Figure 118. Boxplots with scatter comparing the distribution of alpha diversity measured with Shannon between the recipients before (red) and after the transfer (grey).
- Figure 119. The library size for the samples (blue points), the negative controls (red points) and the positive controls (green points) varied.
- Figure 120. Summarises the taxonomy at the genus level as a taxa bar plot of relative abundances for a) positive controls, b) negative controls, and c) samples (ordered with donors on the left and recipients on the right by donor). There are clear differences in taxonomy between the samples and controls. Samples are a subset of the 20 most abundant taxa at the genera level, low_abundances is the less abundant taxa combined.
- Figure 121. Comparison of 20 samples randomly selected a) Pre-decontam and b) Post-decontam. Shows the top 20 genera relative abundances grey is used for the other taxa not in the 20 most abundant.

1) Chapter 1: Introduction

1.01) Statement of multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper	I researched the literature and wrote this entire chapter. Some sections were incorporated into a review on human attractiveness to mosquitoes, “Differential attraction in mosquito-human interactions and implications for disease control”, published in Philosophical Transactions (Martinez et al., 2020).

Student signature:

1.02) Vector-borne disease

Vector-borne diseases (VBDs) account for more than 17 % of all infectious diseases, resulting in over 700,000 deaths each year [1]. Arthropod vectors have a significant role in transmitting various pathogens. Mosquitoes are the deadliest vector of disease [2]. Anthropophilic female mosquitoes strongly prefer human odour making them important vectors of many human pathogens. The three main disease-carrying genera of mosquitoes are *Aedes*, *Anopheles* and *Culex*. *Aedes* mosquitoes transmit arboviruses, including Dengue virus (~96 million cases per year), Zika virus (~500,000 cases per year) and yellow fever virus (~130,000 cases each year). *Anopheles* transmit malaria parasites (~212 million cases each year), while *Culex* transmits Japanese encephalitis virus (~42,500 cases each year), lymphatic filariasis parasites (~38.5 million cases each year) and West Nile Virus (~2,600 cases each year) [3].

Approximately 80 % of the world's population is at risk of VBDs [3]. Tropical low- and middle-income countries carry a disproportionate burden of VBD deaths. Malaria is the biggest killer (619,000 deaths estimated in 2021), with a high burden in sub-Saharan Africa due to African *Anopheles* sp.'s long lifespan and strong human-biting habit [4,5]. Dengue has the greatest burden in Asia (around 70 % of VBD cases).

Climate change is creating more environments where mosquitoes can thrive, extending the transmission season and changing the mosquitoes' behaviour. Estimates suggest that by 2050 half a billion more people will be exposed to day-biting *Aedes* mosquitoes alone [6].

1.03) Human detection and attraction

1.03.01) How mosquitoes detect human odour

Olfaction is a major component of mosquito feeding behaviour in humans. As female mosquitoes mature, they develop an enhanced responsiveness to odours, becoming primed for blood feeding. Notably, they begin demonstrating a preference for human scent five days after emerging [7]. It is generally understood that only female mosquitoes exhibit short-range attraction to humans, seeking the proteins in our blood to produce eggs. However, there are anomalies, a review of human landing traps recorded that the proportion of males captured exceeded 10 % for *Aedes* mosquitoes [8].

Mosquito preference is affected by the mosquito's physiological state [9]. Electro-antennography and behavioural studies have been used to look at mosquito response to olfactory cues, but plasticity in mosquito behaviour can make identifying volatiles of interest difficult [9]. Mosquitoes have diverse sensilla sensory receptors located mainly in the antennae and mouthparts, which detect odorous molecules, as shown in Figure 1 [10]. The antenna is the primary detector of odour compounds. Hydrophobic odorants diffuse through pores on the surface of the sensilla. They are solubilised and transported by odorant-binding proteins (OBPs) through the water-based sensillar lymph to membrane-bound odorant receptors on the dendrites of olfactory receptor neurons (ORNs), ORN axons project into the antennal lobe. They convert chemical odorants into electrical signals that transmit information to the brain [10].

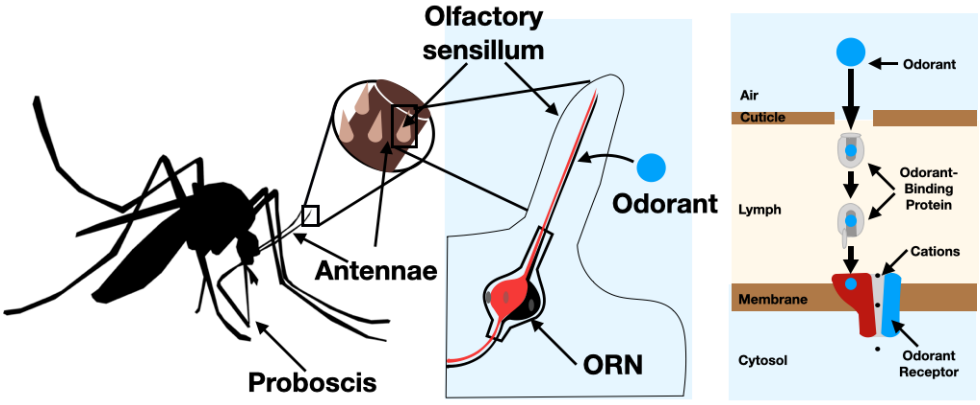

Figure 1. Insect chemosensory pathway adapted from (https://jonathanbohbot.weebly.com).

Olfaction is controlled by numerous chemosensory genes, including those encoding olfactory receptors and odorant-binding proteins (McBride et al., 2014). Insects use three multigene families of receptors to detect odour: odorant receptors (ORs), ionotropic receptors (IRs) and gustatory receptors (GRs) [10]. GRs on the maxillary palps detect carbon dioxide (CO_2) and long-range attractants [11]. IRs detect odour and heat from the host and respond to amines and carboxylic acids [12]. ORs respond to heterocyclic or aromatic compounds containing a benzene ring [13].

Olfaction was generally thought to be one receptor to one neuron, distinctive smells trigger receptors that match different odours. However, recent evidence shows extensive co-expression of sensory receptors within a neuron, suggesting redundancy in the smell of human odour, which may explain why mosquitoes are hard to disrupt [14,15]. Removing IR co-receptor reduces overall attraction to humans, but they can still detect differences between people, they can choose between highly- and poorly-attractive people [16].

1.03.02) How mosquitoes are attracted towards people

Host seeking can be divided into four main phases, as shown in Figure 2 (activation, attraction, landing and probing), although, in reality, it is a continuum of behaviour. This sequence of behaviours leads to a mosquito bite that can result in the transmission of disease when the vector carries pathogens or parasites. Activation and attraction are long-range, landing and probing are short-range.

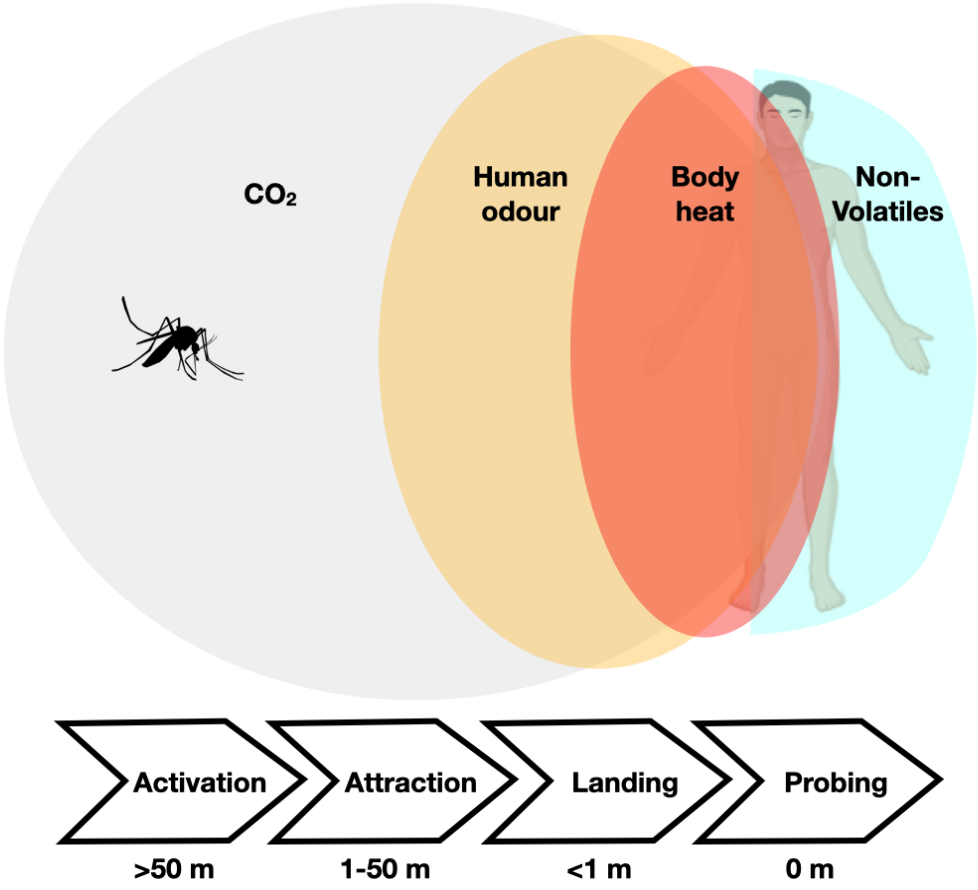

Figure 2. Summary of the main components contributing to mosquito host location and steps of host-seeking adapted from (Raji and DeGennaro 2017).

Long range: Activation

At long range, up to 90 m from the host, CO_2 acts as a catalyst for mosquito response, guiding mosquitoes towards potential hosts [17]. Although not exclusive to humans, CO_2 is estimated to account for 50 % of the anthropophilic attraction of *An. gambiae* to humans [18]. The optimal range for CO_2 to activate host-seeking is between 55-70 m from the host [19]. Mosquitoes follow a concentration gradient towards the host [20]. CO_2 acts

synergistically with other odourants [21–23]. *Anopheles coluzzii* has been reported to have exhibited minimal landings when exposed to clean air or CO_2 alone, whereas when foot odour is added, a significant increase in landings is shown [24].

Intermediate-range: Attraction

Near the host, volatile organic compounds (VOCs) can act as attractants or repellents [22,23,25]. Ammonia, lactic acid, ketones, sulphides, 1-octen-3-ol and carboxylic acids are released from human skin and have been shown to be attractive [26–28]. Subtle changes in ratios and concentrations of many VOCs are essential in human odour being attractive to mosquitoes. Giraldo et al. showed with a semi-field study that body odour impacts attractiveness at distances of up to 20m from the host [29]. Additionally, mosquitoes use visual cues for attraction. They have compound eyes that contain a lens and light-sensitive cells, which make them adapted for detecting movement. The eye allows them to discriminate between form, movement, light intensity, contrast and colour [30]. *Anopheles'* visual systems are suited to gathering light in low-light conditions. Therefore, the angle at which light is captured is more than double the angle of diurnally active mosquitoes (>40^o compared to 12^o *Ae Aegypti*), thus increasing sensitivity at the expense of resolution [31,32].

Short range: Landing

At short range, visual, olfaction, heat and humidity cues allow the mosquito to find the host [33,34]. Landing is a complex, flexible behaviour [35]. Carnaghi et al. showed that host odour is crucial for landing. A synergistic effect was seen when two or more stimuli of the tested visual, olfactory and thermal cues were presented [35]. Mosquitoes preferred black traps at 35 ^{o}C supplemented with odour (host odour and CO_2) [35]. Host volatiles have been shown to be more important than heat for successful landing behaviour [36]. Mosquitoes land less frequently on heat than body odour alone with no visual cue. *Anopheles coluzzi* have been shown to fly near visual cues without landing [37]. Several studies have shown upwind responses to human skin odour alone [38,39].

Heat is an essential cue for landing when combined with CO_2 or human body emanations [40,41]. Mosquitoes have extremely sensitive thermal receptors on their antennae that allow them to locate heat. Odour can stimulate mosquitoes to use visual cues, enhancing visual navigation towards the host at short distances. Nocturnally active mosquitoes are responsive to visual cues in starlight [37]. Mosquito landing is improved in lab experiments when the relative humidity rises compared to constant or falling humidity [42], suggesting that mosquitoes use humidity as a cue when approaching the host.

Probing

Once the mosquito has landed, probing occurs. Probing refers to the behaviour exhibited by female mosquitoes when they search for a blood vessel to feed on, using their fascicle to penetrate the host's skin [43]. The highly specialised piercing and sucking mouthparts as shown in Figure 3, can penetrate intact skin (Lehane, 2005). During feeding, the mouthparts divide into the fascicle (labrum, maxillae, hypopharynx and mandibles) and labium that encloses it (Lehane, 2005). Probing is affected by heat, surface texture and odour [44–46]. Olfaction plays a significant role in determining if a mosquito proceeds to feed after landing on a potential host [44].

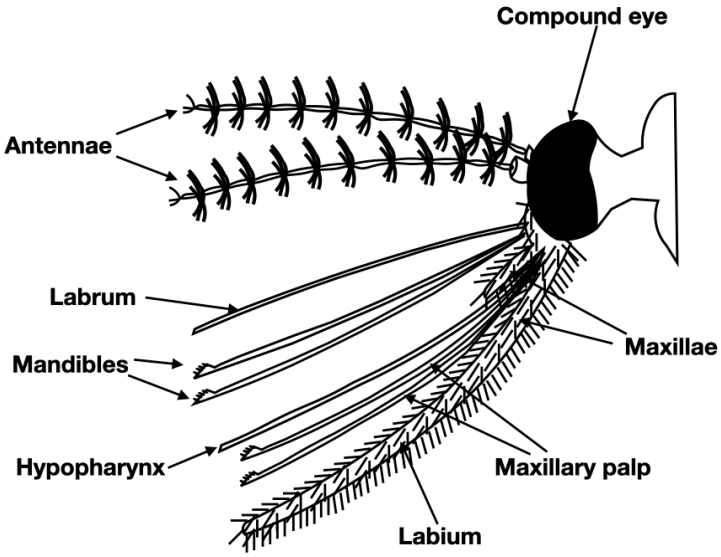

Figure 3. The mosquitoes mouthparts adapted from Encyclopaedia Britannica (2011).

1.04) Current measures to control mosquito-borne diseases

The main practices for preventing mosquito-borne diseases rely on chemicals to reduce the mosquito population and/or prevent bites, these vector control techniques are summarised in Figure 4. Personal protective measures include mosquito repellents, mosquito nets, and wearing clothes that cover exposed skin, such as long sleeves and trousers help reduce the risk of bites and subsequent disease [47–49]. Further external preventative measures include using larvicides to kill mosquito larvae (larviciding) [50] and environmental management, such as covering/draining standing water sources where mosquitoes breed. Vaccines have also been developed to prevent diseases transmitted by mosquitoes, including Japanese encephalitis [51].

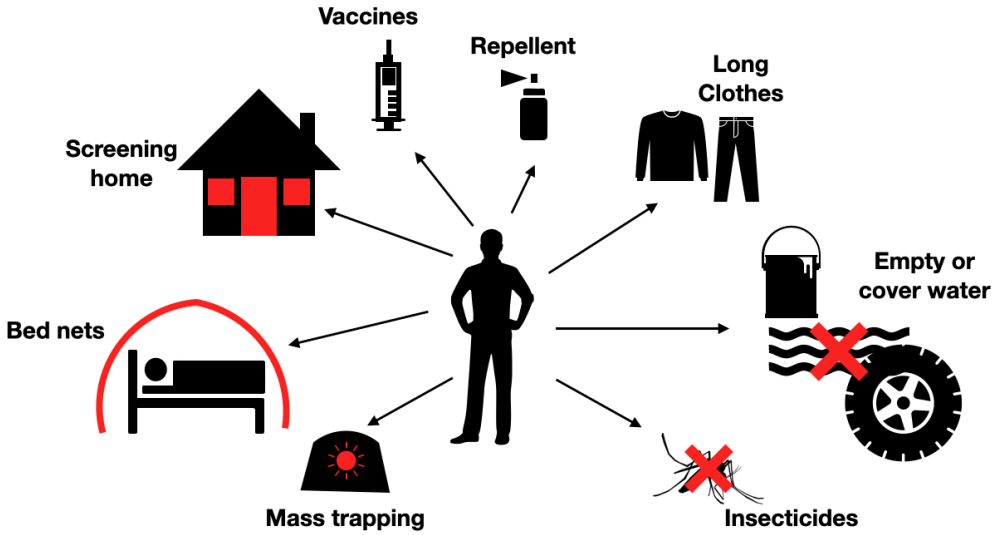

Figure 4. Overview of vector control strategies. Options include personal protective measures like repellents and long clothing for bite prevention, environmental management techniques and insecticides targeting both larval and adult mosquito stages.

In the 1940s, dichlorodiphenyltrichloroethane (DDT) became widely used in vector control programmes [52]. Indoor residual spraying (IRS) with DDT has an excito-repellent effect that reduces house entry, while sufficient contact, through landing or resting on the walls of sprayed homes, also kills adult mosquitoes [53]. Overreliance and widespread use of DDT, however, has led to mosquito resistance [54]. In addition to the reduced efficacy of DDT as vector control, there are some health concerns linked to the use of DDT, it has been shown to harm fish, and there is some evidence of increased breast cancer and preterm birth risks [52]. Insecticide-treated nets (ITNs) were first introduced in the 1980s. They provide a protective physical barrier and lethal insecticide on contact. WHO guidelines for vector control recommend an integrated vector management (IVM) approach to achieve disease

control targets by providing efficacious, cost-effective and sustainable vector control with available resources [55,56].

Malaria, a life-threatening disease transmitted through the bite of infected *Anopheles* mosquitoes, continues to be a global health concern. Artemisinin combination therapy (ACT), a combination of two or more anti-malarial drugs with different modes of action, is used to treat uncomplicated malaria [57]. The combination of IRS, ITNs and ACT has been very successful, together estimated to have averted 663 million malaria cases from 2000 to 2015 [58]. Delivering a core intervention, ITNs or IRS at high coverage for malaria is recommended [57]. Only once this is achieved may supplementary interventions be advised, such as the use of larvicides [57].

Mass trapping, using specialised traps to reduce adult mosquito populations can be an effective strategy to reduce mosquito-borne infections and has been used successfully to control Tsetse [59]. Traps are designed to attract, capture and kill the adult mosquitoes [60]. Reducing adults can reduce the number of mosquitoes that can lay eggs, reducing subsequent generation numbers. The efficiency of the traps is increased by using attractants such as CO_2, chemical lures that mimic human scent and sometimes light to make them more appealing to the mosquitoes [60]. However they are costly and labour intensive [61]. Mass trapping is used as part of an integrated control system but can also be used for monitoring insecticide resistance and mosquito behaviour. Different attractants, delivery systems and trap types can be needed to control diverse mosquito populations [59].

1.04.01) Shortfalls of current vector control tools

Current tools are failing to eliminate VBDs [62]. The combination of declines in funding, rising insecticide resistance, residual transmission and the COVID-19 pandemic means new tools are needed [62]. There are few effective options to prevent bites from day-biting mosquitoes. Repellents only last a few hours as the chemicals are very volatile [63], leading to low adherence in endemic countries [64]. Transmission inside the home can be prevented by screening windows and doors and using IRS, but it requires investment [5,65]. It is logistically difficult to reach rural areas and hard to sustain vector control programmes long-term.

Over-reliance on several insecticides has led to widespread insecticide resistance to multiple classes [66]. Resistance has been reported to pyrethroids, the only insecticide currently recommended for use in long-lasting insecticide-treated nets (LLINs) [67,68].

Pyrethroid resistance could result in a resurgence of malaria, as ITNs were responsible for averting approximately 68 % of cases between 2000 and 2015 [58]. Resistance to all four classes of insecticide (carbamates, organochlorines, organophosphates and pyrethroids) used for vector control has been reported [66]. To tackle multi-class insecticide resistance, Innovative Vector Control Consortium (IVCC) recommended that insecticides be used in rotation, mosaics, mixtures or combinations [69]. However, this recommendation is not universally put into practice.

The COVID-19 pandemic significantly impacted VBD prevention, diagnosis, and treatment. Widespread lockdowns and reduced travel disrupted vector control programmes. Additionally, the symptom overlap between COVID-19 and VBDs, such as difficulty breathing, body pain, fever, and headaches, complicated diagnosis. Consequently, when a patient was co-infected with malaria and COVID-19, one infection often went undetected [70]. Although initial studies proposed antimalarial drugs like chloroquine and hydroxychloroquine as potential treatments for COVID-19 (Meo, Klonoff, and Akram, 2020), these were later found to be ineffective (Singh et al., 2021). This reduced the available antimalarial supply and diverted resources. Additionally, materials previously used for malaria Rapid Diagnostic Tests (RDTs) were redirected to cater to COVID-19 diagnostics [71]. Furthermore, people exhibiting symptoms akin to COVID-19 were discouraged from visiting health centres, leading to delayed care seeking.

Residual transmission, the continued transmission of VBDs after the implementation of control measures, has been reported where high coverage of ITNs and IRS has been accomplished [72]. This transmission would persist if universal coverage with ITNs and IRS was achieved [72]. To be effective, ITNs rely on indoor feeding behaviour and IRS on the indoor resting behaviour of mosquitoes. High coverage with ITNs and IRS has led to mosquito behavioural changes, earlier exit from homes to avoid insecticide contact, and opportunistically feeding on humans outdoors when the humans are unprotected, feeding on animals or resting outdoors [72]. Observed behavioural changes, e.g. indoor biting mosquitoes beginning to bite outdoors, are thought to have occurred across Africa [73]. ITNs and IRS have led to shifts in behaviour to outdoor resting, feeding and zoophilic behaviours in several settings that have achieved high ITN/IRS coverage [74]. Previous programmes aimed at eliminating malaria have failed due to not targeting outdoor, day-biting mosquitoes [74]. Therefore, an effective and efficient scale-up of existing programs is needed, alongside novel tools to target the outdoor biters.

1.04.02) Novel tools

New vector control tools are needed to combat both the behavioural and physiological resistance of mosquitoes that has resulted from the widespread use of LLINs and IRS [68].

Insecticides

New insecticides are being developed, and it is essential not to be over-reliant on these and use them correctly. As previously shown, new insecticides are a short-term solution due to resistance. ITNs which contain pyrethroid and piperonyl butoxide (PBO) outperform current nets in areas with high pyrethroid resistance [75]. IRS products for malaria control containing Broflanilide, a new insecticide, are also being developed [76].

Vaccines

Vaccines are unlikely to be a silver bullet that can eradicate all VBDs due to the low efficacy and difficulty of administering multiple dose regimes in rural areas. There have been huge efforts to produce an effective vaccine against malaria since 1910 [77]. The RTS, S vaccine was recommended for use in sub-Saharan Africa in 2021 despite a four-dose regime having an efficacy of only 39 % in 5-17 months [78] [79]. Other vaccine candidates include R21/Matrix-M, whole-cell *P. falciparum* sporozoite and an mRNA vaccine [80]. Early R21/Matrix-M vaccine results suggest a four-dose regime is 80 % effective in the high-dose group, which is above the WHO-specified target of 75 %+ efficacy over 12 months in African children [81]. Safe, effective vaccines have the potential to make a big impact as part of an integrated vector control programme.

Dengue is difficult to develop a vaccine for due to immunity from one of the four serotypes tending not to give long-lasting immunity from the other three [82]. Denguevax was approved and later suspended due to the increased risk of severe disease, including haemorrhagic fever, that may be caused by antibody-dependent enhancement (ADE) in those that are seronegative before immunisation [82]. Denguevax is only recommended for seropositive people and therefore requires testing before vaccination. A new vaccine Qdenga has been developed that is suitable for those that have not previously contracted dengue. However, there are some concerns about the possibility of ADE as the vaccine does not protect against all serotypes [83].

Genetic control

Various tools based on genetic control have been developed to create mosquitoes unable to reproduce or resistant to certain diseases. Field trials have investigated the mass release of sterile males and gene drives of lethal or self-limiting genes with some success [84]. A natural symbiotic bacterium, *Serratia ureilytica* Su_YN1, was recently identified that has been shown to drive refractoriness of *Anopheles sinensis* to Plasmodium infection through secretion of an antimalarial lipase, which could potentially be further developed to reduce malaria infection [85].

Strains of *Wolbachia*, a bacterial endosymbiont, have been transferred into *Aedes aegypti,* where they block the transmission of dengue and Zika. The long-term success of this strategy depends on the *Wolbachia* being maintained at high frequencies to inhibit the virus without the re-release of mosquitoes [86]. Field studies have shown that *Wolbachia*-infected *Aedes aegypti* are stable after 20 months of co-evolution in the field [86].

Other techniques: Exploiting variations in attractiveness to mosquitoes

Innovative methods such as the utilisation of volatile organic compounds (VOCs) in a dynamic push-pull mechanism have been explored to combat mosquitos [87]. This approach leverages repellent VOCs to 'push' mosquitoes away from homes while using attractants to 'pull' them into traps, which has been shown to result in a significant reduction in house entry [87]. However, in the field, the pull component had limited efficacy, trapping less than 1 % of released mosquitoes in the presence of a human host, signifying the need for further research and development in the scale-up of microbial vector control techniques [88]. Scale-up has also been shown to be problematic with microbial VOCs used for trapping other insects, such as stable flies [89].

It has also been suggested to develop microbiome-based repellents for long-lasting, effective mosquito protection [90]. Several propositions are being considered, ranging from the topical application of repellent probiotic bacterial strains to bioengineering probiotics that produce less attractive or even deterrent odours [90]. Moreover, identifying natural repellent metabolites from skin microbes could lead to potent alternatives to DEET, with chemical innovations potentially extending their duration of effectiveness. These microbial innovations present another component that has the potential to strengthen the current vector control toolkit.

The exploration, development, and integration of microbial solutions into existing mosquito control strategies could provide prolonged protection against mosquito bites, particularly at

the individual level. As such, this thesis aims to investigate variations in human attractiveness to mosquitoes, along with associated differences in microbiome composition and volatile profiles. This will add to our existing knowledge and could pave the way for the development of additional vector control tools in the future.

1.05) Natural differences in attraction

1.05.01) Body odour

Human body odour comprises a complex mixture of over 500 VOCs, but few are considered human-specific [91]. VOCs are carbon-based compounds known for their high vapour pressure and low molecular weight, produced as by-products of both central and secondary metabolic processes [92]. These volatile microbial derivatives give characteristic smells to foods like cheese and yoghurt [92]. However, mosquitoes only detect a portion of these VOCs, constrained by the limited sensitivity of their chemosensory receptors. They respond to specific combinations of VOCs, which can either work together synergistically or counteract each other's effects [93]. Mosquitoes are tuned to detect certain VOCs. For instance, aldehydes and carboxylic acids have proven influential across various mosquito life stages, including plant-seeking, host-seeking, sexual communication and oviposition site selection [9]. Importantly, a compound's attractiveness can vary depending on the mosquito's physiological state and the environmental context [94]. Dormont et al. (2013) published a list of the 25 compounds most commonly isolated from human skin, featuring substances from a few specific chemical classes: carboxylic acids, ketones, alkanes, fatty acid methyl esters, and alcohols [95]. Among these, 13 volatiles were found to be common attractants for three major disease vector species, namely *Aedes aegypti, Ae. albopictus,* and *An. gambiae*. These shared attractants include 1-octen-3-ol, sulcatone, propanoic acid, lactic acid, and heptanal [90].

Attractants and repellents

The behavioural effects of potential repellent volatiles like sulcatone are unclear due to contradictory findings within and between species that may be due to differences in concentrations tested. Low concentrations were reported as a repellent to *Aedes aegypti [25,96]*, but not attractive to *Culex pipiens pallens [97]* and attractive to *An gambiae [96]*. 6-Methyl-5-hepten-2-one has also improved trap catches with *Aedes [98]*. It is emitted from the skin at a low release rate that varies between people [99]. While at high concentrations, much higher than naturally produced in human odour, but comparable to DEET, 6-methyl-5-hepten-2-one has been reported as a repellent to *An gambiae [96]* and *Culex quinquefasciatus [100]*. Logan et al. showed that 6-methyl-5-hepten-2-one combined with geranylacetone 1:1 has potential as a spatial repellent with activity against *Anopheles gambiae*, *Culex quinquefasciatus* and *Aedes aegypti* [93]. Complex attractive and repellent odours are unlikely to be due to one volatile. An effective natural repellent probably needs

a mixture of repellent odours at the correct concentrations to be repellent and may need to be optimised for the species of mosquito targeting.

Some attractive human-specific volatiles have been identified, including 3-methylbutanoic acid, lactic acid and octanal [101]. Bacteria emanations have been compared to ammonia (used as a positive control) to identify the attractiveness of VOCs produced by specific bacteria to mosquitoes [23]. The VOCs produced by individual bacterial species - their 'headspace' - have been utilised in behavioural experiments to gauge mosquito response [23]. The study identified six compounds produced by bacteria that affect *An gambiae* behaviour: butyl 2-methyl butanoate, pentathiane, 2-pentadecanone, butyl butyrate, acetoin and 3-methyl-1-butanol [23].

Mechanisms

Skin VOCs are mainly derived from gland secretions and the metabolism of the skin microbiome [102]. There are four ways volatiles can be generated from the skin [103], as shown in Figure 5. Type 1 VOCs are endogenous, originating from inside the body, while types 2, 3, and 4 are exogenous VOCs, which commonly dominate skin emissions.

1) Type 1 are endogenous VOCs that originate from inside the body through metabolism, food or medication and bacterial metabolism in the body [103]. 125 endogenous VOCs have been previously characterised using human cell lines, including 2-phenyl ethanol, acetic acid and 3-Methylbutyric acid [104].
2) Type 2 are exogenous VOCs from environmental exposures and the use of cosmetic products [103]. Even with a wash-out period only using odour-free soap, there will be volatiles from the environment and products such as shampoo, which result in non-biological differences between people [103]. Soap volatiles dominate the odour profile of people after washing, and more natural soaps can be distinguished from more chemically heavy soaps [105]. Different soaps have also been shown to influence human attractiveness to mosquitoes [105].
3) Type 3 are exogenous VOCs from the skin reacting with ozone. Skin lipids contain unsaturated organic compounds (unsaturated fatty acids and squalene) that react with ozone in the air to produce oxidation products (ozonolysis) [106]. Sebum is a complex mixture of lipids, approximately 12 % squalene [107]. The primary skin ozonolysis products are produced from squalene: propan-2-one (acetone), 6-methyl-5-hepten-2-one (sulcatone) and geranylacetone [108]. 6-methyl-5-hepten-2-one and geranylacetone have been suggested as repellents to mosquitoes at some doses [25]. Unsaturated fatty acids ozonolysis derives dodecanal, undecanal,

decanal, nonanal, octanal and hexanal, of which octanal, nonanal, and decanal have been found in higher concentrations in poorly-attractive people in some studies [25,106].

4) Type 4 are exogenous VOCs that originate from microbes on the skin surface, breaking down skin secretions. Microbial metabolism is associated with alcohols, acids, ketones, nitrides and sulphides [95].

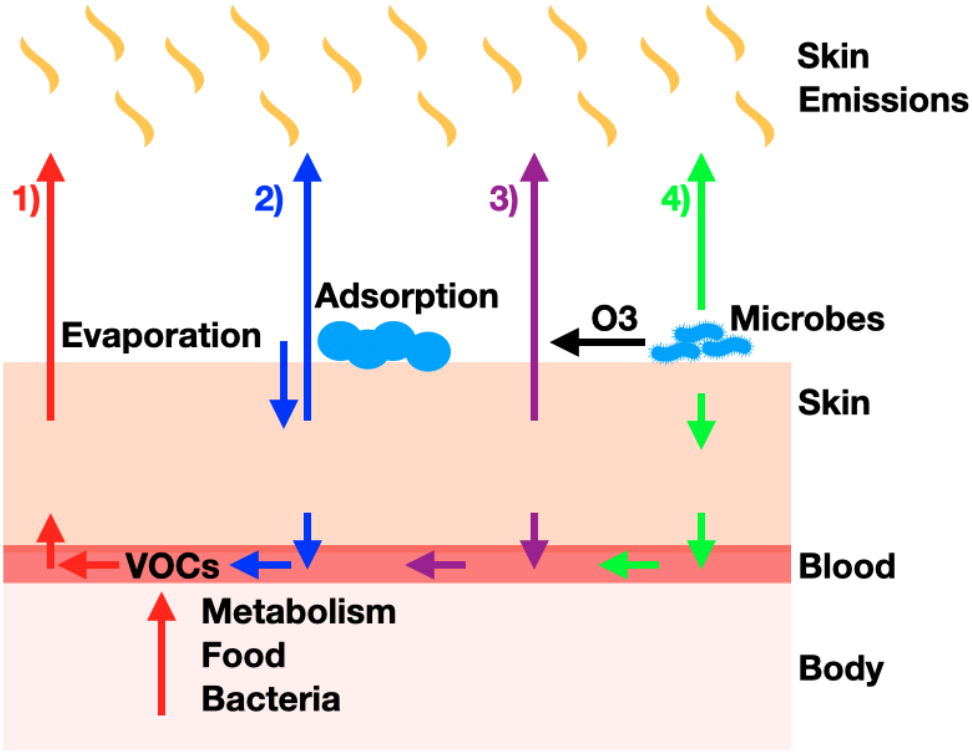

Figure 5. The four main mechanisms for how skin emissions (VOCs) are produced. These include processes within the body and interactions with external factors such as cosmetics, ozone, and microbes. Adapted from (Zou and Yang, 2022).

Some mechanisms by which microorganisms commonly found on the skin contribute to body odour by breaking down long-chain non-volatile compounds to VOCs have been suggested. These have mainly been studied in the context of axillary odour, which is of interest for deodorants. James et al. (2004) showed the role of bacteria in breaking down non-volatiles into VOCs involved in axillary malodour. *Propionibacterium* and *Staphylococci* ferment glycerol and lactic acid into short-chain (C2–C3) VOCs, e.g. acetic and propanoic acid [109]. These carboxylic acids are involved in attractiveness to mosquitoes [95]. A lipase active sub-group of *Corynebacterium* termed “corynebacteria (A)” converts unusual fatty acids that originate from triacylglycerol in sebum into short-medium chain volatile fatty acids (C2-C11) [110]. Ara et al. later identified that isovaleric acid produced by *Staphylococcus*

epidermis contributes to foot odour [111]. Several factors impact the release of VOCs by the microbe the VOC originates from, including the density and diversity of other microorganisms and environmental conditions such as carbon and nitrogen source, pH and temperature [102].

1.05.02) The skin microbiome

The skin microbiome refers to all living organisms, including bacteria, fungi and viruses that colonise the surface of the skin [112]. Human skin is the interface of the body with the external environment. The surface layer of the skin includes microbial communities as shown in Figure 6 [112]. The distribution of bacterial species across different regions of human skin depends on local skin site characteristics, including sebum, moisture content and hair follicle density [23,113]. The composition of the skin microbiome is modulated by physiological and environmental factors. Physiological factors associated with the skin microbiome include age and sex [112]. While environmental factors include occupation, antibiotic use and cosmetics [112]. Human genetic variations have been identified as contributing to the skin microbiome. Some skin microbes, including *Corynebacterium*, *Brevibacterium* and *Propionibacterium,* have been shown to have very high heritability [114].

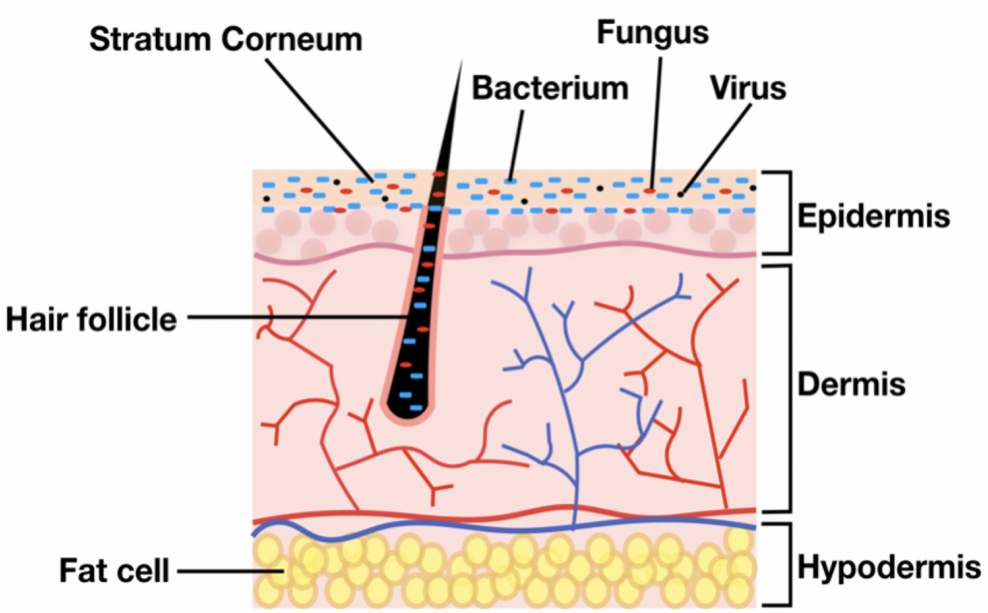

Figure 6. The three primary layers of human skin: the epidermis, dermis, and hypodermis. The figure shows the location of the skin microbiome within the epidermis and hair follicles. Adapted from (Grice and Segre, 2011).

Inside the uterus, the foetus is sterile. The colonisation of the skin by bacteria occurs at birth, and the baby acquires a microbiome [115]. If the baby is delivered, naturally, the baby's skin microbiome resembles the mother's vaginal microbiome, dominated by *Lactobacillus*, *Prevotella* or *Sneathia* [116]. Whereas if delivered by c-section, the baby's skin microbiome resembles the skin microbiome of the mother and is dominated by *Staphylococcus*, *Corynebacterium* and *Propionibacterium* species [116]. The skin

microbiome of infants become less similar to their mothers over the first year of life [117], likely to be driven by the environment and lifestyle. The skin microbiome in adults is unique for each individual and remains relatively stable over time [118]. Oh et al. (2017) demonstrated that healthy adults maintain both the most abundant microbial species and some less abundant species for up to two years. Furthermore, the long-term composition of the skin microbiome is more consistent within an individual than between different people [118].

Sweat glands shape microbiome composition

Different gland secretions and the microenvironment selects distinct microbiota in different body parts [119,120]. Mammals have sebaceous glands and two types of sweat glands: apocrine and eccrine [120]. Figure 7 shows the distribution, and Table 1 summarises the distribution, function and secretions. Skin glands secrete ammonia and lactic acid, whilst skin bacteria convert skin gland secretions into carboxylic acids [121]. The presence of different glands and the microenvironment selects for a distinct microbiota in different parts of the body, which leads to different volatile profiles [119,120]. Human feet have high numbers of eccrine glands. They are significant producers of body odour [122].

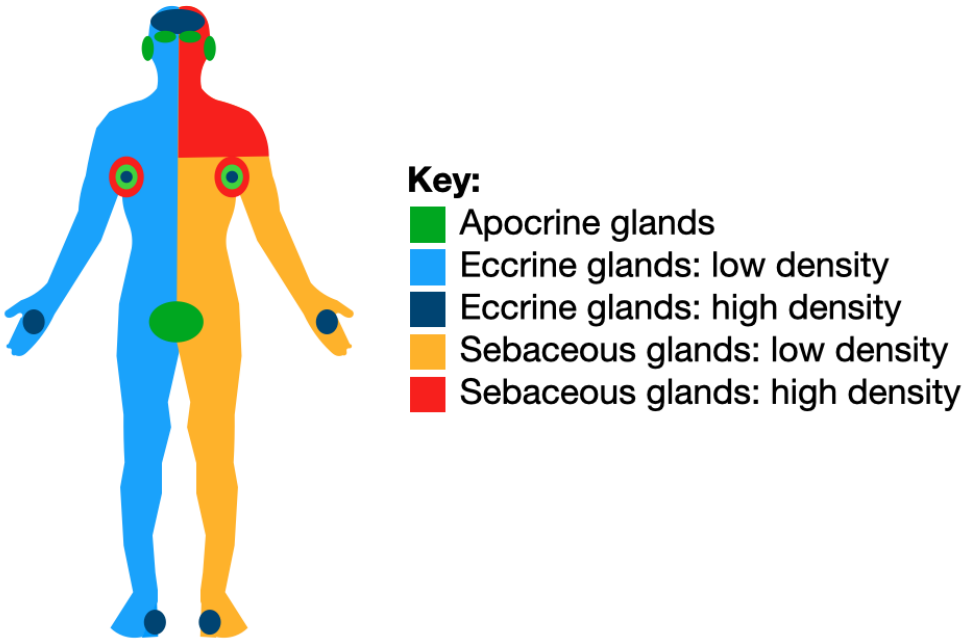

Figure 7. The distribution of the sebaceous, eccrine and apocrine glands on the human body adapted from (Smallegange et al. 2011).

Eccrine glands are found throughout the body, particularly numerous on the hands and soles of feet, the focus of the sample collection in this thesis. Eccrine sweat is made up of water, salts, protein, amino acids, urea, ammonia and lactic acid. In contrast, apocrine glands are mainly found in the armpits and genital areas. Apocrine secretions are major

contributors to body odour due to the microbial breakdown of these sweat components producing VOCs [123]. Meanwhile, sebaceous glands are located all over the body except palms and soles [112]. Eccrine and sebaceous glands emit secretions microbes break down into volatile fatty acids, such as isovaleric acid (Ara et al., 2006). While lactic acid secreted by eccrine glands is notably attractive to mosquitoes [124].

Table 1. The distribution, function and secretions of eccrine, apocrine and sebaceous glands adapted from (Smallegange, Verhulst and Takken, 2011).

Type of gland	**Eccrine**	**Apocrine**	**Sebaceous**
Distribution	Numerous in palms and soles of feet. In decreasing order on the head, trunk and extremities.	In the dermis of the skin. Associated with hair follicles. Mainly situated in the axillae, perineal and genital areas.	All over the body except palms and soles. The largest and most numerous are in the skin of the head.
Function	Thermoregulation.	Suggested to contain pheromones and play a role in chemical communication.	Produce sebum.
Secretion	Water, some trace salts, protein, amino acids, urea, ammonia and lactic acid.	Lipids, steroids and proteins.	Sebum (consisting of lipids).

Skin microbiome and attractiveness to mosquitoes

When sweat, which contains organic compounds like lactic acid, ammonia, and other carboxylic acids, is metabolised by bacteria on the skin, it creates volatile compounds that can attract mosquitoes [124]. An early study showed that freshly secreted sweat is odourless; the characteristic “sweat smell” is only present after incubation with bacteria [125]. It was later hypothesised that the skin microbiome explained differences in attractiveness to mosquitoes between freshly washed hands and those left unwashed for several hours [126]. They hypothesised that the microorganisms produced unknown attractive metabolites on the skin, which were attractive to mosquitoes [126]. Braks and Takken (1999) examined sweat's attractiveness to *An. gambiae* mosquitoes. They collected

fresh sweat from three individuals and compared it to sweat incubated aerobically at 37 °C for 1 or 2 days using a dual-port olfactometer, with distilled water as a control [127]. Their results showed that while freshly secreted sweat was unattractive to mosquitoes, the incubated sweat proved to be attractive [127]. This study had some limitations: the small sample size, the potential impact on microbes from directly testing sweat droplets instead of bacterial plates, and variations in attractiveness among samples, possibly due to individual differences in microbial presence or sweat composition. Despite these shortcomings, the gross effects provided compelling experimental evidence that skin bacterial communities alter human sweat secretion in ways that affect mosquito attraction, as fresh sweat was shown to be unappealing.

Anopheles mosquitoes prefer to feed close to the ground and are therefore attracted to humans' feet and lower legs [128–130]. In a study examining the volatiles and attractiveness of 8 individuals, Verhulst et al. (2016) observed that *Anopheles coluzzii* mosquitoes showed a reduced attraction to armpits in comparison to hands or feet. However, when participants refrained from using scented products for 24 hours, there was no difference in the attractiveness of hands, feet, and armpits, despite variations in volatile profiles associated with differences in the skin microbiome. This change in preference was linked to the repelling odours found in deodorants [131]. The resident bacteria of the feet tend to include *Firmicutes,* such as *Staphylococcaceae* and *Actinobacteria,* while having a high fungal diversity, including *Aspergillus*, *Cryptococcus* and *Candida* species [112,119]. VOCs are released when microorganisms on the skin metabolise skin secretions produced by the glands [132]. Ara et al. (2006) reviewed microorganisms involved in foot odour in detail and screened compounds for activity against isovaleric acid, a key foot odour produced by *Staphylococcus epidermidis* [111]. In brief, high numbers of *Staphylococci* and aerobic *Coryneform* were associated with foot odour [133]. Strongly odoured feet tend to have a higher population density of microorganisms, producing exo-enzymes, e.g. lipase and protease [133]. The smell of feet is often described as “cheesy”, and *An. Gambiae* are attracted to Limburger cheese. This is due to *Brevibacterium* bacteria contributing to the smell of both [134]. *Staphylococcus epidermis* and *Bacillus subtilis* have been associated with foot odour [23,135].

It is now generally established that the skin microbiome plays a role in human attractiveness to mosquitoes, with variations between individuals. Verhulst et al. (2009) demonstrated that skin microbes cultured from the foot and subsequently incubated on blood agar plates at 34°C for 12 hours were more attractive to *Anopheles gambiae* than the control plates. This was evidenced in a two-choice olfactometer test and further verified using an MM-X trap in

a testing room [136]. They showed that the VOC profile of these incubated foot microbes had 14 compounds in greater abundance than control blood agar plates. By experimentally combining 10 of these compounds, they observed a higher mosquito catch rate than controls (LDPE sachets filled with distilled water) in an MM-X trap despite the agar drying out after a couple of hours [136]. This gave further evidence that skin microbes are producing VOCs attractive to mosquitoes. However, the microbial and VOC profiles of the five volunteers were not compared to investigate differences between people. Additionally, Verhulst et al. (2010) compared the attractiveness of five common species of skin microbes: *Bacillus subtilis, Brevibacterium epidermidis, Corynebacterium minutissimum, Pseudomonas aeruginosa* and *Staphylococcus epidermidis* to *Anopheles gambiae* using a two-choice olfactometer against the media on identical glass slides [23]. Out of these, four microbes were attractive, while *Pseudomonas aeruginosa* was not attractive, showing there are differences in attractiveness between microbes isolated from the skin [23]. One potential caveat of this study is that the microbial strains tested were commercially obtained rather than directly isolated from human skin, which might lead to differences in their volatile profiles compared to skin-derived strains.

Through molecular methods (16S amplicon sequencing of V2 region), Verhulst et al. (2011) have also shown differences in skin microbiome composition based on relative abundances of microbes between poorly-attractive and highly-attractive people [137]. Several microbes of interest were identified as associated with poorly-attractive and highly-attractive groups, including *Pseudomonas* and *Variovorax* with the poorly-attractive and *Delftia*, *Leptotrichia* and *Acidobacteria* with highly-attractive [137]. There were some microbes identified that were likely environmental contaminants, including *Variovorax,* a soil microbe [138], which may be attributable to low DNA yield from skin samples or not controlling for kit and environmental contamination through including negative controls. There are inherent limitations in choosing to only compare the poorly-attractive and highly-attractive people while excluding the middle range. Specifically, it leads to a loss of data and may not offer insights that are generalisable to the broader population. Moreover, excluding the middle range could introduce potential biases, and determining which values constitute 'extreme' can be somewhat arbitrary. On the positive side, contrasting the extremes amplifies the differences, potentially strengthening the statistical power to discern variations between the groups. This approach also simplifies interpretation by providing clearer contrasts and can reduce data variability The existing body of research predominantly centres on *Anopheles* mosquitoes, known carriers of malaria. In this thesis, the differences in the skin microbiome between individuals who are unattractive and attractive to both *Anopheles* and *Aedes* species will be investigated.

1.06) Factors associated with inter-individual differences in attractiveness to mosquitoes

Some people are more attractive than others to mosquitoes [29,38]. Those highly attractive get bitten more and consequently are at higher risk of VBDs. Absolute attractiveness is dependent on the people around you. Unattractive people will still attract some mosquitoes, whereas, in the company of more attractive people, they may not get bitten. Figure 8 shows a summary of the main factors known to contribute to differential attractiveness. Natural differences in human attractiveness to mosquitoes have been shown to be stable for over a year [139]. Skin characteristics contribute to how highly- or poorly-attractive a person is to mosquitoes.

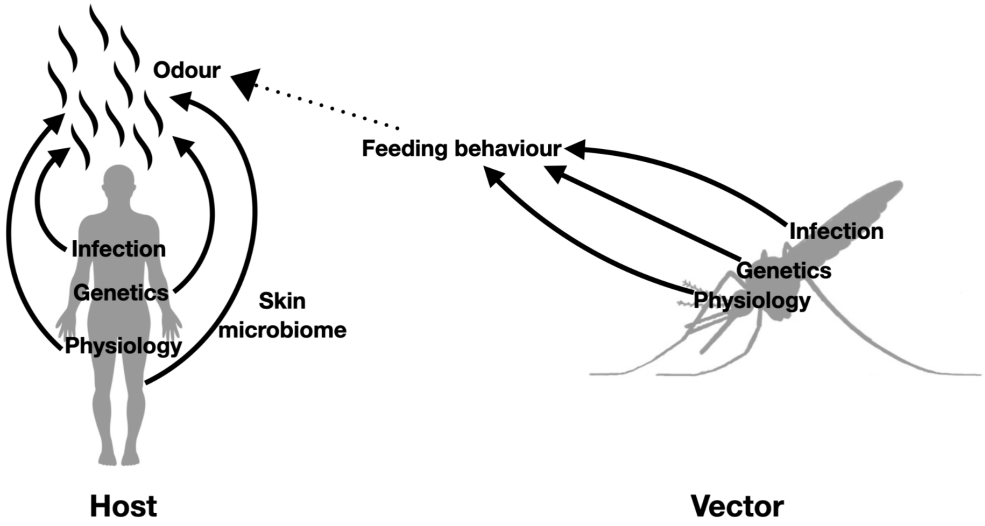

Figure 8. Key determinants influencing human attractiveness to mosquitoes include both human-derived odour factors and innate mosquito host-seeking behaviours.

1.06.01) Microbiome and body odour contribute to variations in attractiveness

Human body emanations result in differential attractiveness that remains stable over time [16,23,29,38]. Mosquitoes may locate humans using the human-specific carboxylic acids they emit [121]. The attractiveness of VOCs produced by specific bacteria to mosquitoes has been tested [23]. People who are more attractive to mosquitoes tend to have a higher abundance and less diversity of bacteria compared to those less attractive [137]. Verhulst *et al.* have suggested that higher skin bacterial composition correlates with lower

attractiveness to mosquitoes, suggesting that high microbial diversity on the skin may be protective against bites [137]. They identified individual bacteria genera that are in higher abundance in poorly attractive people, e.g. *Pseudomonas* and highly-attractive people, e.g. *Staphylococcus.*

Experimental studies have demonstrated that variations in body odour contribute to differential attractiveness [38,137,140–142]. Schreck et al. (1990) conducted a study on the attractiveness of *Aedes aegypti* mosquitoes using eight volunteers. Their findings highlighted variations in attraction levels: one individual was significantly more appealing to the mosquitoes, while another was notably less so [140]. This variation was determined by collecting skin emanations from the participants using a clean dish, suggesting that the mosquitoes' attraction was influenced by individual skin substances [140]. Lindsay et al. (1993) conducted experimental hut trials over a span of 2.5 years in The Gambia, revealing consistent rankings in human attractiveness to mosquitoes among the male participants. Despite the trials being limited in size, the huts being no more than 12 metres apart, and no control for residual odour, they demonstrated evidence of persistent differences in relative attractiveness over time [141]. Knols et al. (1995) further corroborated the existence of differences in attractiveness among individuals. In a study conducted with Tanzanian men, the researchers controlled for residual odour by utilising tents at varied locations. Over the course of nine testing days, one volunteer consistently exhibited a significantly higher attractiveness to *Anopheles gambiae*, *Anopheles funestus*, and *Culex quinquefasciatus* compared to the other two participants [142]. Brady et al. (1997) observed consistent differential attractiveness within three groups of four men over a span of ten weeks during human-biting catch (HBC) experiments [143]. They identified a regular pattern of relative attractiveness amongst the test subjects over this period [143]. In laboratory studies, skin emanations have been collected and evaluated using a dual-port olfactometer. Qiu et al. (2006) gathered these emanations onto glass beads from 27 individuals. The beads, which were supplemented with ammonia, were compared to control beads, which were also supplemented with ammonia. Out of the 27 participants, emanations from 22 were found to be more attractive than the control, based on six replicates. Conversely, the emanations from the remaining five participants were deemed unattractive. [38]. More recently, De Obaldia et al. (2022) adapted a two-choice olfactometer to test odour collected from forearm samples of eight participants onto nylon sleeves with *Aedes aegypti* over 3.1 years with over 2330 behavioural assays. They found one subject to be significantly more attractive than two other subjects stable over many months [16]. The initial VOCs that attract mosquitoes were identified over 100 years ago [144], and subsequent research has further highlighted various attractive and repellent compounds [25,136,145]. At long range, 60

metres from the host, *Anopheles gambiae* demonstrates differential attraction when subjected to multiple hosts odours [29]. These findings underline the intricate role of human odour in mosquito behaviour and attraction.

Low attractiveness to mosquitoes may be due to the production of repellent odours that "mask" the attractive odours or lower production of attractive odours [25]. Candidate repellents have been tested in behavioural studies with *An. gambiae*. Surprisingly higher concentrations of attractive compounds, such as 3-methyl-1-butanol, have been found to be repellent [146]. Logan et al. (2008) identified a chemical basis for repellency or low attractiveness to *Aedes* mosquitoes. Using a Y-tube they compared the attractiveness of 9 "test" individuals' hands against a "standard" hand that had an 80 % relative attractiveness. As shown in previous studies there were differences in mosquito attractiveness among people tested, which led to classification into attractive and unattractive groups [25]. They then selected two participants from each group to compare further, applying GC-EAG to identify physiologically active compounds and GC-MS for compound identification. Of the 33 peaks associated with EAG activity, 23 were identified by peak enhancement on GC-MS. A comparison of mean amounts between the groups revealed 8 compounds with significantly greater amounts in the unattractive group than attractive group: benzaldehyde, 6-methyl-5-hepten-2-one, octanal, nonanal, naphthalene, decanal, geranylacetone and an unidentified compound. This suggests these compounds may play a role in the less attractive participants' attractiveness to the mosquitoes [25]. Five of these compounds: 6-methyl-5-hepten-2-one, octanal, nonanal, decanal, and geranylacetone, were further tested behaviourally applied in five doses between 1×10^{-4} g to 1×10^{-8} g to the "standard" hand. Notably, slightly increased levels of 6-methyl-5-hepten-2-one reduced probing activity; all doses inhibited flight activation but not relative attraction [25]. However, the comparison of relative amounts of compounds between unattractive and attractive groups indicated fewer compounds that were significantly different (only 3 compounds) and there was no adjustment for multiple testing as well as small sample sizes. De Obaldia et al. (2022) utilised a modified two-choice olfactometer to compare the attractiveness of 64 participants to *Aedes aegypti*. They selected 11 highly attractive and 7 weakly attractive participants to compare using GC-MS with 4 replicates and identified differentially abundant compounds between the groups [16]. They showed highly-attractive tended to have higher levels of three carboxylic acids: pentadecanoic, heptadecanoic, and nonadecanoic acids, plus ten other compounds from the same group compared to poorly-attractive people [16]. There were differences in the body odour blend between the highly-attractive people, meaning there may be multiple mosquito-attractive phenotypes [16]. Although, no chemicals that

were consistently higher in the poorly attractive group compared to the unattractive group have been identified, which contrasts with Logan et al. (2008)'s findings, suggesting that these individuals lack attractive odour rather than having increased amounts of repellent compounds [16]. Interestingly, they identified one participant that had high levels of carboxylic acids but was weakly attractive, this participant may have been producing compounds that masked these highly attractive compounds but it was not tested [16]. It is unclear if people are unattractive to mosquitoes or truly repellent and whether there are compounds that mask or repel mosquitoes in less attractive people's body odour. Further studies delving deeper into molecular microbiology and metabolomics could reveal further understanding of variations in the abundance of bacteria and volatiles between attractive and unattractive individuals. This could lead to innovative mosquito control strategies utilising these natural differences to identify mosquito attractants and repellents.

1.06.02) Covariates that contribute to variations in attractiveness to mosquitoes

There are several covariates that are known to affect the attractiveness of humans to mosquitoes. These include genetic and non-genetic factors, physiological, environmental and infections. Some of these factors may influence attractiveness through their impact on the microbiome.

Environmental factors: Diet, alcohol and antibiotic use

Several environmental factors, including diet, alcohol consumption, and antibiotic use, have been linked to variations in human attractiveness to mosquitoes. While the full impact of these factors on mosquito attraction is yet to be fully understood, there's evidence supporting the role of dietary choices. For instance, certain diets can modify skin structure and functionality, potentially reducing the recurrence of skin diseases [147]. Consuming spicy foods, for example, is believed to modify body odour. Eating bananas has been associated with increased attractiveness to *Anopheles* mosquitoes, underscoring the potential influence of diet on mosquito attraction [148]. Furthermore, beer consumption not only makes an individual more appealing to mosquitoes[149]. but has been linked to a drop in body temperature and a rise in CO_2 exhalation. However, neither of these changes fully explains the enhanced attraction observed [149]. The mechanisms for how diet influences attractiveness to mosquitoes have not been fully elucidated.

Antibiotics have been shown to reduce skin microbiome diversity. Therefore most skin microbiome studies exclude participants that have received antibiotics up to 12 months before [150,151]. Both general and cutaneous medical conditions are associated with changes in microbiome composition [152], and medicines used to treat atopic dermatitis are also associated with changes in the diversity of bacteria [153]. There are significant differences in the skin microbiome between those living in urban and rural locations in China [154]. The people and animals with whom we co-inhabit shape our microbial community, and those living with pets have an increase in their shared skin microbiota [155]. There is also a shift during contact sports. The skin microbiota of teams become more similar after playing contact sports, e.g. roller derby [156]. Washing alters the bacterial communities present on the skin. Therefore skin preparation before sample collection should be standardised [157]. The exact biological mechanisms for how these environmental factors change body odour and affect attractiveness to mosquitoes require further investigation.

Physiological: age, gender, and pregnancy

Previous studies have also shown that physiological factors: age, gender and pregnancy affect the attractiveness of humans to mosquitoes. Adults are more attractive to mosquitoes than children. This differential attraction is only seen when hosts are nearby, e.g. mother and child sleeping in the same bed [158]. Compared to adults, children have a distinct skin microbiome [153]. Adults have a greater bacteria alpha diversity, meaning they have more species of bacteria present on the skin [154]. A sex difference in attractiveness to mosquitoes has been shown in two trials where adult women were less attractive to mosquitoes than men [159,160]. Gender effect on bacterial abundance is body site dependent. Disparities between genders are thought to be due to differences in the production of sweat, sebum, hormones, and the use of cosmetic products between genders [114]. Males have a greater sebum secretion associated with a greater relative abundance of *Propionibacterium* with age [154]. Women have a significantly higher alpha diversity of the palm than males [157]. However, other studies have shown no association between gender and bacterial diversity [114] or gender and attractiveness [161]. There is some evidence that age and sex are confounders and should be considered and adjusted for in future studies.

Pregnant women are twice as attractive to *An. gambiae* due to physiological and behavioural changes associated with pregnancy [162]. Pregnancy results in behavioural change as women need to urinate more, meaning they are more likely to leave the safety of their bed net during the night. There are also physiological changes, including 21 % more exhaled breath and temperature increases which can increase emanations from the skin, activating mosquito host-seeking at long range [162]. Pregnancy results in some immune suppression, making the mother more susceptible to parasite infections like malaria [163]. Pregnant women have also been shown to be more attractive to mosquitoes at short ranges. Pregnant women have a larger surface area and are heavier and hotter, which may produce a stronger host signal through changes in host emanations [164]. It is hypothesised that pregnant women produce pregnancy-specific volatiles, which makes them differentially attractive [165]. Increased body temperature and hormone changes may affect the abundance of bacteria on the skin, causing changes in the VOCs emitted, but this has not been fully investigated. Additionally, changes in the skin microbiome may occur during pregnancy, throughout the menstrual cycle or with contraceptives, which impacts human attractiveness to mosquitoes.

Infection status (malaria)

Malaria infection is linked to increased attractiveness, suggesting malaria parasites make changes to the host that encourage mosquitoes to approach for a blood meal and subsequently take up circulating parasites [166,167]. The association between *Plasmodium* infection and attractiveness may be due to physiological changes such as an increase in temperature, sweat or change in the composition of breath [122]. Alternatively, *Plasmodium*-induced changes in attraction may be mediated by changes in the skin microbiome's richness or composition, which could be controlled genetically. However, studies to date have neglected to investigate the role of the microbiome.

The gametocyte stage was identified as early as the 1980s as important in mosquito feeding on infected hosts [168]. A mice study showed *Plasmodium-associated* changes in odour profiles: chronically infected mice were differentially attractive and showed clear differences in volatile blends compared to uninfected mice [169]. Similarly, in field studies, children infected with *Plasmodium* gametocytes are more attractive than uninfected children [167]. Children with non-infective malaria (asexual stage) were no more attractive than non-infected children [167]. Longitudinal studies in controlled human malaria infection (CHMI) trials identified changes in volatiles and people becoming less attractive during the asexual stage of infection [170]. CHMI do not allow infections to reach the gametocyte stage, which may explain these results. Another human study in South American adults showed that *Plasmodium* gametocytes are associated with increased attractiveness; patients tended to have higher body temperatures suggesting a synergistic effect of *Plasmodium* gametocytes and temperature on attractiveness to *An. darlingi* [171]. Smallegange et al. showed that *An. gambiae* infected with transmissible malaria (sporozoite stage) had significantly more landing and probing attempts in response to human odour than uninfected mosquitoes (Smallegange et al., 2013). Robinson et al. (2018) identified quantitative differences in specific VOCs between infected and uninfected individuals. These included aldehydes being produced in greater amounts (heptanol, octanal and nonanal) [172]. Changes in the skin microbiome between infected and uninfected people may explain the differences in VOCs identified by Robinson et al. but were not explored.

Plasmodium falciparum has been shown to produce (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), a metabolite which increases the production of CO_2, aldehydes and monoterpenes in infected cells. In lab studies, HMBPP has been shown to increase vector attraction and feed on infected blood [173]. The effect of this metabolite has not yet

been replicated in a living host. Evidence to show that HMBPP reaches the skin surface and is in the skin emanations has not yet been shown.

Infection status (flaviviruses)

It has been hypothesised that infection with flaviviruses, including dengue and Zika, would produce host cues that would make the host more attractive to mosquitoes, as shown for malaria. Zhang et al. showed that *Aedes* are more attracted to flavivirus-infected mice (dengue and Zika) than uninfected mice [174]. Infection with flaviviruses leads to more than 1.5-fold changes in 20 compounds common between both dengue and Zika-infected mice (11 upregulated and 9 downregulated) [174]. Three compounds had significant electrophysiological responses: acetophenone, decanal and styrene [174]. The highest response was seen for acetophenone, which attracted more mosquitoes when applied to animal and human skin [174]. This result shows that acetophenone is upregulated by flavivirus infection and is attractive to mosquitoes. Acetophenone is mainly produced by microbes in the intestine or on the skin [174]. Depleting the gut microbiome did not affect attractiveness, whereas depleting the skin microbiome made infected mice less attractive than infected mice [174]. After infection with DENV2 or Zika, there was a ~10-fold increase in the abundance of culturable bacteria from the skin [174]. *Bacillus* was shown to be a producer of acetophenone, mice treated with *Bacillus spp* were more attractive to mosquitoes than those treated with bacteria downregulated in the infected mice *(Lactobacillus spp*.) [174]. Flavivirus infections have been shown to modulate the skin microbiome composition of mice by altering the expression of a host gene, reducing the production of an antibacterial compound that increased acetophenone production and increased attractiveness to mosquitoes [174].

Genetics

The extent to which genetics play a role in human attractiveness to mosquitoes through body odour remains uncertain. Several early studies used a dog's remarkable sense of smell as a proxy for differences in body odour profile between twins [175]. Dogs can discriminate twins if they differ in genetic relatedness or environmental factors but struggle to differentiate the body odour of identical twins with similar environmental factors [175]. Additional research with human sniffers found non-cohabiting MZ twins could be matched by body odour more accurately than expected by chance, but DZ twins could not [176]. Kuhn and Natsch applied two-dimensional gas chromatography-mass spectrometry (GCxGC-MS) to analyse volatile carboxylic acids. They found that twins had a more similar axillary volatile profile than unrelated pairs [177]. However, the small sample sizes and the requirement to enrich carboxylic acids due to difficulties analysing small quantities in body odour samples limit the strength of these studies. Additionally, genetic factors may indirectly affect mosquito attraction by influencing characteristics such as BMI. Mosquitoes prefer to feed on individuals with a higher BMI, as larger individuals have a larger surface area with more sweat, moisture, heat and semiochemicals attractive to mosquitoes [158]. Therefore, genetic factors directly influencing body odour and indirect factors such as BMI may play a role in determining how attractive individuals are to mosquitoes.

There are few studies on the direct significance of human genetics in human attractiveness to mosquitoes. Initial survey-based studies were focused on ordinal variables that gave little evidence of a genetic component due to low repeatability. Kirk et al. (2000) found greater concordance between MZ than DZ adolescent twins in the reported frequency of mosquito bites [161]. There was low repeatability between the surveys conducted at 12 and 14 years, possibly due to physiological changes during puberty, including increased activity of sweat glands that are likely to change human attractiveness to mosquitoes. However, the comparative question “Compared with your twin, who is bitten by mosquitoes more often?” provided some evidence for a genetic component. Logan et al. (2010) later reported a significant interaction between the number of midge bites received by parents and their firstborn child in a similarly sized study (N=325) [178]. Survey-based studies are limited by response, non-response, and recency biases and this study is limited by its reliance on perceived rather than measured attractiveness. Moreover, people's bite reactions can affect their perception of their attractiveness to mosquitoes. Experimentally twin attractiveness to *Aedes aegypti* mosquitoes was investigated, and the heritability of attractiveness was estimated to be between 62 - 67 % [179].

One specific set of genes implicated to have a role in human attractiveness to mosquitoes is the Human Leukocyte Antigen (HLA) genes [101]. These genes code for proteins that are crucial to the immune system's ability to recognise and respond to foreign pathogens [180]. Some early studies indicated that humans prefer the body odours of individuals with MHC-dissimilar genes, suggesting that MHC or related genes may influence mate choice [181,182]. However, other studies have found no evidence of MHC-dissimilar mate preference, possibly due to differences in experimental methods and small sample sizes [183,184]. Studies investigating the relationship between HLA genes and odour have found no significant differences in carboxylic acid release between siblings, indicating that HLA genes do not influence volatile carboxylic acid production [185].

Verhulst et al. (2013) investigated the correlation between human attractiveness to *Anopheles gambiae* and HLA profiles by typing HLA-A, -B, -C, -DRB1 and -DQB1 for individuals that were highly and poorly attractive to mosquitoes. They found some evidence that HLA C*07 alleles occurred more frequently in highly attractive (N=9) than in poorly attractive individuals (N=7) (P=0.064) [101]. More recently, a study identified associations between HLA alleles and the relative abundance of skin microbes, including *Corynebacterium* and *Staphylococcus [186]*. Several mechanisms have been proposed to explain how MHC genes affect human odour profiles, as reviewed by [187]. The peptide-microflora hypothesis suggests MHC molecules bind to subsets of the available peptides, which are carried to body sites where microorganisms metabolise them to produce an individual’s odour [187]. *In vitro*, experiments suggested an interaction between HLA peptides and the microbiome in the production of 3-methylbutanal, although it is unclear the extent to which HLA alleles impact the odour profile [188]. Overall, evidence in humans suggests that if present, MHC-dependent preferences for human body odour are weak, and other factors such as diet, hygiene or the skin microbiome are likely to explain more of the variation observed in human body odour. There remains a gap in knowledge about whether there is a genetic component to human attractiveness to mosquitoes.

1.07) Microbiome Editing

Microbiome editing is focused on altering the human microbiome to improve health. It includes various approaches that manipulate the microbial communities in the body.

The first step in microbiome editing of the skin is Skin Microbiome Transplant (SMT) from a healthy “donor” to a “recipient” with the aim to improve the skin of the recipient [189]. This is similar to the concept of a faecal microbiome transfer from a healthy donor to a recipient with digestive issues. Once the positive impact of a SMT is established, scientists can identify key microbial targets by analysing the differences between healthy and imbalanced microbiomes and the recipients pre- and post-transfer. The benefit is the microbiome is transferred as a community giving it a better chance of proliferating on the recipient [189]. The introduction of a complex and diverse array of beneficial microbes, alongside postbiotics, can be particularly effective at restoring a state of dysbiosis. However, challenges include maintaining microbial viability during storage and application, as well as ensuring that the introduced microbes interact with the native microbiome to produce the desired effect. It is difficult as the skin microbiome has a low number of microbes that can be collected and the approach is not scalable [189]. There is demand for more precise modification as introducing a mixture of bacteria that is not standardised or quantified from a donor is not reproducible and carries safety risks.

Specific microbial targets can be identified for enhancement or removal to promote a healthy microbiome balance. Skin bacteriotherapy applies beneficial microbes, or probiotics to the skin, which can be one or multiple strains with the intention of having beneficial effects [189]. Phage therapy offers a more precise approach by using bacteriophages, viruses that target specific bacteria, to selectively remove disease-causing bacterial species while sparing beneficial ones. The specificity of phage makes them attractive for removing individual species in a microbiome, if target specific microbes linked to attractive VOCs could potentially reduce attractiveness to mosquitoes without broad alterations of the skin microbiome [90].

The debate on the use of prebiotics, probiotics and postbiotics in products is ongoing. Probiotics can be administered as single strains, which, while easier to formulate and standardise, may yield inconsistent results depending on the individual's unique skin microbiome and may struggle to colonise the skin effectively. Consortia of multiple strains can offer a greater chance of success and broader applicability but present challenges in

formulation and potential competition among the strains. Prebiotics encourage the growth of beneficial bacteria already present on the skin, and synbiotics, which combine prebiotics and probiotics, enhance the overall balance and function of the skin microbiome. Postbiotics, which are the by-products of probiotic bacterial activity, can also be utilised for skin health. Postbiotics can include the VOCs known to be involved in mosquito differential attraction, manipulating them could have immediate repellent impact.

In summary, microbiome editing tailors interventions for the skin, aiming to restore balance, enhancing health. Microbiome editing approaches could be utilised in the future for the development of novel mosquito repellents by altering the skin's microbial profile to reduce attractiveness to mosquitoes.

1.08) Aim and Objectives

Aim

This project aims to determine the contribution of the skin microbiome, body odour and genetics in human attractiveness to mosquitoes.

Objectives

1) Review the literature on skin microbiome and human attractiveness to mosquitoes, and detail the experimental approach (Chapter 1).
2) Investigate differences in attractiveness to mosquitoes between identical and non-identical twin pairs (Chapter 2).
3) Investigating differences in skin microbiome composition between highly- and poorly-attractive people (Chapter 3).
4) Investigating differences in odour profile between highly- and poorly-attractive people (Chapter 4).
5) Investigate if transferring skin microbiome from poorly- to highly-attractive participants can reduce human attractiveness to *Aedes* mosquitoes (Chapter 5).

Anticipated outcomes

- Estimate heritability of attractiveness to *Anopheles coluzzi* mosquitoes in both cohorts
- Identify differences in microbial composition between poorly- vs highly-attractive
- Identify microbes that explain differences in attractiveness poorly- vs highly-attractive
- Identify differences in odour profiles between poorly- vs highly-attractive
- Associate microbes of interest with volatiles of interest to identify microbial volatiles candidate bacteria produce select putative repellents
- Screen participants behaviourally with *Aedes aegypti* to select poorly-attractive “donors” and highly-attractive “recipients”
- Establish skin microbiome transfers method, investigate if there is a reduction in attractiveness to *Aedes aegypti* and which microbes it appears to be correlated with

1.09) General Methodology

Throughout this PhD project VOC, microbiome and behavioural data have been collected and analysed. The main methods for each of these will be covered in detail in the respective chapter, chapter 2 behavioural assays and genetics, chapter 3 microbiome, chapter 4 volatile analysis and Chapter 5 skin microbiome transfers (SMT). Some of these methods were previously established in the Logan research group. The microbiome and SMT methods required optimisation at LSHTM for this project.

Overview of the study

Datasets from twins in the UK and The Gambia are part of a larger project, GenoScent, which aimed to recruit 50 monozygotic (MZ) and 50 dizygotic (DZ) twin pairs in both settings. Full ethical approval for each study was received from the relevant committees. UK ethics was approved by the LSHTM ethics committee on 13/12/17 with reference 14500. Gambian ethics was approved by The Gambian government and medical research council (MRC) Gambia on 12/02/2020 with reference 17537, then by the LSHTM ethics committee on 13/02/2020 with approval number 17537. Additionally, ethical approval was received for a secondary data analysis of all GenoScent data from the LSHTM ethics committee on 15/01/2020 with reference 18044. In 2021 my MSc student gained ethical approval 25937 on 05/07/2021 for a pilot SMT study and later received further ethical approval to repeat this with reference 26659 on 15/03/2022.

The UK cohort of MZ and DZ same-sex twin pairs was recruited from the TwinsUK database. The twins were identified and invited to come to LSHTM in London for sample collection. The Gambian cohort of MZ and DZ same-sex twin pairs were recruited from and around Basse, eastern The Gambia. These twins were identified from a Demographic Surveillance Survey and discussion with community leaders.

All participants provided written or thumbprint informed consent or assent to provide socks (for attractiveness screening), foot odour (for volatile analysis) and microbiome swabs (for microbiome sequencing). Additionally, in The Gambia, blood samples were collected to test for malaria infection by a rapid diagnostic test, PCR and to determine zygosity.

2) Chapter 2: Human genetics and attractiveness to mosquitoes

2.01) Statement of multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper	The design of sample collection occurred before I started my PhD. My PhD uses the mosquito behaviour data collected during the GenoScent project. UK attractiveness samples (sock) were collected in 2018 by Catherine Oke and Dr Julien Martinez (during my MSc). The UK wind tunnel assays were run by Catherine Oke and Elizabeth Pretorious in 2018/19 during my MSc. I was involved in running some wind tunnel behavioural assays with Catherine Oke to learn the methodology. I co-led setting up fieldwork in the Gambia with Catherine Oke. The majority of the Gambian attractiveness samples were collected by a field team in The Gambia and shipped back to the UK due to COVID-19 disruption. Dr Robert Jones analysed the zygosity data for the UK cohort and supervised research assistants working on the project as well as supporting with manual peak alignment of peaks. There were issues with the wind tunnel in 2020, me and Scott Tytheridge designed and tested the cage assay we used to measure mosquito behaviour of Gambian samples. Lauren Marchant ran most of the cage assays, I supported with training and rearing. I conceived the experimental and statistical approach to create attractiveness groups and investigate contribution of human genetics to attractiveness. I received advice and feedback on the analysis from Dr Julien Martinez, Dr Ernest Diez Benavente, Dr Salvador Gezan and Dr Chrissy Roberts.

Student signature:

2.02) Introduction

The genetic basis of attractiveness to mosquitoes could have important implications for mosquito control strategies. If it is determined that genetic factors make a contribution to attractiveness, then it may be possible to identify those genes that make some individuals less attractive to mosquitoes. These genes may be involved in the production of natural repellents or modulate the skin microbiome, resulting in microbiome-mediated repellent effects. If such genes are identified, it may be possible to develop drugs that increase the production of natural repellents, which could reduce biting rates and, subsequently, the transmission of pathogens.

It is well documented that there is differential attractiveness to mosquitoes between individuals, with some individuals being more attractive than others to mosquitoes [38,142,178,190]. These differences in attractiveness are due to the production of natural repellents or attractants. Various factors, including diet and pregnancy, have been recognised as co-variates that explain some of the inter-individual variations in attractiveness to mosquitoes [148,162]. The role of genetics in human attractiveness to mosquitoes remains less well-explored. In this chapter, MZ, DZ and unrelated pairs will be compared to disentangle the shared genetic and environmental factors.

Several genetic studies have been conducted to explore whether there is a genetic component of attractiveness to mosquitoes. One such twin study used distance matrices to indicate that the blend of carboxylic acids in human odour is more similar in MZ twins than in random individuals, suggesting a genetic component to odour production [177]. In another twin study (the pilot to the study presented in this thesis) attractiveness of twins to *Aedes aegypti* mosquitoes was experimentally investigated, and the heritability was estimated to be between 62 - 67 % [179]. The study used a Y-tube olfactometer, a Y-shaped plastic chamber, to test Twin A vs control (clean air), Twin B vs control and Twin A vs Twin B, where Twin A and B belong to the same twin pair. However, it did not make a comparison of the Twin A vs Twin B data to determine if there is a larger difference in attractiveness for DZ twins compared to MZ twins.

The study presented in this thesis aims to fill some of the research gaps by comparing the attractiveness of MZ and DZ twins to the malaria vector *Anopheles coluzzii.* Additionally, it incorporates a cohort of twins from the Gambia, a natural human-*Anopheles* population. This chapter aims to unravel the role of genetics in attractiveness to mosquitoes by

comparing the differences in attractiveness between twin pairs. A higher concordance for mosquito attraction in MZ twins than DZ twins would suggest a significant genetic component to human attractiveness to mosquitoes. This is, to my knowledge, the first investigation that employs a twin study to begin deciphering the genetic contribution to human attractiveness to *Anopheles* mosquitoes.

2.03) Aim and objectives

This chapter aimed to identify, through observed mosquito behaviours, whether host-seeking was substantially influenced by human genetic factors.

This aim was achieved through the following objectives:

1) To collect odour samples (socks) from the feet of two cohorts of twins (in the UK and The Gambia) and measure attractiveness to *Anopheles coluzzii* mosquitoes behaviourally.

2) To adjust the resulting measures of attractiveness to mosquitoes for covariates also measured in the behavioural experiments and investigate if adjusted attractiveness to mosquitoes is normally distributed.

3) To select unattractive and highly attractive groups to compare in further analysis.

4) To examine if there is a relationship between proportional gene sharing and similarities in attractiveness to mosquitoes between MZ and DZ twins.

5) To estimate the heritability of attractiveness to mosquitoes.

2.04) UK cohort

In the UK, female twin pairs were recruited from the Twins UK cohort (N = 176, 38 MZ and 50 DZ pairs), and written informed consent was obtained from all human volunteers. Twins were white European, post-menopausal, female twin pairs aged between 50 and 90 years, who were recruited from the TwinsUK database https://twinsuk.ac.uk/ from the Department of Twin Research, Kings College London (KCL ethics reference E892). Post-menopausal women were selected to reduce effects that may be seen with hormonal fluctuations during the menstrual cycle that are likely to impact odour profile and, therefore, attractiveness to mosquitoes [191].

2.04.01) Measuring participant's attractiveness to mosquitoes using a wind tunnel

Expected hypothesis (H0): There is no difference in attractiveness to mosquitoes among individuals. This means that all individuals, on average, are equally attractive to mosquitoes.

Alternative hypothesis (H1): There is a difference in attractiveness to mosquitoes among individuals. This means that some individuals are more or less attractive to mosquitoes than others.

Figure 9 shows the expected progression of mosquitoes over time. The set-up, pre-release from the release chamber (Figure 9A). During the flight, after the release gate is opened when most mosquitoes are in the main chamber of the tunnel (Figure 9B) and after the experiment when most mosquitoes have made a choice of which trap to enter (Figure 9C).

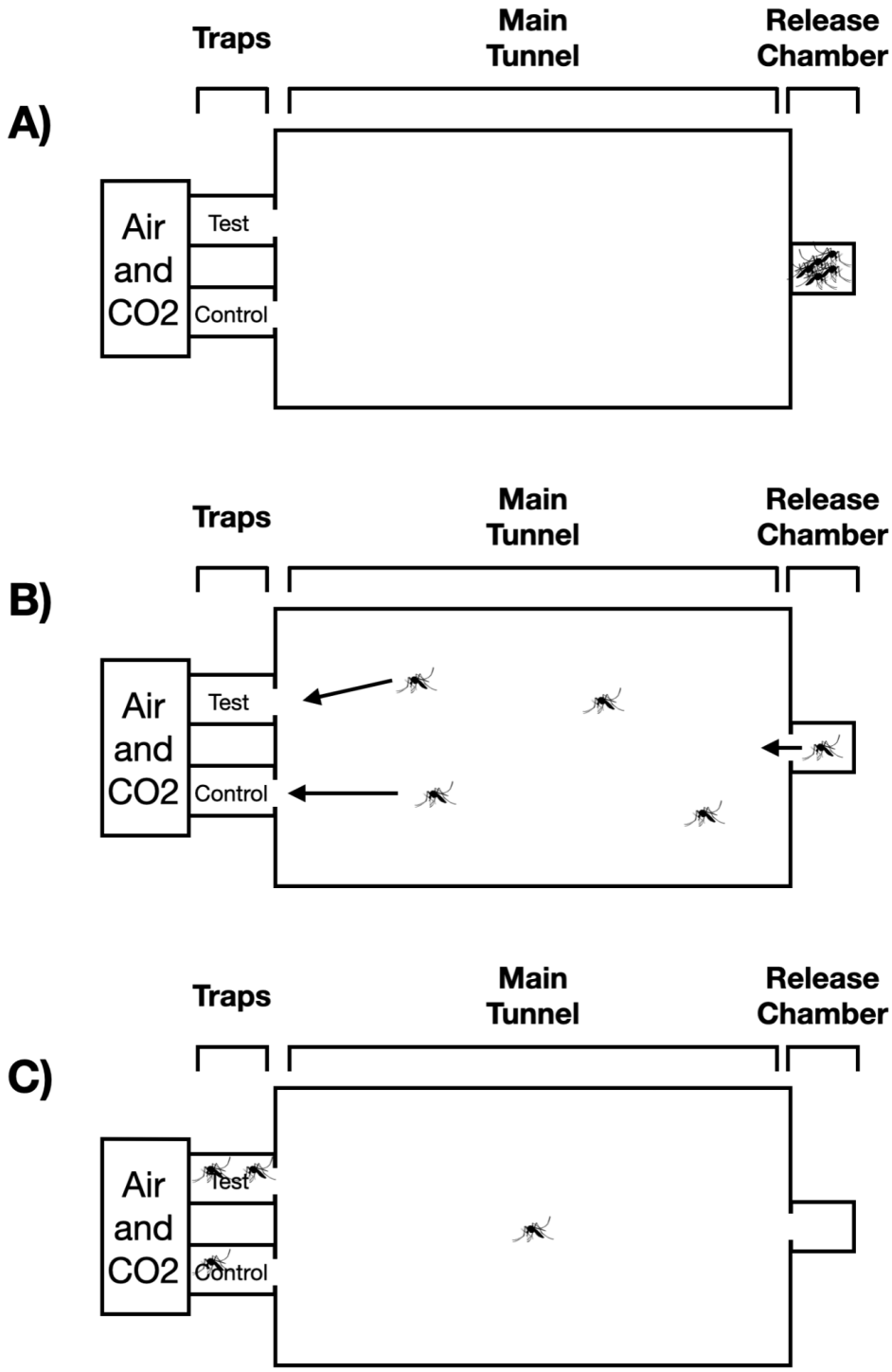

Figure 9. Expected progression of mosquitoes through the wind tunnel A) Pre-release from the release chamber (before the gate is opened), all mosquitoes are in the release chamber B) During the assay, mosquitoes fly from the release chamber into the main tunnel where they can follow the odour plumes from the traps and make a choice to enter either the trap containing a test or control sock C) Most mosquitoes are expected to make a choice by the end of the 20-minute assay but some mosquitoes may remain in the main tunnel or the release chamber.

Body odours were collected from participants using a pair of nylon socks (100 % polyamide, 15 deniers), prewashed with 70 % ethanol and air-dried. Participants were asked to avoid consuming alcohol or strong-smelling foods for 48 hours before sample collection and avoid applying cosmetic products to the feet and lower legs except for odour-free soap provided (Simple, Unilever). Participants wore the socks overnight for 7-8 hours in their own homes, removed them and placed them into sterile glass jars. Participant socks were brought to LSHTM in the glass jar the day they were removed. We stored the socks at -20 ^{o}C until used in behavioural experiments.

All mosquito behavioural studies using *Anopheles* mosquitoes were carried out using non-blood-fed *Anopheles coluzzii* mosquitoes (Ngousso strain, originated in 2006 in Yaoundé, Cameroon (#MRA-1279, BEI Resources, Maryland, United States) maintained in a colony at LSHTM. Before the experiment, mosquitoes were maintained at 26±1°C and 70 % humidity under a 12/12 light/dark cycle. Larvae were reared using tap water and pond pellets (Dr Clarkes Pond Pellets, Pettex Ltd, Ilford, UK). The colony was maintained using human blood collected from volunteers at LSHTM. The stock cage was blood fed 1-2 times a week using a hemotek, human breath and odour (worn sock) were used to encourage blood feeding.

Non-blood-fed experimental adults, 5-8 days old were fed on 10 % glucose solution and housed in 15 x 15 x 15 cm fabric cages (BugDorm-4M1515 Insect Rearing Cage, BugDorm, Taichung, Taiwan). Cotton gloves were worn while handling the socks to prevent odour contamination. The participant's socks were removed from the freezer 1-2 hours before assays, remaining in the glass vials to defrost to room temperature. Mosquitoes were collected before the experiment and given 1 h to acclimate in the assay room and a further two minutes after the lights were switched off to acclimatise before being released into the wind tunnel. Experiments were performed during the last 4 h of the mosquito scotophase (i.e. between 9 am and 1 pm for mosquitoes on an L:D cycle from 1 am to 1 pm) in a controlled environment with no light sources (27 ± 2 °C; 70 ± 10 % RH).

The attractiveness of human odour collected from the UK twins on each sock to *Anopheles coluzzii* mosquitoes was tested in a dual-choice wind tunnel assay based on published methodology [192]. Twenty non-blood-fed female mosquitoes (5-8 days old) were used in each replicate. Mosquitoes were given 1 hour in the assay room.

The two-choice wind tunnel based on Knols et al. (1994) is shown in Figure 10. This was done using two identical tunnels. Each sock was measured once in each tunnel. Two tunnels allowed for two assays to be run simultaneously at any given time. Mosquitoes were

released into the tunnel on the right-hand side, flew across the flight chamber and into the small holes on the left-hand side of the flight chamber.

Figure 10. Overview of the dual choice wind tunnel used for behavioural experiments to test attractiveness to Anopheles coluzzii mosquitoes using worn socks in the UK cohort. On the right-hand side is the release chamber. Traps to catch the mosquitoes are behind the white panel on the left-hand side.

Participant or control socks were placed on a wire frame (~8cm diameter), as shown in Figure 11.

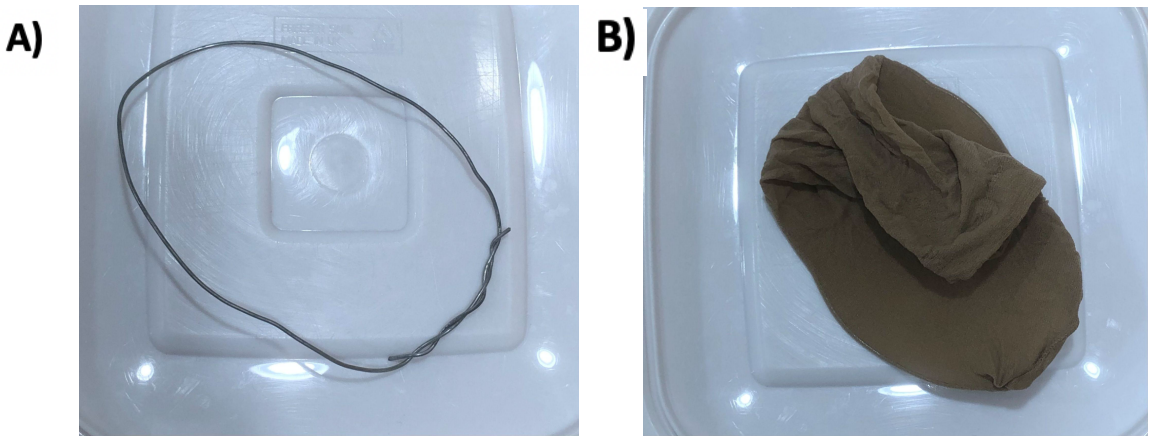

Figure 11. A) The metal wire frame and B) Metal wire frame inside a cotton sock.

The sock on the wireframe was then placed inside a trap, as shown in Figure 12. The trap consisted of a cylinder measuring 12 cm in diameter. The funnel where the mosquitoes enter connects to the main tunnel. The funnel measured 5 cm in diameter on the right-hand side closest to the tunnel where the mosquitoes enter and 1 cm at the smallest point of the funnel. The funnel meant the mosquitoes could not escape once they entered the trap. The other end had a lid that fitted to the airflow. There were two traps. In one, the sample was placed, and in the other, a control (clean, unworn sock). Each sock was tested twice against an unworn sock with a fresh batch of mosquitoes. Samples were block randomised.

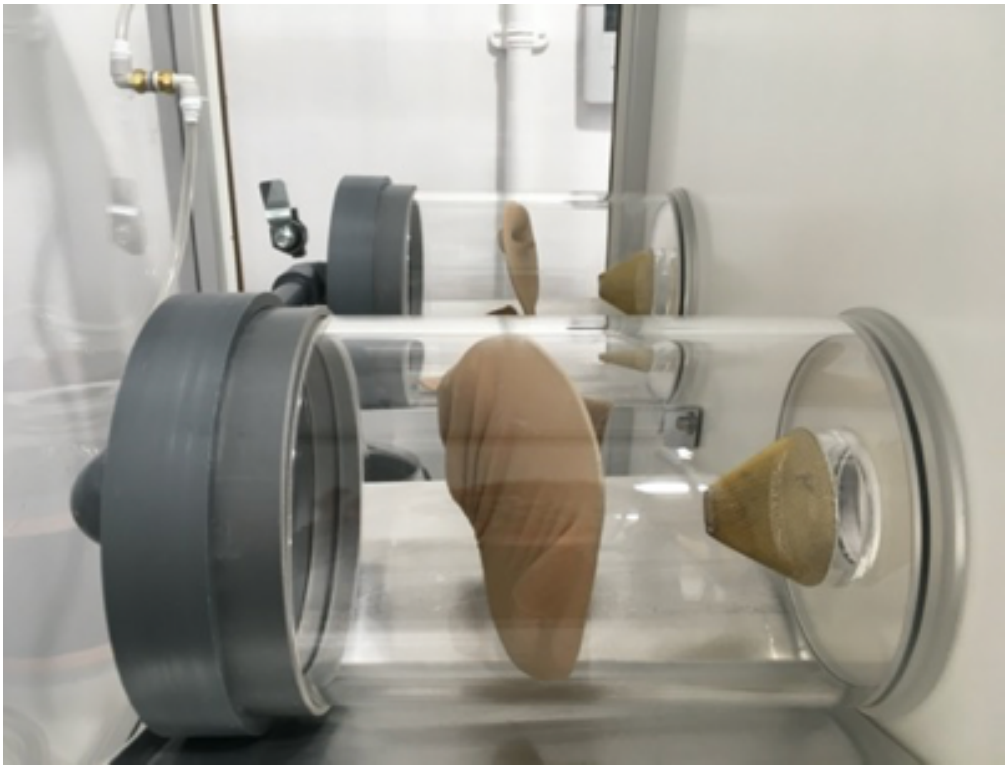

Figure 12. The trap behind the white panel of the wind tunnel (shown on the right-hand side of the image). Socks are placed inside each of the two traps on wireframes. Air flows from the left to the right through the trap carrying the body odour from the sock into the wind tunnel. Mosquitoes enter the hole on the right-hand side of the trap through the funnel, the entry point from the main wind tunnel.

Charcoal-filtered, humidified air (60 % humidity) was supplied to the flight chamber at 0.20 ± 0.01 m/s and warmed by blowing the air through a jar of water that was heated using a heating pad, as shown in Figure 13. This heated air flows through the trap in Figure 12.

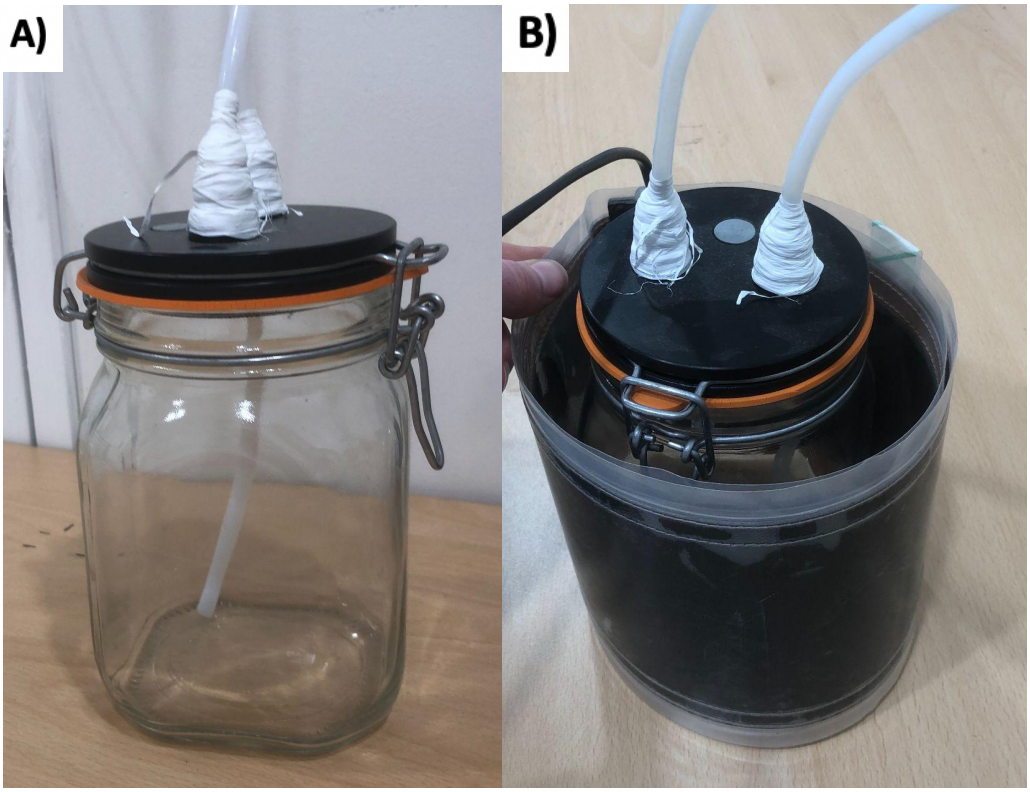

Figure 13. A) The mason jar to which water is added has an in and out air outlet, and B) the jar was wrapped in a heating pad.

A labelled figure for the trap set-up and the direction of airflow from the heated jar through the trap carrying the odour from the sock into the wind tunnel is shown in Figure 14.

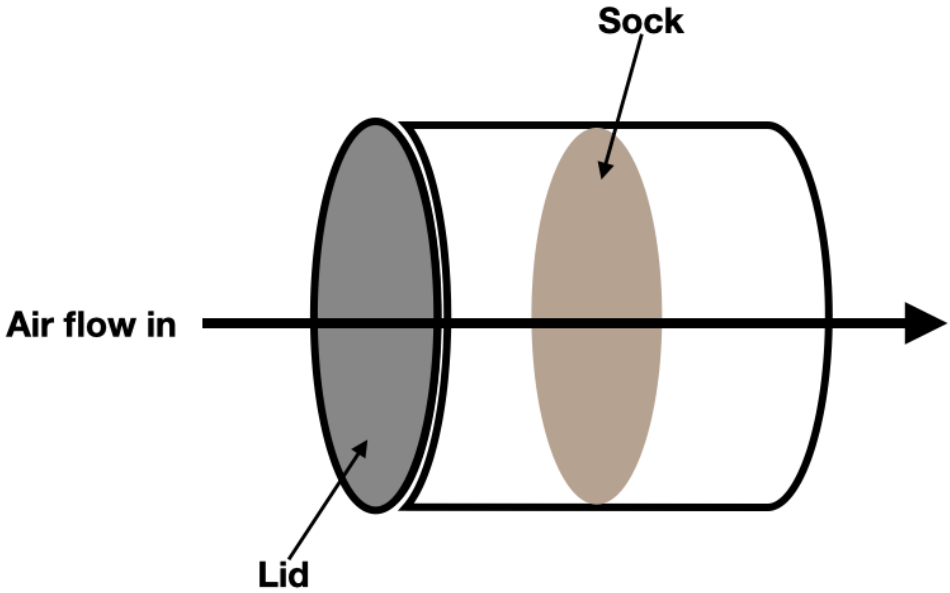

Figure 14. The direction of airflow through the trap into the wind tunnel was used for behavioural experiments to test the attractiveness to Anopheles coluzzi mosquitoes using worn socks in the UK cohort.

Additionally, 5 % CO_2 was supplied at 175 ml/min to activate mosquito host-seeking (De Boer et al. 2017). Figure 15 shows the CO_2 released into the release chamber below the trap/ host odour through a small hole for each trap.

Figure 15. The entry from the main tunnel to the traps is through the top large hole, the CO2 is released from the pipe below the trap entry hole. There are two holes and CO2 releases, one for each trap.

Figure 16 shows the release chamber used to release 20 female mosquitoes into the tunnel. It measures 8 cm in diameter and has a release gate. The hole at the top is used to aspirate the mosquitoes into the release chamber and is sealed with cotton wool to prevent them from escaping into the testing room. Mosquitoes are released from the left-hand side into the main tunnel of the wind tunnel using the release gate.

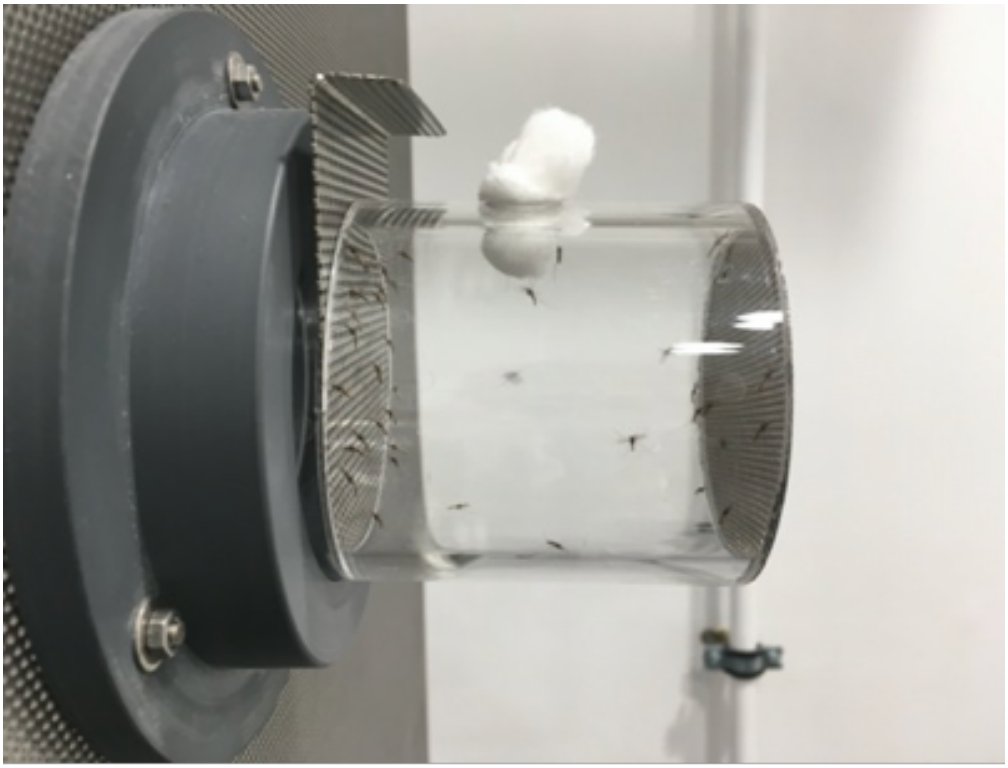

Figure 16. Release chamber containing 20 female Anopheles coluzzii mosquitoes. The release gate on the left-hand side releases them into the main wind tunnel. Mosquitoes are added to the release chamber using a pooter into the hole at the top, which is then sealed with cotton wool.

Mosquitoes were released from the release chamber and given 20 minutes to choose an odour source in dim light, after which the choice was recorded. The mosquitoes were anaesthetised using CO_2. No people were present in the experiment room while the assays were running. Traps were cleaned with 30 % ethanol between assays, and the wind tunnel was cleaned at the end of each day with 70 % ethanol.

2.04.02) Calculating participant attractiveness scores

Four measures of attractiveness were calculated: relative response (RR), relative attractiveness (RA), absolute attractiveness (AA) and flight activity (FA). The equation for each is shown in Table 2.

Table 2. Equations for calculating each of the attractiveness measures in the wind tunnel for the UK cohort.

Attractiveness measure	**Equation**
Relative response (RR)	$RR = \frac{\text{Number of mosquitoes which entered any trap}}{\text{Total number of mosquitoes released}}$
Relative attractiveness (RA)	$RA = \frac{\text{Number of mosquitoes that chose human trap}}{\text{Number of mosquitoes which entered any trap}}$
Absolute attractiveness (AA)	$AA = \frac{\text{Number of mosquitoes that chose human trap}}{\text{Total number of mosquitoes released}}$
Flight activity (FA)	$FA = \frac{\text{Number of mosquitoes that left release chamber}}{\text{Total number of mosquitoes released}}$

2.04.03) Exploring and adjusting attractiveness scores for covariates

All twin data were first filtered to have a RR >35 %, i.e. at least 35 % of the mosquitoes chose to enter one of the traps. A low RR would indicate an issue with the replicate. In this study, we will not be considering FA as a measure of mosquito attraction as all tests had high flight activity.

The main focus of my investigation is on the attractiveness of the trap containing a participant's sock to mosquitoes. There are two key measures of interest: relative attractiveness and absolute attraction we chose to investigate. Relative attractiveness refers to the proportion of mosquitoes that select the trap with the participant's sock out of those that have made a choice. In contrast, absolute attraction refers to the proportion of mosquitoes that select the trap with the participant's sock out of all the mosquitoes that are released. It is important to note that these measures are interconnected and were influenced

by factors such as the total number of mosquitoes (which is approximately 20 in our experiments).

Raw attractiveness measures in the wind tunnel are potentially influenced by external variables like temperature and wind speed. Covariates that improved the adjusted R-squared of linear regression models were corrected for by adjusting the raw attractiveness.

Definitions of covariates measured and tested as covariates in the wind tunnel:

- Temperature = Temperature in the wind tunnel room at the time of the run
- Humidity = Humidity in the wind tunnel room at the time of the run
- Time of the day = Time of the day the replicate was run
- Average wind speed = Average wind speed for the run (usually 0.19 – 0.21 m/s)
- Tunnel = Tunnel used, two identical tunnels in the same room (tunnel 1 and 2)
- Number released = Total number of mosquitoes released from the release chamber for that run

2.04.04) Comparing absolute and relative attractiveness to mosquitoes

The Pearson correlation analysis between absolute and relative attractiveness showed an R-squared value of 0.801 with a 95 % confidence interval of [0.761, 0.835]. This suggests that 80.1 % of the variability in relative attractiveness can be accounted for by absolute attractiveness, as illustrated in Figure 17. The two attractiveness measures display a moderate positive correlation. In investigating attractiveness among the UK cohort, the relative attractiveness measure was selected. This measure represents the fraction of mosquitoes that were drawn towards the human trap relative to the total number of mosquitoes that entered any trap, thereby indicating the preference of mosquitoes that made an active choice.

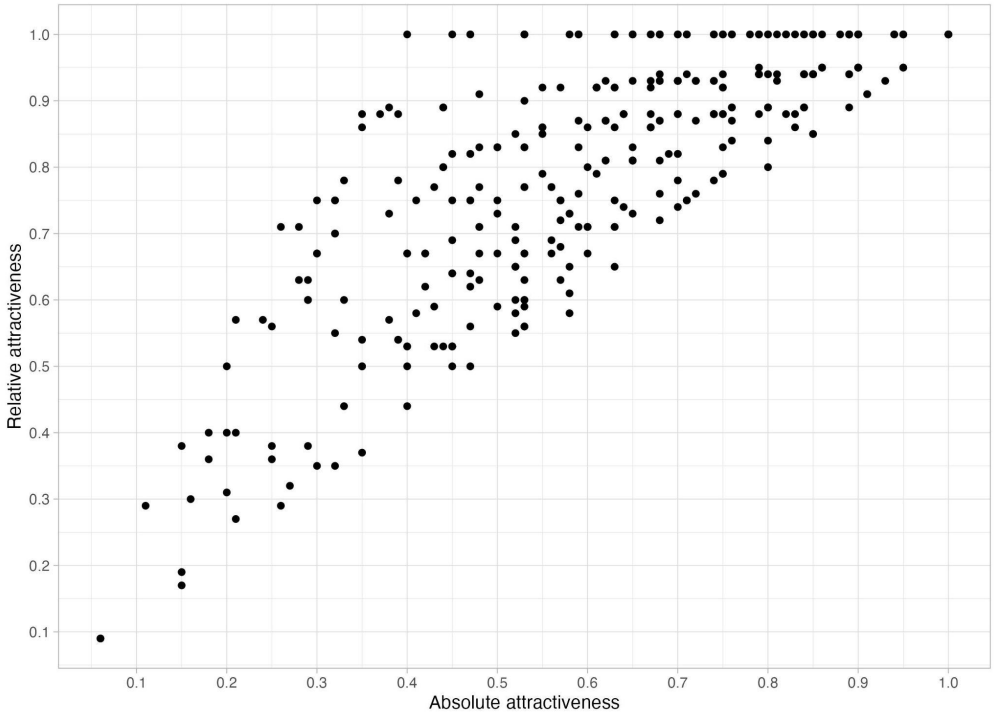

Figure 17. Positive Pearson correlation between raw absolute attractiveness and raw relative attractiveness to mosquitoes.

Initial exploratory analysis of the covariates was conducted by plotting each covariate against the raw attractiveness measures to assess trends. There were similar relative and absolute attractiveness trends, including increasing humidity with increasing attractiveness. Next, differences in covariates between MZ and DZ twins were investigated. Table 3 shows there is a significant difference in BMI. There were more obese DZ twins than MZ twins. No significant differences in the other covariates.

Table 3. Baseline covariate comparison between MZ and DZ twins for the UK cohort. The age group labelled 'unknown' is a result of unrecorded data. All women were over 50 and post-menopausal. P-value fishers exact test.

Characteristic	**MZ, N=76**	**DZ, N=100**	**P-value**
Age category			0.7
50-59	6 (13%)	6 (8.6%)	
60-69	18 (39%)	27 (39%)	
70-79	18 (39%)	33 (47%)	
80-89	4 (8.7%)	4 (5.7%)	
Unknown	30	30	
BMI category			0.02
Underweight	0 (0%)	0 (0%)	
Normal	37 (62%)	35 (42%)	
Overweight	19 (32%)	32 (38%)	
Obese	4 (6.7%)	17 (20%)	
Unknown	16	16	
Sex			>0.9
Male	0 (0%)	0 (0%)	
Female	76 (100%)	100 (100%)	
Smoker			0.4
Yes	1 (1.4%)	4 (4.0%)	
No	73 (99%)	93 (93%)	
Vape	0 (0%)	2 (2.0%)	
Occasionally	0 (0%)	1 (1.0%)	
Unknown	2	0	
Statin user	18 (24%)	27 (27%)	0.6
Antibiotic used	1 (1.3%)	0 (0%)	0.4
Hormone drug user	4 (5.3%)	9 (9.0%)	0.3
Mood endocrine drug user	14 (19%)	19 (20%)	>0.9

I conducted multivariate linear regression to control for significant covariates to refine the measure of individual relative attractiveness to mosquitoes in the UK cohort. This approach allowed me to adjust for factors other than inherent attractiveness that could impact the attractiveness measure, yielding a more precise estimate of attractiveness for each individual. The regression model's results are presented in Table 4. Additional outputs from the regression included the adjusted R-squared, which quantifies the proportion of total variation in relative attractiveness that can be accounted for by the model's predictor variables. The residual standard error, another important metric, estimates the standard deviation of the model's unexplained errors (or residuals). This represents the variation in relative attractiveness that the selected predictor variables could not account for.

Table 4. Regression results after adjusting for relative attractiveness, incorporating all covariates measured in the wind tunnel.

Term	Estimate	Standard error	Statistic	P value
(Intercept)	-2.385	0.876	-2.721	0.007
TimeC	0.436	0.201	2.164	0.031
Temp	0.092	0.032	2.860	0.004
Humid	0.009	0.002	5.430	0.000
Tunnel	0.009	0.021	0.430	0.668
AvgWindSpeed	1.729	1.291	1.339	0.181
Nmos	-0.006	0.007	-0.911	0.363

The most effective selection of covariates, which led to the greatest improvement in the R-squared value, excluded average wind speed, as depicted in Table 5. Thus, for a more accurate measurement of relative attractiveness to mosquitoes in the UK cohort, the time of day, temperature, humidity, tunnel, and number of mosquitoes were adjusted for. This process resulted in a more refined measure of attractiveness after adjusting for these covariates.

Table 5. Regression results after adjusting for relative attractiveness, including all covariates included in the final model.

Term	Estimate	Standard error	Statistic	P value
(Intercept)	-2.086	0.848	-2.459	0.014
TimeC	0.441	0.202	2.185	0.029
Temp	0.094	0.032	2.931	0.004
Humid	0.009	0.002	5.360	0.000
Tunnel	0.009	0.021	0.432	0.666
Nmos	-0.007	0.007	-0.961	0.337

The adjusted attractiveness was estimated using the formula:

$$Adjusted\ Att\ =\ Raw\ Att\ +\ \beta 0\ +\ (\beta 1 * covariate\ 1)\ +\ (\beta 2 * covariate\ 2)\ +\ (\beta X * covariate\ X)$$

Here, β0 is the intercept term, the baseline level of the relative attractiveness (response variable) when all predictors are zero. β1, β2, and βX are the coefficients assigned to each respective covariate added to the model. In order to negate the effects of positive covariates, the covariates were added to the raw attractiveness and intercept terms, and the signs of their coefficients were inverted.

Therefore, for relative attractiveness, the formula was:

$$Adjusted\ Att\ =\ Raw\ Att\ -2.085924\ +\ (-0.44050\ *Time)\ +\ (-0.008963 * Tunnel)\ +\ (-0.093884\ *\ Temperature)\ +\ (-0.008958 * Humidity) + (0.006608 * Number\ of\ mosquitoes)$$

I then visualised how the adjusted attractiveness correlated with the raw attractiveness as shown in Figure 18. The final model for relative attractiveness gave an adjusted R-squared 0.897 [0.879, 0.914], which indicates that 89.7 % of the variation in the adjusted relative attractiveness is explained by the raw relative attractiveness.

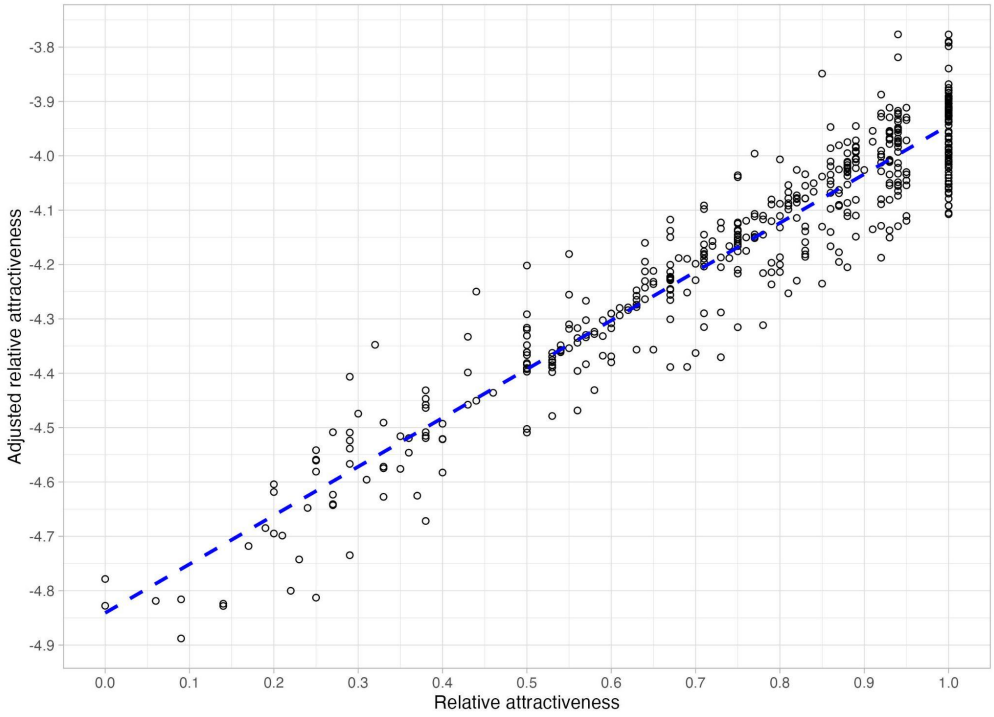

Figure 18. Scatter plot to show the strong positive correlation between raw relative attractiveness and adjusted relative attractiveness. The blue lines show linear regression.

After obtaining the adjusted relative attractiveness scores for each participant from their two replicates of the behavioural experiment using the same participant sock, the average score were calculated to obtain a single measure of relative attractiveness for each participant for use in Chapters 3 and 4.

2.04.05) The difference in attractiveness between positive and negative controls

In the behavioural experiments, unworn socks cleaned with 70 % ethanol and allowed to dry were used as multiple controls. In the negative control trials, two identical socks were compared: a clean, unworn sock was set against another clean, unworn sock. Every sample tested from a participant was similarly compared to a clean, unworn sock. For the positive control tests, a researcher wore a previously cleaned sock. The positive control was tested against a clean, unworn sock.

The attractiveness of positive and negative controls was tested in the wind tunnel against an unworn sock. The relative attractiveness results are shown in Figure 19 (Raw scale) and Figure 20 (adjusted scale). The findings showed that the positive controls were significantly more attractive than the negative controls (t = -11.61, df = 90.45, p-value < 2.2e-16), with a mean of -4.06 for the positive control and -4.44 for the negative control. The measure used was the number of mosquitoes that chose the trap containing the control out of the total number that entered either trap. As both traps contained unworn, clean socks in the test of negative controls, this would be expected to be 50 % for the negative control and higher for the positive control, which on the adjusted scale would be -4.418. With the negative control, we saw 6/72 tests of the negative are above 0.75 (i.e. 15 mosquitoes choosing trap A over trap B), which is 8.3 %.

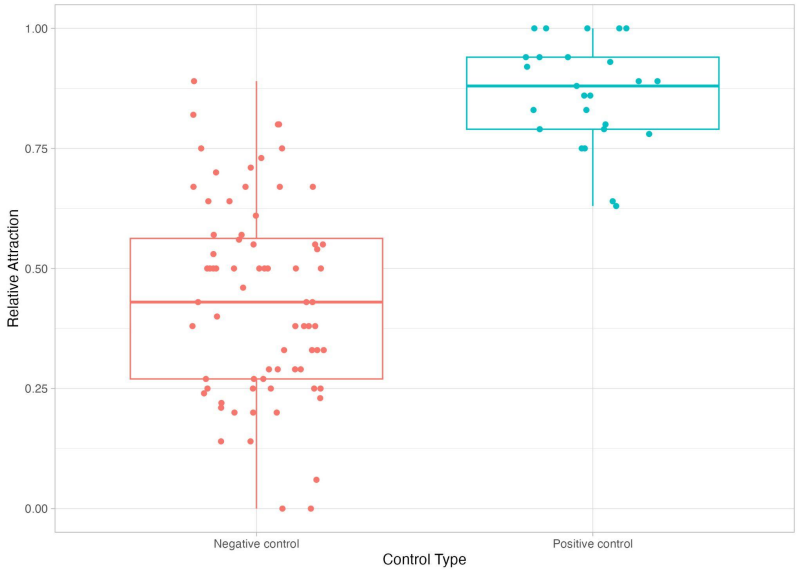

Figure 19. Comparison of the raw relative attractiveness of negative (red) and positive controls (blue) tested throughout the period samples were tested. Each point represents a test with a control compared to an ethanol-washed sock in the wind tunnel on the raw scale.

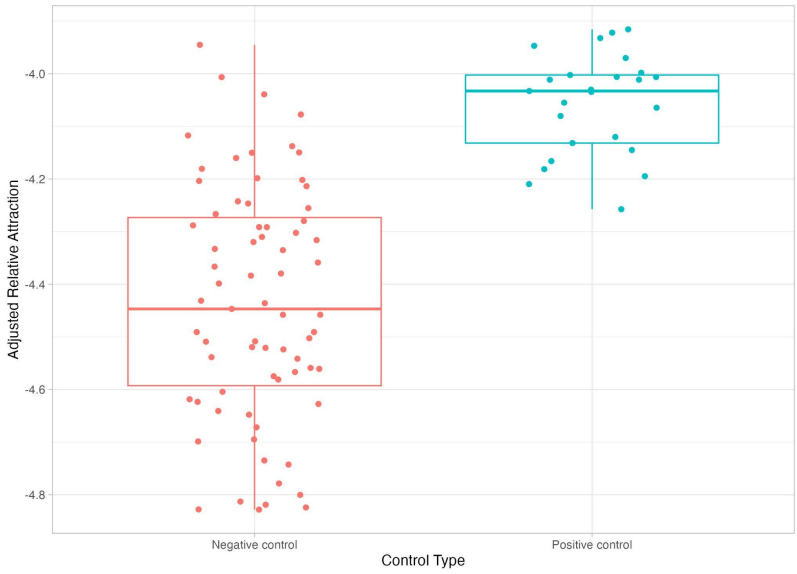

Figure 20. Comparison of the raw relative attractiveness of negative (red) and positive controls (blue) tested throughout the period samples were tested. Each point represents a test with a control compared to an ethanol-washed sock in the wind tunnel on the adjusted scale.

The same control socks, using a fresh control prepared and frozen at a minimum of overnight, are shown in Figure 21. There is a consistent trend of the positive control (blue points) being more attractive than the negative control (red points) on each day of testing. The second replicate begins on 7th December 2018. In the second replicate, the positive control was less attractive than the first replicate. The positive control socks were from the same person in both replicates and treated the same as samples, i.e. frozen and defrosted before testing. Therefore, the positive control sock in replicate two is fresh socks from the same participant who may have declined in attractiveness to mosquitoes between the replicates due to environmental factors. There was more variation in the attractiveness of the negative control in the second replicate.

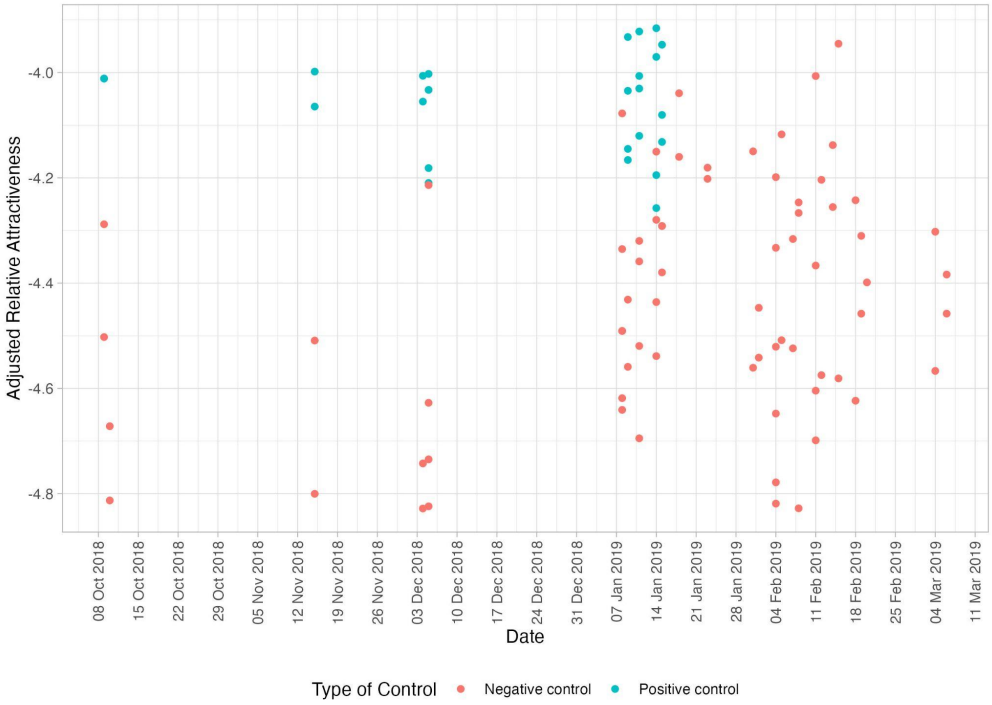

Figure 21. Comparison of the raw relative attractiveness of negative (red) and positive controls (blue) tested over time. The second replicate begins on 7th December 2018. More controls were run in the second replicate.

2.04.06) The difference in attractiveness between unattractive and attractive groups

To check that there were consistent differences between the controls in both replicates, they were compared, as shown in Figure 22. The same trend is seen in both replicates. In replicate 1 the positive control is consistently more attractive than the negative control. There is a smaller difference in the means for positive and negative control for replicate 2 compared to replicate 1. In replicate 1 (t = -8.76, df = 14.33, p-value 3.91e-07), with a mean of -4.06 for the positive control and -4.63 for the negative control. In replicate 2 (t = -8.74, df = 42.57, p-value 4.709e-11), with a mean of -4.06 for the positive control and -4.40 for the negative control. As seen in the second replicate, the results for the negative control are more variable. Overall, there are consistent differences in attractiveness between the positive and negative controls in both replicates.

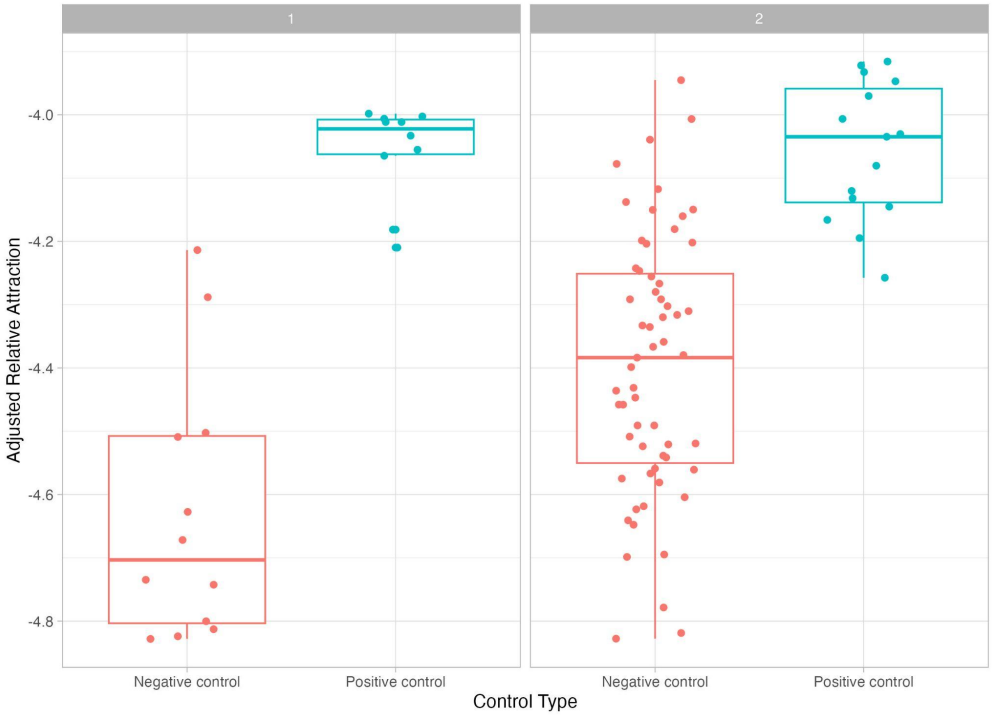

Figure 22. A comparison of the raw relative attractiveness of negative (red) and positive controls (blue) tested throughout the period samples was tested. Faceted by replicate, replicate 1 on the left panel and replicate 2 on the right.

2.04.07) Selecting attractiveness groups

Began by comparing the adjusted relative attractiveness scores for both replicates for each participant to check if any differences in attractiveness between participants were consistent between the replicates, i.e. different batches of mosquitoes tested on a different day with the same participant sock. Figure 23 shows there are some differences between replicates which is unsurprising in a behavioural assay using different cages of mosquitoes as mosquitoes are known to exhibit individual variations in their host preferences.

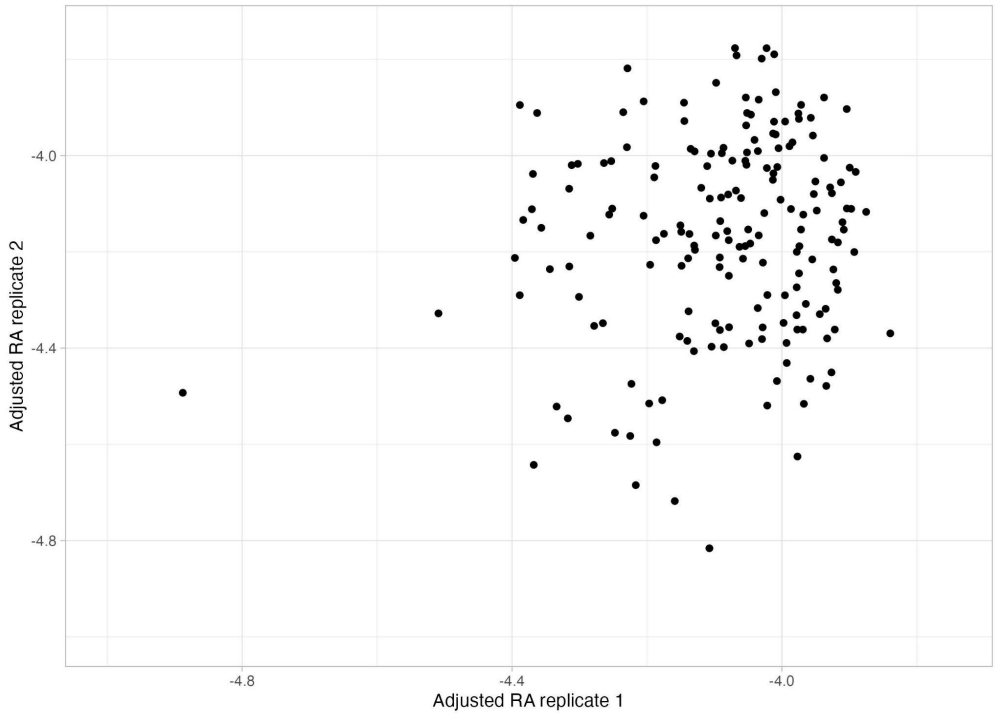

Figure 23. Scatterplot of the adjusted mean relative attractiveness score for each participant in replicate 1 (x-axis) and replicate 2 (y-axis).

Low and high attractive groups were created based on relative attraction scores from both behavioural replicates using the wind tunnel. The highly attractive group was made up of samples that scored above -4.1 in both replicates (N=51). In contrast, the low attractive group was composed of samples that scored below the 95th percentile of the negative control (Adjusted RA =-4.098), with those scoring below that threshold in both replicates classified as low (N=45). All other participants were grouped as Mid (N=84). The selection criteria were designed to check if low and high-scoring participants were consistent across replicates. Figure 24 demonstrates the scores of each person in the low, mid and high groups for both replicate 1 (red points) and replicate 2 (blue points), with the
left facet showing variability in scores in the low group, middle facet variability in the middle group and right facet showing consistently high scores in the high group. Interestingly, the variance between replicates was smaller at the higher end of scores than at the lower end,

suggesting that active attraction played a more significant role in determining the attractiveness of highly attractive samples, while random chance was a more significant determinant for low attractive participants. Notably, no “repellent” group was identified, indicating that no group consistently scored low in attractiveness compared to the negative control. The red dashed line indicated the value for a 0.5 probability adjusted for the average of co-variates. Mosquitoes have no preference for these individuals in the low group compared to the negative controls.

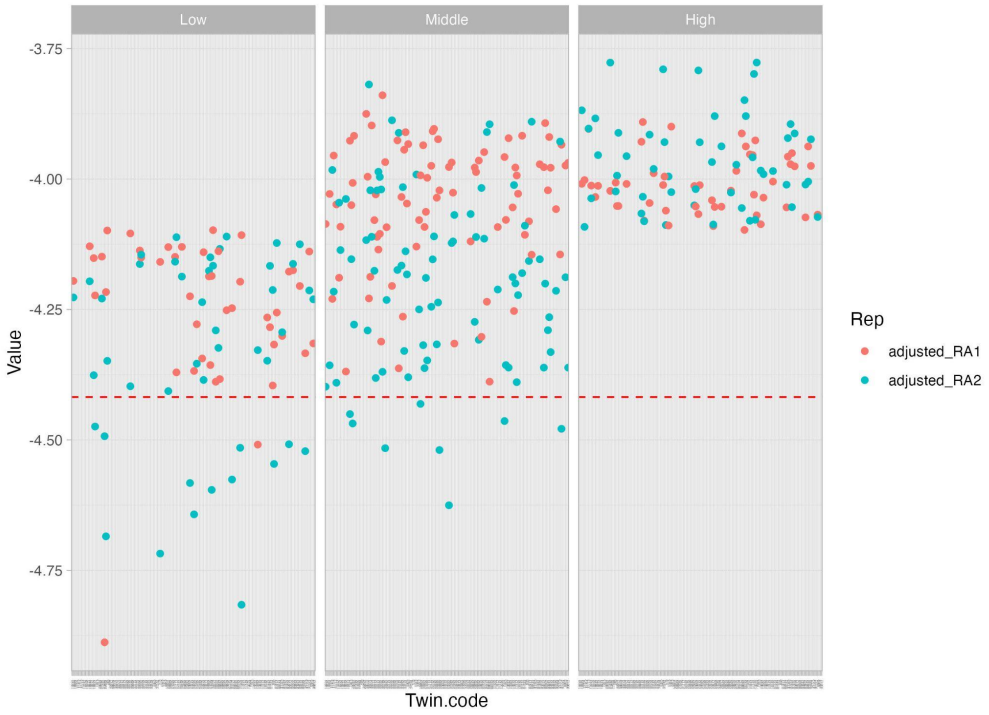

Figure 24. Comparison of the relative attractiveness of replicate 1 (red) and replicate 2 (blue) for each participant faceted by attractiveness group, low on the left and high on the right. The red dashed line shows the adjusted 0.5 probability threshold on the adjusted scale.

The variability between the replicates shows that attractiveness scores are not consistent between replicate 1 and replicate 2 for some of the samples, indicating it is not robust to select the low and high groups based on the extremes and exclude those in the middle. More behavioural replicates were needed. Therefore, two groups were created based on their scores. The 'attractive' group consists of those that consistently scored high in both replicates. Conversely, the 'unattractive' group is for those that did not achieve consistently high scores, specifically those scoring less than the 95th percentile of the negative control (Adjusted RA =-4.096), as depicted in Figure 25. There were N=50 attractive participants and N= 130 unattractive participants.

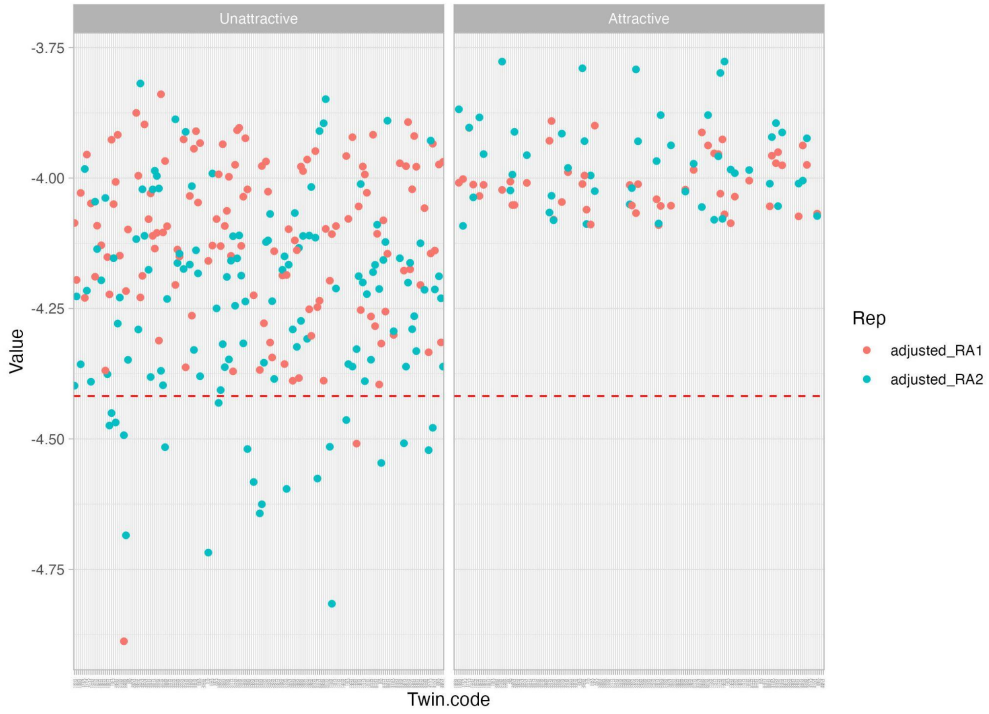

Figure 25. Comparison of the relative attractiveness of replicate 1 (red) and replicate 2 (blue) for each participant faceted by attractiveness group, unattractive on the left and attractive on the right. The red dashed line shows the adjusted 0.5 probability threshold on the adjusted scale.

The adjusted absolute attractiveness scores (adjusted for wind tunnel covariates) for both replicates were combined to get average adjusted absolute attractiveness scores. These are compared for the unattractive and attractive groups, as shown in Figure 26.

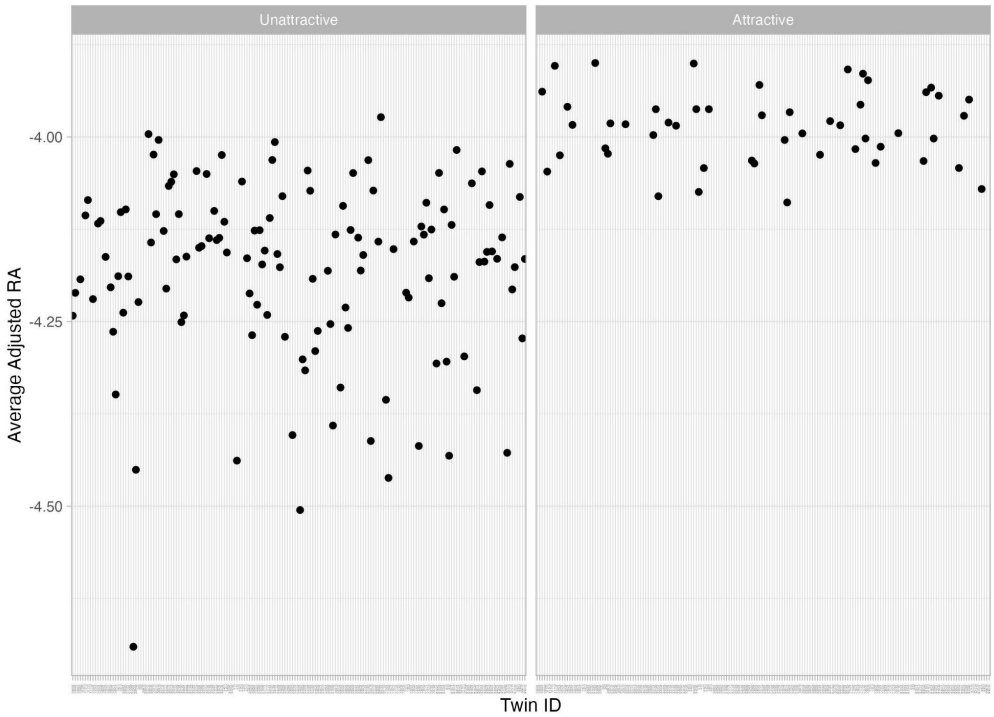

Figure 26. Comparison of the adjusted relative attractiveness across both replicates for each participant faceted by attractiveness group, unattractive on the left and attractive on the right.

The average adjusted RAs for the unattractive (dark blue) and attractive (dark green) groups are summarised in Figure 27. There is a clear difference in attractiveness between the groups. The attractive group is statistically significantly more attractive than the unattractive group (t = -15.41, df = 177.42, p-value < 2.2e-16), with a mean of -4.18 for the unattractive group and -3.99 for the attractive group.

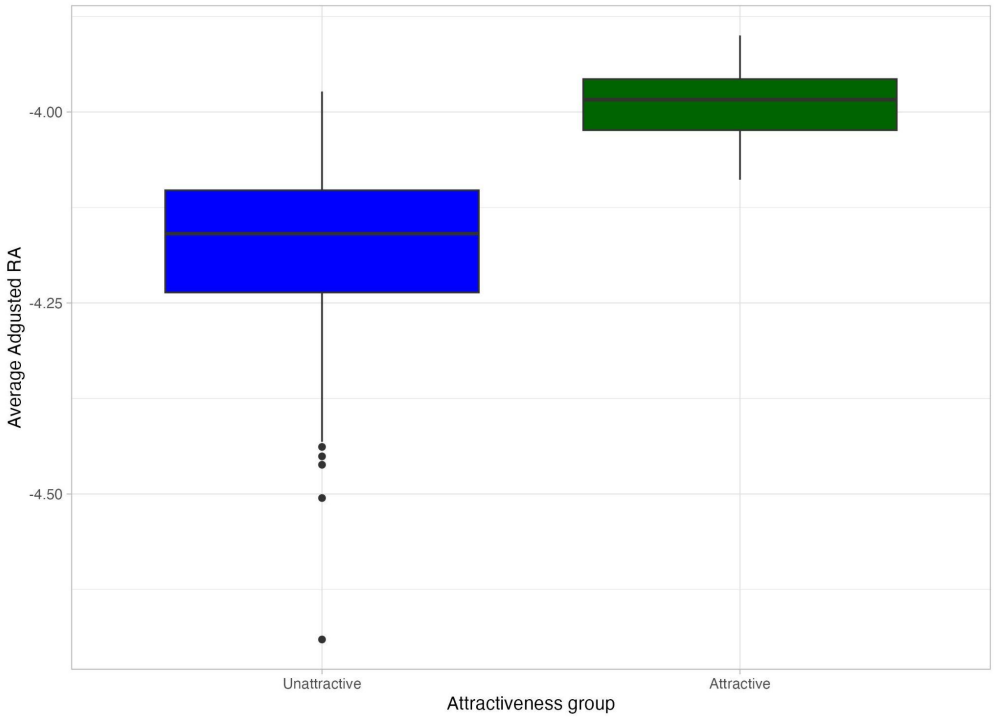

Figure 27. Comparison of the average adjusted absolute attractiveness score across both replicates for participants in the unattractive (dark blue) and attractive groups (dark green).

2.04.08) Individual level: Distribution of participants' attractiveness to mosquitoes

The next step was to investigate the overall distribution of adjusted average relative attractiveness for all participants. Figure 28 shows a density plot of the adjusted relative attractiveness. This is left-skewed. There appears to be fewer poorly attractive participants, while most participants have a relative attractiveness score near the median -4.12.

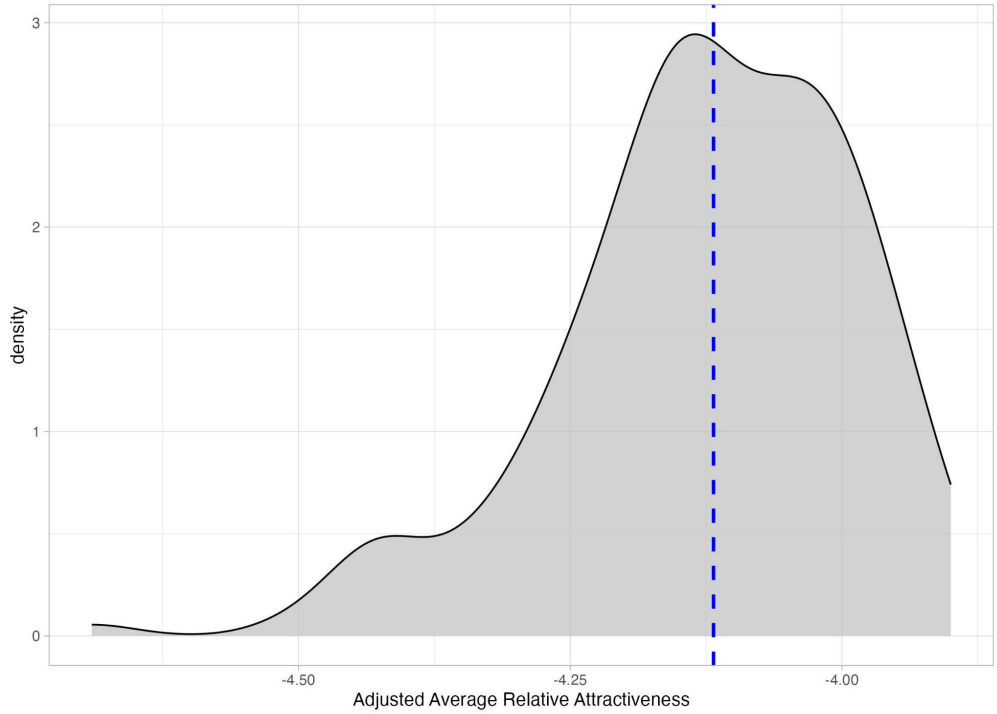

Figure 28. The distribution of relative attractiveness of UK twins to mosquitoes. The median is displayed with a dashed blue line.

2.04.09) Twin pair level: Differences in attractiveness between related and unrelated pairs

Expected hypothesis (H0): There is no difference in the similarity of attractiveness to mosquitoes between MZ and DZ or unrelated pairs.

Alternative hypothesis (H1): MZ twins will exhibit greater similarity in attractiveness to mosquitoes compared to DZ or unrelated pairs.

Next, the correlation in attractiveness for each twin pair were explored between MZ and DZ twins. Scatterplots of each twin in the twin pair against the other twin in the pair (Twin A vs Twin B) were plotted to check for any correlation, as shown in Figure 29. Spearman's correlation coefficients were used to estimate the strength of correlation between twins of the same pair and compared using Fisher's z-transformation test. One-sided p-values are reported, testing if MZ is more correlated than DZ pairs. The scatterplot shows correlations for relative attractiveness to mosquitoes between MZ twins (rho = 0.135, 95 % CI = -0.184 - 0.428) and DZ twins (rho = 0.299, 95 % CI = 0.022 - 0.533). A Fisher's z-test found no

statistical evidence that the MZ twin's relative attractiveness correlation was stronger than that of the DZ twins (z=-1.13, one-sided p=0.258).

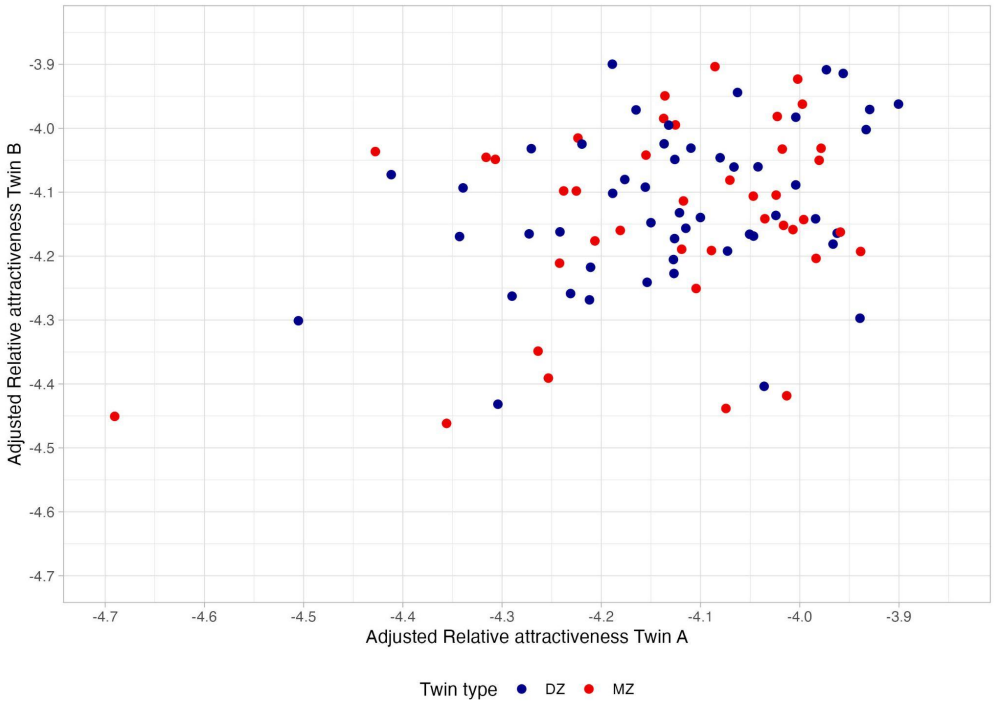

Figure 29. Scatter plot of the relative attractiveness of monozygotic (red) and dizygotic (blue) twin pairs. Dots represent a twin pair where Twin A and Twin Bs attractiveness within the pair are compared.

The investigated pairwise the hypothesis that twins have a smaller difference between their corrected attractiveness scores than unrelated individuals by calculating absolute differences between pairs using the equation:

$$Attractiveness(Twin\ A, Twin\ B) = |\ Adjusted\ attractiveness(Twin\ A) - Adjusted\ attractiveness(Twin\ B)\ |$$

Here, "Adjusted attractiveness" refers to the measure of relative or absolute attractiveness adjusted for the covariates as described above. The absolute difference between the adjusted raw attractiveness scores for Twin A and Twin B in the pair are used to calculate the attractiveness of the twin pair as a whole.

Modelled a group of unrelated pairs based on every participant being unrelated to every other participant except for their twin. This analysis allowed me to examine if there is a relationship between gene sharing and similarities in attractiveness to mosquitoes between

MZ, DZ and unrelated pairs. Gene sharing could impact on attractiveness in either direction, using absolute differences accounts for this.

The distribution of absolute differences in relative attractiveness between the MZ twin pairs (N=40), DZ twin pairs (N=50) and unrelated pairs (N=31916) as shown in Figure 30. Then ANOVA was used to compare the means of the three groups which gave no evidence of a difference (F value = 2.04, P value = 0.13). There is no difference in the distribution of the difference in attractiveness to mosquitoes per pair between MZ, DZ and unrelated pairs. The unrelated pairs have a much larger sample size and some extreme outliers, resulting in the left-tailed distribution. Q scores, i.e. the proportion of MZ (or DZ) pairs closer to one another than the unrelated pairs, were calculated as an estimate of the proportion of unrelated scores that had a closer relationship to one another than the MZ/ DZ twin pairs with 95 % confidence intervals. For MZ twins: Q = 0.46 (95 % CI: 0.372 - 0.548) and DZ twins: Q = 0.43 (95 % CI: 0.349 - 0.508), which gives no evidence genetics are contributing to differences in human attractiveness to mosquitoes.

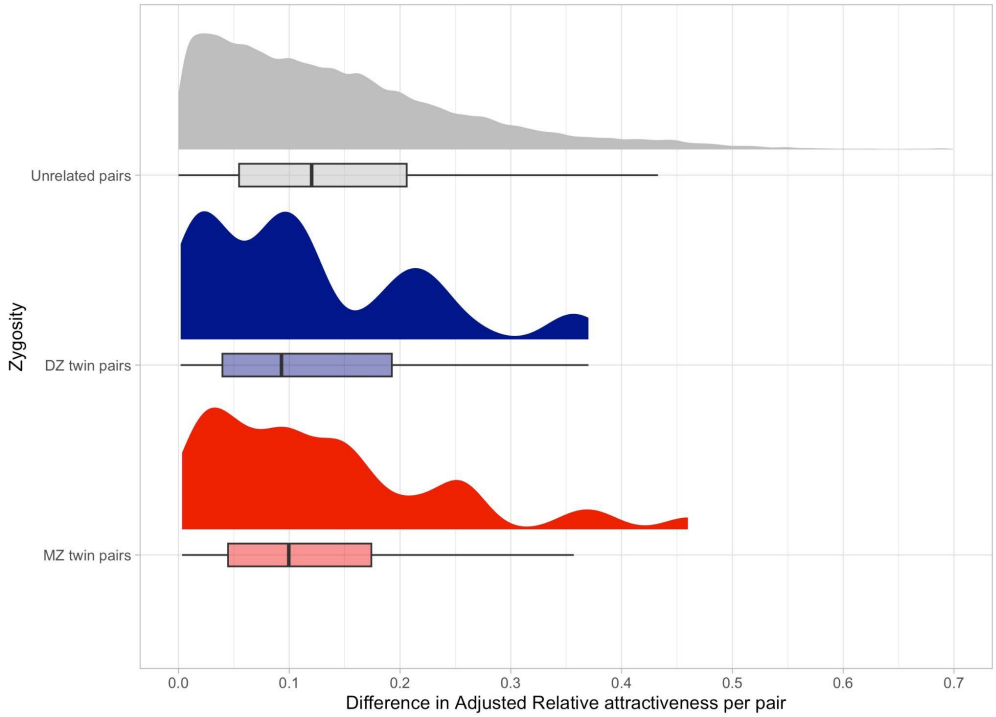

Figure 30. Boxplot and density plot showing the distribution of the difference in adjusted relative attractiveness per pair separated by pair type MZ (red), DZ (blue) and unrelated (grey).

2.04.10) Heritability Estimates

Expected hypothesis (H0): All coefficients in the model are equal to zero, indicating that the predictors are not significant in predicting the response variable of log-transformed relative attractiveness.

Alternative hypothesis (H1): At least one coefficient in the model is not equal to zero, indicating that at least one predictor is significant in predicting the response variable of logit-transformed relative attractiveness.

Next, the narrow sense heritability of both attractiveness measures was investigated, i.e. the proportion of the phenotypic variance explained by additive genetic effects [193]. To do this, linear mixed models were fitted to the logistic transformed attractiveness data using the ASReml package (4.1.0.126) [194] in the R software (3.6.3). Standard errors for the narrow sense heritability estimates were calculated using the Delta method approximation [195].

In the UK wind tunnel study, the additional fixed effects of testing room temperature, humidity, and time of day during the behavioural assay, tunnel and treatment side were tested using a Wald test. The fixed effects of testing room temperature, humidity, and time of day during the behavioural test were chosen to be included in the model based on the results of a Wald test, which assessed the significance of the coefficients. The full fixed effect model includes the predictors of humidity, time and temperature that were selected using the Wald test and additional random effect structure as a variance matrix of twin.code (a term for the twin pairs ID) and AINV (a matrix of genetic relatedness between subjects, where the genetic relationship between DZ twins is assumed to be 0.5 and MZ twins 1.0). The equation is, therefore:

$$logitRA = \beta 0 + \beta 1 * Humidity + \beta 2 * Time + \beta 3 * Temp + uTwin.code + \mathrm{e}$$

Where:

- logitRA is the response variable (logit-transformed relative attractiveness to mosquitoes)
- β_0, β_1, β_2 and β_3, are the fixed effect coefficients corresponding to the intercept, humidity, time and temperature

- Humidity, time and temperature are the explanatory variables (co-variates measured in wind tunnel)
- $u_{Twin.code}$ represents the random effect for Twin.code, assumed to follow a multivariate normal distribution with a variance-covariance matrix, vm specified using AINV structure
- e representing the residual error term

The summary function calculates the variance components of the linear mixed-effects model, including the residual variance and variance due to the random effects structure. This information is used to estimate the proportion of variance in the response variable attributable to the model's fixed and random effects. The predict function was used to estimate the heritability of the relative attractiveness based on the variance components obtained in the model, as explained above. The narrow-sense heritability of relative attractiveness was estimated to be 0.134. The heritability estimate indicates the proportion of variance in relative attractiveness attributable to genetic factors. This estimate indicates that genetics play a limited role in determining human relative attractiveness. Traits with a similar heritability estimate include breeding traits such as litter size and bull fertility [196]. In comparison, traits like human height have much higher heritability, approximately 0.9 [197]. The standard error of the estimate is 0.061, which indicates the precision around the estimator. 95 % confidence intervals were estimated around the heritability estimates. The 95 % CI is 0.014 - 0.254. This means we can be 95 % confident that the true difference in heritability of relative attractiveness falls within this range. The confidence interval is wide as we do not have the power to get a precise estimate of heritability with this method.

Then used a likelihood ratio test to compare the full and restricted models with the random effects removed. The p-value p=0.004 indicates that the more complex model provides a significantly better fit to the data than the simpler model. This suggests that the random effects of the twin ID of the individual and the matrix of genetic relatedness between the subjects are necessary for capturing the underlying structure of the data.

2.04.11) Summary

After conducting a comprehensive analysis of the UK cohort, focused on categorising attractiveness to mosquitoes into two groups: attractive and unattractive. The pairwise analysis between MZ, DZ and unrelated groups, combined with the absence of evidence from heritability estimates, revealed there is no evidence of heritability. To further explore the role of genetics and the grouping of human attractiveness to mosquitoes will now be investigated in the Gambian cohort. This population experiences natural exposure to *Anopheles* mosquitoes and *Plasmodium* parasites. The differences in human attractiveness to mosquitoes between groups and if attractiveness to mosquitoes is a heritable trait will be investigated.

2.05) Gambian Twins Cohort

In The Gambia, we began by recruiting same-sex twin pairs aged over 18 and then reduced the age to 12; once an ethics amendment was approved (N=320 consented). The selection of twins to test experimentally depended on zygosity testing, as described below. Samples were collected from 298 individuals who met the eligibility criteria to participate.

Blood samples were collected from the eligible individuals (N=149 twin pairs) by finger prick onto Whatman paper. Blood samples were used to test for both malaria and zygosity. Twins were tested for malaria as it is known to be a confounder that increases human attractiveness to mosquitoes [172] by the laboratory group at MRC Gambia.

Participant zygosity was determined by identifying Short Tandem Repeats (STRs) in DNA extracted from the blood samples. The laboratory group at MRC Gambia used the ampFLSTR Identifiler PCR kit (Thermo Fisher Scientific), which amplifies 15 loci and Amelognin, to discriminate widely used STRs for human identification. Each individual was scored for known STRs at the following loci: AMEL-4, CSF1PO-1, D13S317-2, D16S539-2, D18S51-3, D19S433-3, D21S11-1, D2S1338-2, D3S1358-2, D5S818-4, D7S820-1, D8S1179-1, FGA-4, TH01-2, TPOX-3, and vWA-3. In the UK cohort, TwinsUK participants had known zygosity, making the STR analysis unnecessary. However, in The Gambia, some twins were unsure of their zygosity, hence the need for DNA analysis. Individuals were scored for known STRs at the 15 loci and Amelognin using OSIRIS software [198]. Amplification was successful in 101 twin pairs, and the remaining samples were discarded due to poor amplification. From the 101 twin pairs, 50 had STR identities ranging from 94.0 % to 100.0 % (mean = 99.5 %) and were considered monozygotic (MZ), while 51 twin pairs had identities ranging from 25.0 % to 70.4 % (mean = 54.4 %) and were considered dizygotic (DZ). Some samples were damaged during transit from The Gambia to UK. The cohort, therefore, included N=94 twin pairs (47 DZ and 47 MZ twins).

2.05.01) Measuring participants attractiveness to mosquitoes using a cage assay

As described for the UK cohort above, we collected body odour samples using cotton socks to ensure we measured the body odour attractiveness independently of temperature or humidity. The socks were stored at -20 ^{o}C in The Gambia until they could be transported on

ice back to the UK. Due to unresolved technical problems with the wind tunnel in 2019 and 2020, we developed an alternative cage-based assay to evaluate the attractiveness of the Gambian socks.

An overview of the Gambian behavioural assay set-up is shown in Figure 31. It shows the participant's sock on top of the cage heated with a hemotek heated insect feeder (Hemotek FU1 feeder, Hemotek, UK).

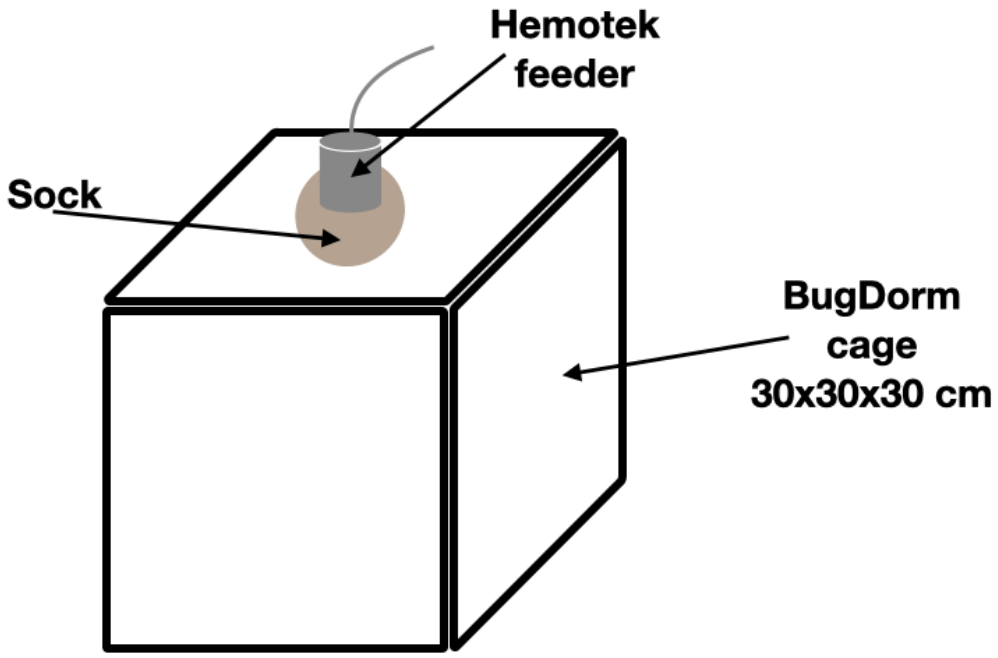

Figure 31. Diagram of the assay used for behavioural experiments to test the attractiveness of worn Gambian socks to Anopheles mosquitoes.

The participant's sock was placed on a wire frame (~8cm diameter), as shown in Figure 32, the same frames as used during the UK cohort.

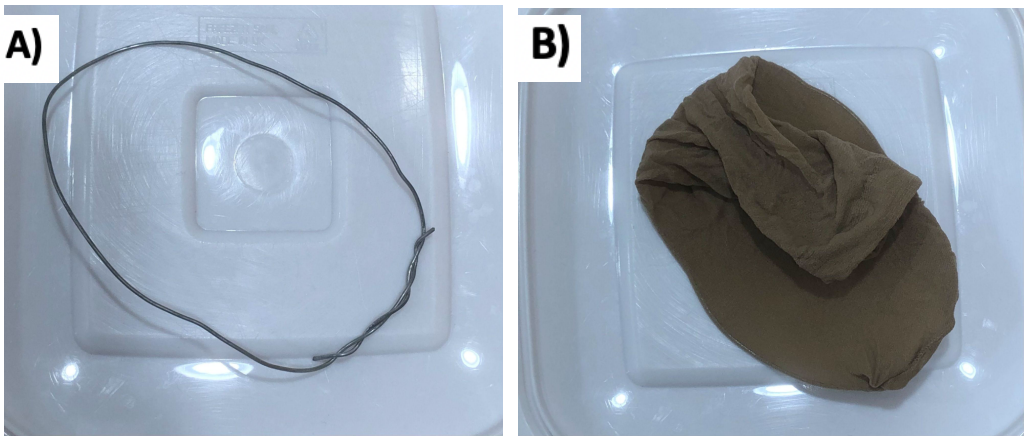

Figure 32. A) The metal wire frame and B) Metal wire frame inside a cotton sock.

Twenty female *Anopheles coluzzi* mosquitoes were released into a 30 x 30 x 30 cm BugDorm cage (BugDorm-4M3030 Insect Rearing Cage, BugDorm, Taichung, Taiwan). The sock on the wireframe was placed on the top, and a Hemotek, set to 37 °C, was placed on top of the sock, as shown in Figure 33. After five minutes, the number of mosquitoes probing the sock area was counted.

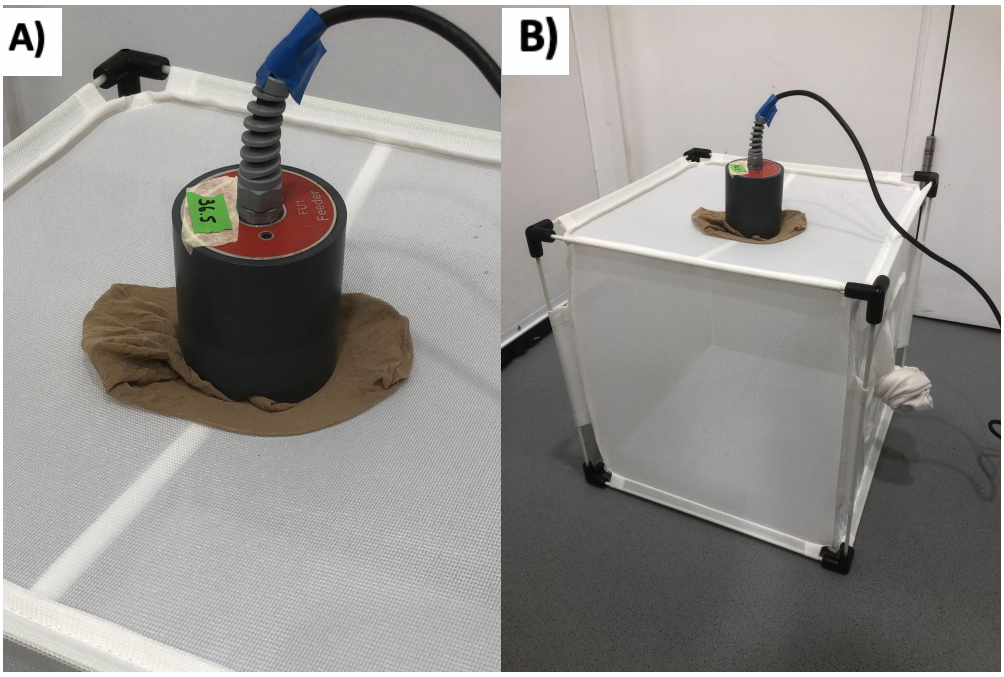

Figure 33. A) The hemotec is placed on top of the sock on the wire frame, on top of a BugDorm. B) Zoomed-out view of the cage assay.

All tests were conducted in the scotophase. Experiments were run once per sample, and the sequence of samples was randomised. Lighting and time of day were consistent with the UK wind tunnel assays previously described. Negative controls comprised a clean nylon sock washed in 70 % ethanol and left to dry. Positive controls worn by a researcher were used throughout the testing period. These control socks were stored under the same conditions as the samples. The set-up required a researcher to be in the room throughout the test. The researcher was kept consistent throughout the testing period.

2.05.02) Exploring and adjusting attractiveness scores for covariates

For the Gambian cohort, there is only one measure of attractiveness due to using a cage assay to measure absolute attractiveness instead of a choice assay. The absolute attraction was calculated as the number of mosquitoes probing the sock area at the time of counting divided by the total number of mosquitoes in that cage. Twin pairs that did not have attractiveness data for both twins in a pair were dropped.

Definitions of covariates measured and tested as covariates in the cage assay:

- Temperature = Temperature in the assay room at the time of the run
- Humidity = Humidity in the assay room at the time of the run
- Time of the day = Time of the day the assay was run
- Number released = Total number of mosquitoes

Comparison of baseline characteristics between MZ and DZ is shown in Table 6. There is a significant difference in the age group between the MZ and DZ twins. Gender splits between MZ and DZ twins are very similar.

Table 6. Comparison of baseline covariates for MZ and DZ twins within the Gambian cohort. P-value from fishers exact test.

Characteristic	**MZ, N=111**	**DZ, N=116**	**P value**
Sex			0.6
Male	51 (46%)	57 (49%)	
Female	60 (54%)	59 (51%)	
Age category			<0.001
10-14	76 (68%)	38 (33%)	
15-19	19 (17%)	53 (46%)	
20-24	8 (7.2%)	12 (10%)	
25-29	2 (1.8%)	2 (1.7%)	
30-34	2 (1.8%)	9 (7.8%)	
35-39	4 (3.6%)	2 (1.7%)	
No fever	111 (100%)	116 (100%)	
Malaria negative	111 (100%)	116 (100%)	

I used linear regression to adjust the measures of attractiveness for significant covariates. Table 7 shows the regression output of the optimised model, which includes the time of the test, temperature, humidity in the testing room, and the number of mosquitoes released.

Table 7. Regression results after adjusting for absolute attractiveness, including all covariates included in the final model.

Term	Estimate	Standard error	Statistic	P.value
(Intercept)	0.864	0.365	2.365	0.019
TimeC	0.147	0.051	2.889	0.004
Temp	0.008	0.011	0.771	0.442
Humid	-0.013	0.003	-3.723	0.000
Total_Mosq	0.004	0.006	0.678	0.499

The absolute attractiveness was adjusted for temperature, humidity, time of the day, and number of mosquitoes released. The equation was:

$$Adjusted\ Att\ =\ Raw\ Att\ \ +0.864+\ (-0.147\ *Time)+\ (-0.008\ *\ Temperature)\ \ +\ (0.013*Humidity)+(0.004*Number\ of\ mosquitoes)$$

The raw and adjusted attractiveness are highly correlated, as shown in Figure 34. Absolute attractiveness R-squared 0.891 [0.866, 0.916]. Indicating that 89.1 % of the variation in adjusted absolute attractiveness is explained by the raw absolute attractiveness.

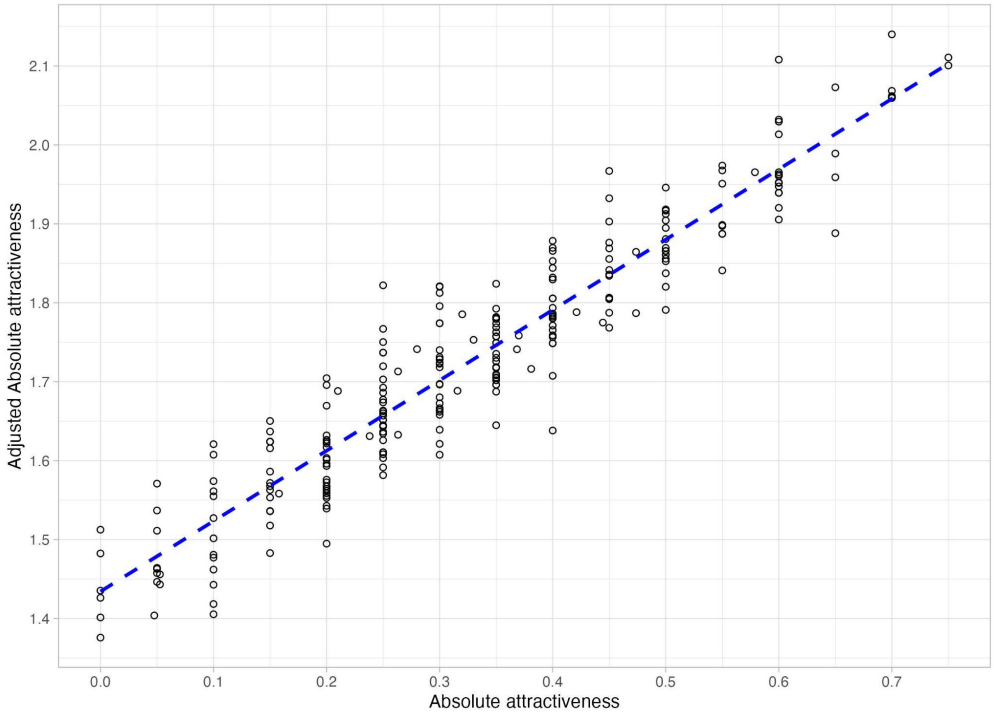

Figure 34. Scatterplots to show the strong positive correlation between raw absolute attractiveness and adjusted absolute attractiveness. The blue line shows linear regression.

2.05.03) The difference in attractiveness between positive and negative controls

During the testing period, two types of controls were used - positive controls (socks worn by the same researcher known to be attractive) and negative controls (clean, unworn socks). The attractiveness of both types of controls was tested in the behavioural assay, and the number of mosquitoes probing the control was used to calculate the absolute attractiveness results shown in Figure 35. The findings showed that the positive controls were significantly more attractive than the negative controls (t = -5.26, df = 54.43, p-value 2.486e-06), with a mean of 1.77 for the positive control and 1.61 for the negative control. The measure used was the number of mosquitoes that probed the sock divided by the number of mosquitoes in the cage. It is not possible to compare the difference in attractiveness between positive and negative controls for the UK and The Gambia cohorts as a different researcher's sock was used as the positive control in the UK cohort versus The Gambia.

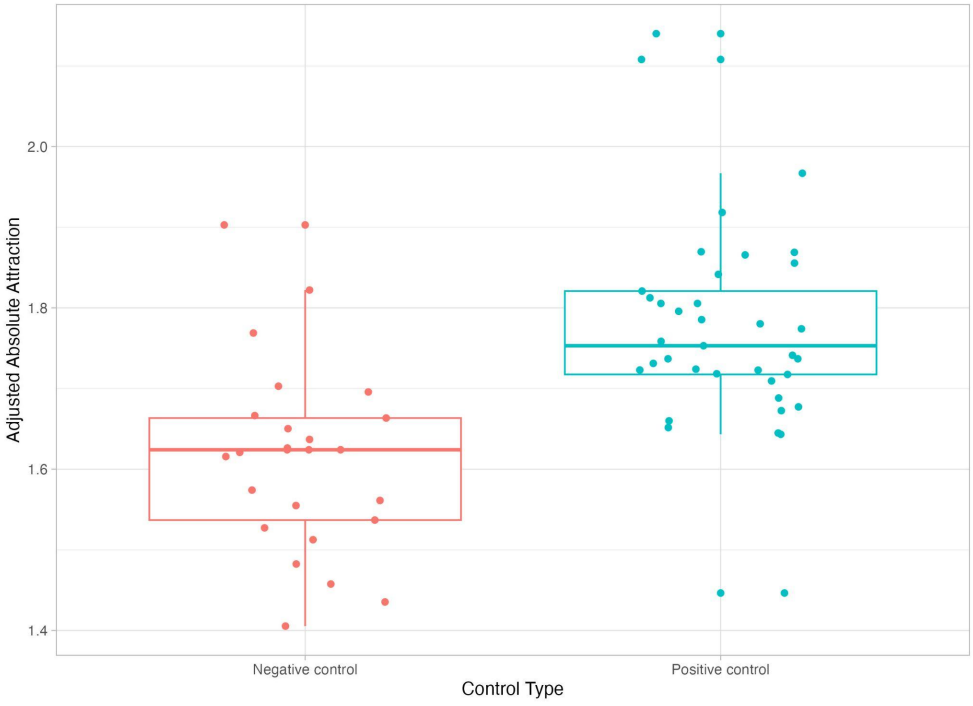

Figure 35. Comparison of the raw absolute attractiveness of negative (red) and positive controls (blue) tested throughout the period samples were tested.

The same control socks are plotted over time in Figure 36. This shows a consistent trend of the positive control (blue points) being more attractive than the negative control (red points) on each day of testing. The positive control socks were from the same person throughout and treated the same as samples, i.e. frozen and defrosted prior to testing.

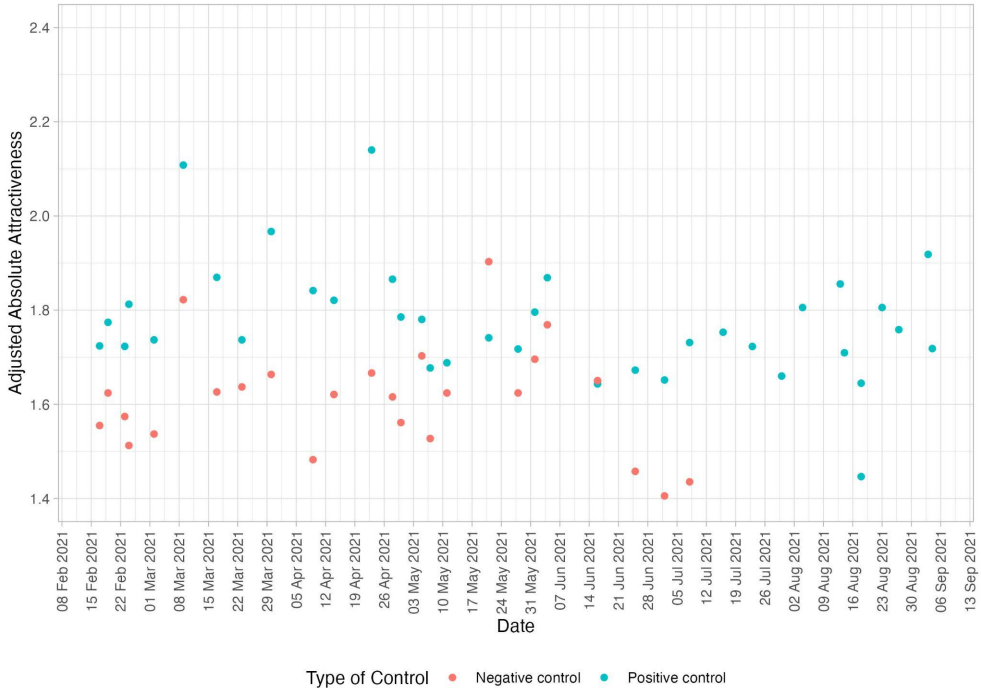

Figure 36. Comparison of the raw absolute attractiveness of negative (red) and positive controls (blue) tested over time of testing.

2.05.04) Selecting attractiveness groups

To use all of the data and to be consistent with the UK analysis. An 'attractive' group for samples that scored high was created. In contrast, the 'unattractive' group consisted of samples that scored similarly to the negative control, i.e., less than the 95th percentile (1.81), as depicted in Figure 37.

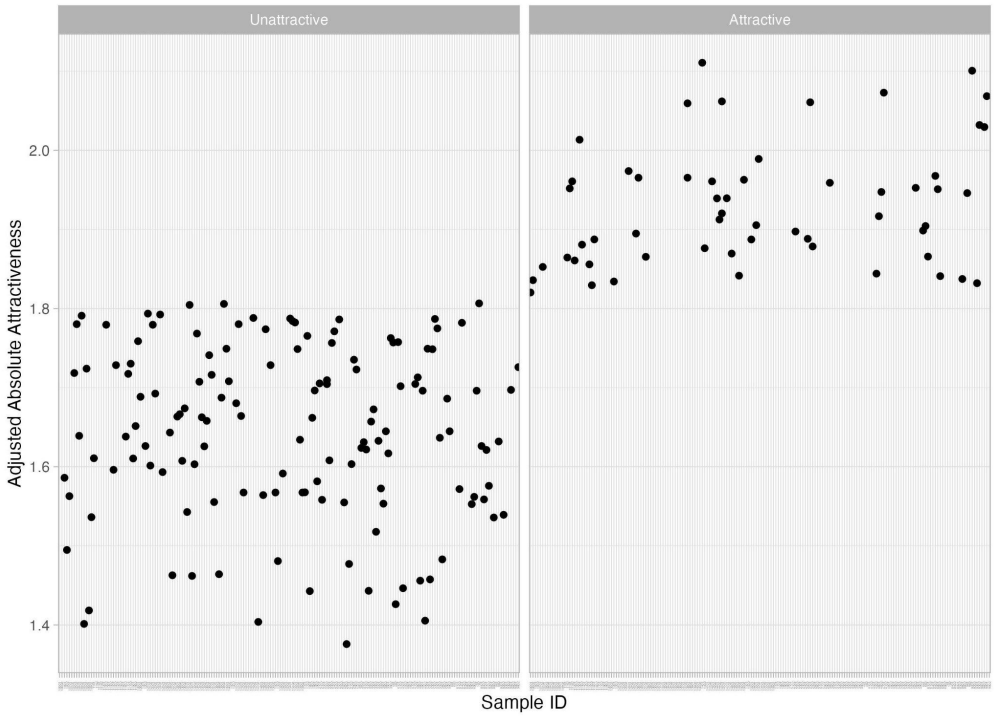

Figure 37. Comparison of the average adjusted absolute attractiveness for each participant faceted by attractiveness group, unattractive on the left and attractive on the right.

The adjusted absolute attractiveness for the unattractive (dark purple) and attractive (dark orange) groups are summarised in Figure 38. There is a clear difference in attractiveness between the groups. The attractive group is statistically significantly more attractive than the unattractive group (t = -20.59, df = 142.49, p-value < 2.2e-16), with a mean of 1.64 for the unattractive group and 1.93 for the attractive group. There are N= 136 unattractive and N=52 attractive.

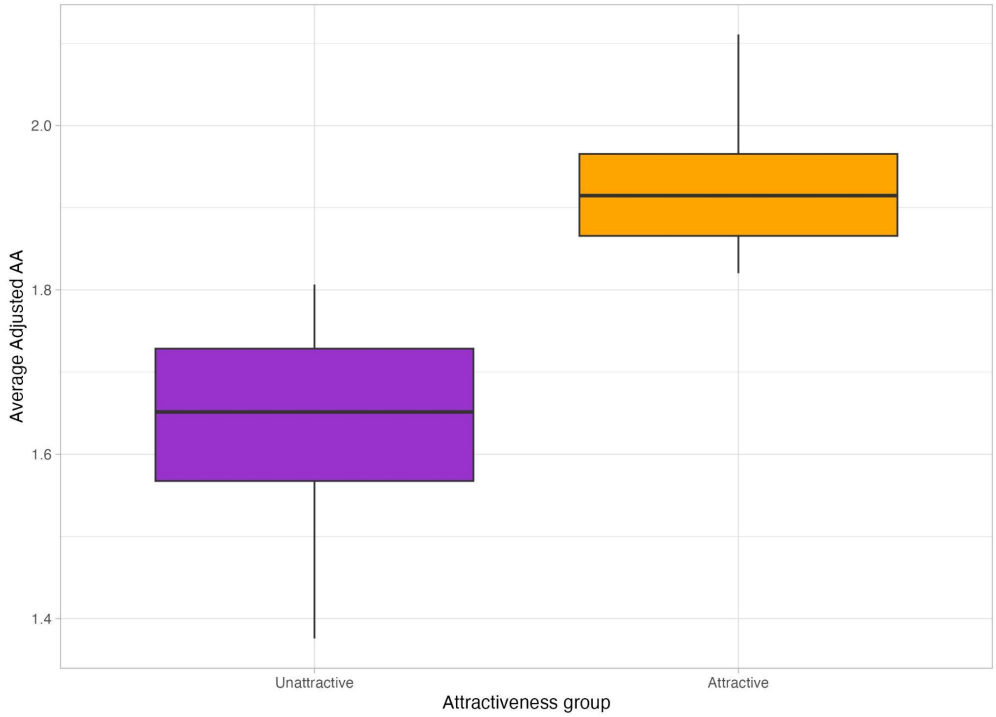

Figure 38. Comparison of the average adjusted absolute attractiveness score for participants in the unattractive (light purple) and attractive groups (dark orange).

2.05.05) Individual level: Distribution of participants' attractiveness to mosquitoes in the Gambian cohort

The Gambian cohort consisted of 94 twin pairs with known zygosity and attractiveness data (94 DZ twins and 94 MZ twins). Figure 39 shows a density plot of the adjusted absolute attractiveness. This is normally distributed. There appear to be some participants that have much lower attractiveness while others are much higher attractiveness, with most participants having a relative attractiveness score near the mean of 1.73.

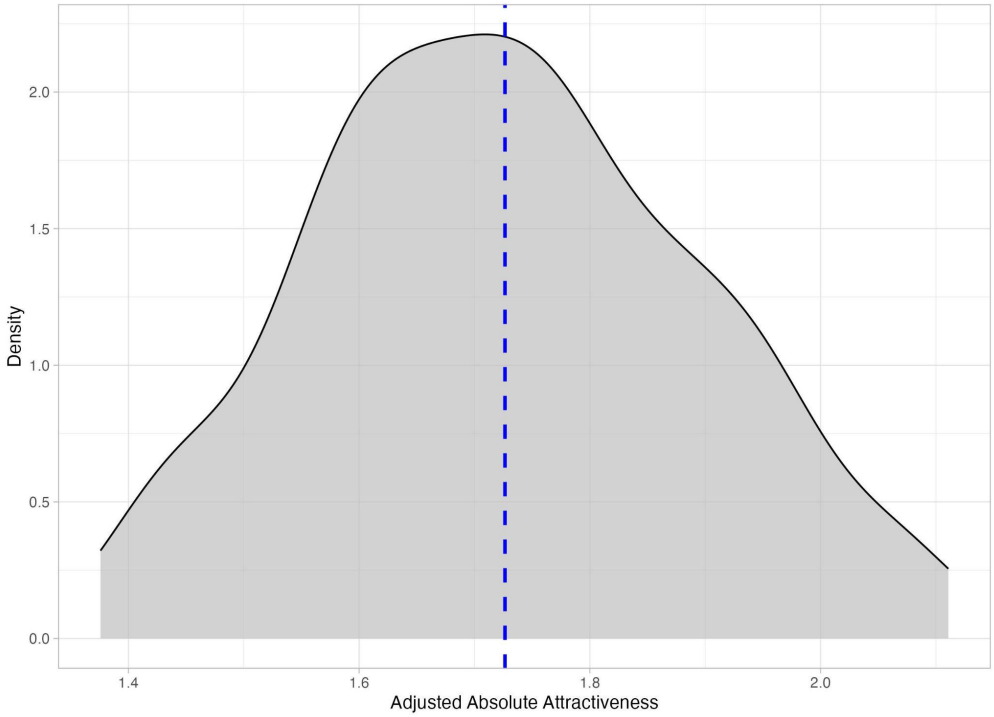

Figure 39. The distribution of absolute attractiveness of Gambian twins to mosquitoes. Mean is displayed with a dashed line.

2.05.06) Twin pair level: Differences in attractiveness between related and unrelated pairs in the Gambian cohort

The correlations for relative attractiveness to mosquitoes between monozygotic twins (rho = 0.178, 95 % CI = -0.115 - 0.442) and dizygotic twins (rho = 0.129, 95 % CI = -0.164 - 0.401) are shown in Figure 40. A Fisher's z-test found no statistical evidence that the MZ twin's relative attractiveness correlation was stronger than DZ twins (z= 0.339, one-sided p= 0.7835). Results do not give evidence that there is a difference in similarity in attractiveness scores for each pair between MZ and DZ twins.

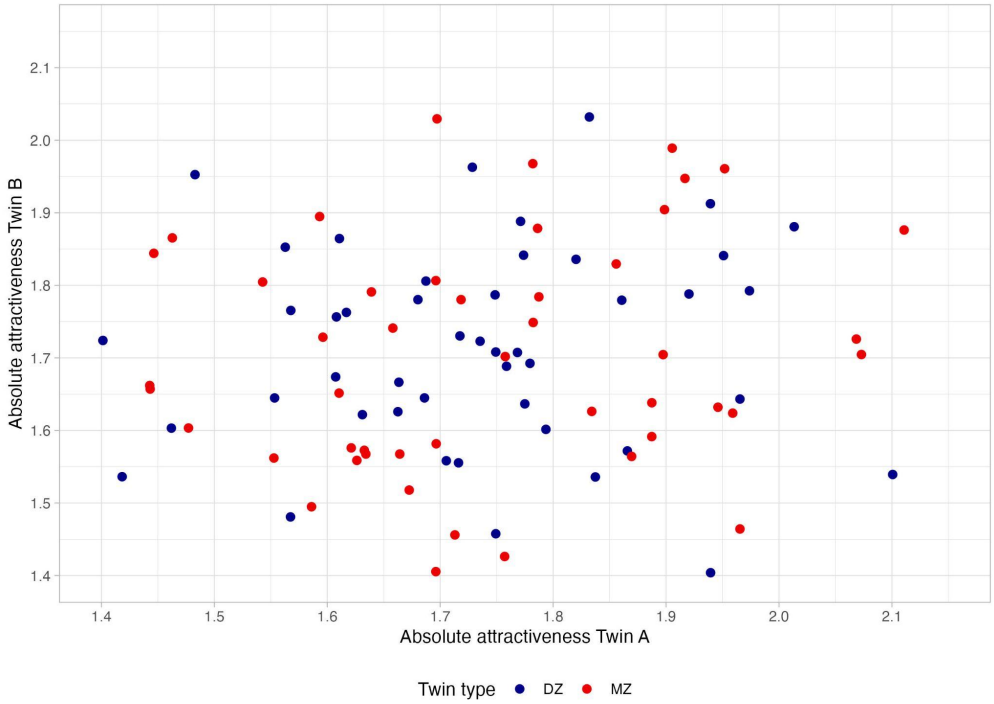

Figure 40. Scatter plot of the absolute attractiveness of monozygotic (red) and dizygotic (blue) twin pairs. Dots represent a twin pair where Twin A and Twin Bs attractiveness within the pair are compared.

Then calculated absolute differences in absolute attractiveness between the MZ twin pairs (N=47), DZ twin pairs (N=47) and unrelated pairs (N=38948). The distribution of differences in relative attractiveness between MZ, DZ and unrelated pairs are shown in Figure 41. ANOVA to compare the means of the three groups gave no evidence of a difference (F value = 2.38, P value = 0.09). There is no difference in the distribution of the difference in attractiveness to mosquitoes per pair between MZ, DZ and unrelated pairs.

The unrelated pairs have a much larger sample size and some extreme outliers, which results in the left-tailed distribution. Q scores, i.e. the proportion of MZ (or DZ) pairs that are closer to one another than the unrelated pairs, were calculated as an estimate of the proportion of unrelated scores that had a closer relationship to one another than the MZ/ DZ twin pairs with 95 % confidence intervals. For MZ twins: Q = 0.44 (95 % CI: 0.355 - 0.53) and DZ twins: Q = 0.43 (95 % CI: 0.352 - 0.504), which gives no evidence genetics are contributing to differences in human attractiveness to mosquitoes.

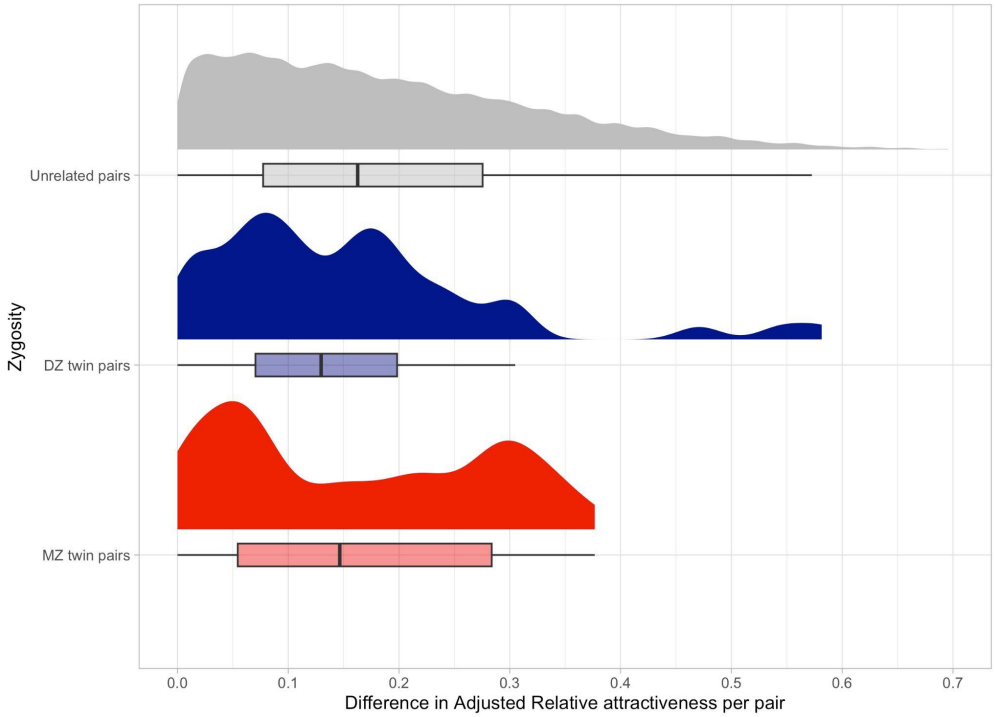

Figure 41. Boxplot and density plot showing the distribution of the difference in adjusted absolute attractiveness per pair separated by pair type MZ (red), DZ (blue) and unrelated (grey).

2.05.07) Heritability Estimates in the Gambian cohort

To investigate the genetic contribution to absolute attractiveness, a similar linear mixed-effect model to the UK cohort was used. The fixed effects of humidity and time are continuous and random effects ID and genetic relatedness. The narrow-sense heritability was estimated to be 0.185, with a standard error of 0.128. The 95 % confidence interval around this estimate was -0.066 to 0.436. This indicated that there is no evidence for a genetic contribution to absolute attractiveness and that the estimate is not precise.

2.06) Discussion

In Chapter 2, explored the role of human genetics on attractiveness to mosquitoes. Our findings indicate that human genetic factors do not play a substantial role in determining attractiveness to *Anopheles coluzzii* mosquitoes. If genetics played a significant role, we would expect MZ to have more similar levels of attractiveness to mosquitoes when compared to DZ twins and DZ twins have more similarities than unrelated pairs. Our data, however, did not support these expectations.

In this chapter, examined the similarities in attractiveness to mosquitoes among MZ twins, DZ twins and unrelated pairs. The comparison revealed no evidence of attractiveness differentiation between either UK or Gambia cohorts, suggesting no genetic influence on human attractiveness to mosquitoes. This observation was further supported using scatterplots of twin A against twin B in each pair, which did not show a stronger correlation between MZ twin pairs than DZ pairs in attractiveness to mosquitoes. Therefore, our results indicate that genetics do not appear to play a significant role in determining human attractiveness to *Anopheles*. However, the conclusions from this study are limited due to the sample size and associated limited statistical power. Additionally, there were fewer replicates of behavioural assays than desired due to variation between replicates that had not been anticipated.

The heritability analysis suggests that between 13.4 % and 18.5 % of the variability in human attractiveness to mosquitoes could be attributed to genetic factors. However, the wide confidence intervals around these estimates indicate that there is uncertainty in these estimates. For the UK cohort, the confidence interval remained above zero, suggesting the presence of some genetic component. In contrast, the Gambian cohort's confidence interval crossed zero, implying no genetic influence. The limited power of our study, and considerable variation in observed attractiveness, influenced the precision of our results. Notably, our findings contrast with a previous pilot study, which found a stronger correlation in attractiveness to *Aedes aegypti* mosquitoes among MZ twins than among DZ twins and a much higher heritability estimate in the range of 62 - 67 % [179]. We utilised a different mosquito species, *Anopheles coluzzii,* rather than *Aedes aegypti,* which have different olfactory responses. Additionally, we used a different behavioural assay, the wind tunnel or cage assay, rather than the Y-tube, with fewer replicates per sample which may have resulted in less precision in our heritability estimates [179].

Interestingly, our heritability estimates are comparable to the findings reported in a GWAS that presented an estimated heritability of 9.1 % based on self-reported attractiveness data [199]. Nonetheless, this study had similar limitations as previous questionnaire-based studies since it relied on reported attractiveness to mosquitoes, which is linked to bite reactions. While there is some indication of genetic influence on human attractiveness to mosquitoes in the UK cohort in this study, the evidence does not indicate a strong influence of genetics on human attractiveness to mosquitoes. The analysis presented gross effects, so it is not possible to rule out strong effects for rare genetic factors linked to specific genes.

Our genetic analysis focused on two distinct cohorts: a western cohort from the UK and a rural cohort from the Gambia. We hypothesised that there might be stronger selection pressure for unattractiveness to mosquitoes in the Gambia, where people are exposed to *Anopheles* and *Plasmodium spp.* at significantly higher frequencies. There were differences in demographics between the cohorts. The UK cohort comprised women aged between 50 and 90 years, while the Gambian twins were much younger, with ages ranging from 12 to 35 (average age 16.5 years). The recruitment strategy in The Gambia meant that these twins were more likely to share living environments, resulting in a higher likelihood of shared environmental factors influencing their attractiveness to mosquitoes. Humidity in the behavioural assay test room was the strongest covariate to adjust for, which is known to be an important cue in mosquito host-seeking behaviour. Additionally, in the Gambian cohort, the DZ twins were younger (majority <15 years) due to recruitment challenges in recruiting twins over 18 years. As zygosity was determined after the samples were collected, keeping the number of MZ and DZ twins balanced in the Gambia was challenging. In the Gambian cohort, developments of hormones due to puberty may have also impacted body odour profile impacting attractiveness to mosquitoes [200]. This is particularly concerning as the genetic correlation could be confounded with other non-separable influences, such as environmental factors, meaning the MZ and DZ twins may not have been directly comparable and resulting in potentially unreliable heritability estimates. The study's generalisability is restricted, with the Gambian cohort predominantly representing a younger demographic, while the UK findings were skewed towards an older group.

In Chapter 2, we have used behavioural assays to measure human attractiveness towards *An. coluzzii* mosquitoes. The assays used measured different behaviours. The wind tunnel presented mosquitoes with a choice. In contrast, the cage assays did not, potentially gauging mosquito hunger instead of odour preference, as they did not make an active choice between two stimuli. Additionally, in the UK analysis, we conducted two replicates, revealing more variation than anticipated. However, logistic challenges caused by COVID-

19 restrictions permitted only one replicate for the Gambian cohort. It would have been better to conduct more replicates to get more precise estimates of human attractiveness to mosquitoes, improving the heritability estimates' precision. The UK cohort was assessed using a wind tunnel assay, which involved multiple stages of attraction, such as initial activation by CO_2, guided flight by the human odour plume, and natural flight within the wind tunnel. The odour plumes in the wind tunnel and cage assays likely differed; the wind tunnel facilitated the formation of a concentration gradient that mosquitoes could track, whereas the cage provided less space for such a gradient [201]. Using larger or rectangular cages for the Gambian cohort might have allowed for better concentration gradients that the mosquitoes could follow. *Anopheles* mosquitoes generally cover wide distances when flying, making the wind tunnel an appropriate environment to evaluate their attraction behaviour, as demonstrated by 3D video tracking [37]. However, visual stimuli may have also influenced attractiveness. *Anopheles coluzzii* has been found to be highly responsive to visual cues in the presence of host odour, even though they are nocturnally active [37]. In the Gambian cohort, a cage assay with a person present to count landing behaviour was used; this assay could have been enhanced by using video tracking technology. The same researcher was present for all tests to minimise differences between replicates. Infrared cameras have been utilised by other groups, but video quality was poor and reliable access to Wi-Fi in the insectary was lacking [24]. Using a Perspex shield, as demonstrated by Lucas-Barbosa et al. [202], could have helped reduce the observer's odour impact and improved the repeatability of results in the Gambian cohort. CO_2 is a significant factor in *Anopheles* attraction, with even 0.015 % CO_2 above ambient increasing landings considerably in a cage assay [24]. We conducted a pilot using CO_2 following the setup described by Webster et al. (2015), but it hindered host-seeking behaviour and did not yield clear distinctions between positive and negative controls. Consequently, CO_2 was not used in this assay. In future, we would conduct further work to optimise the cage assay with CO_2 supplied at the correct concentration and optimise the pulsing of CO_2 instead of a continuous stream as we tested. Despite this limitation, we demonstrated a normal distribution between samples and consistently higher attractiveness for the positive control compared to the negative. In the Gambian assay, we used a sock heated with a hemotek to replicate a human. Direct heat application could have affected odour sublimation from the sock, unlike the UK assay, where heated air was blown through the sock. Despite the limitations and changes in the assay between cohorts, we observed similar inter-individual differences in attractiveness using both assays. We encountered difficulties rearing enough *Anopheles* mosquitoes, and natural variation between days and mosquito batches overtime was expected. Despite some differences between testing days, the positive control consistently exhibited higher attractiveness than the negative control.

Several key issues require further consideration and deeper investigation. Whilst the experiments presented in this chapter provided an indication that some individuals were consistently attractive to mosquitoes, there was no evidence for a counterpart group which was consistently repellent to mosquitoes. Another consideration is the design of more efficient behavioural assays. It would be beneficial to develop choice assays that are quicker and do not require complicated setups like the two-choice olfactometer. There are current bottlenecks and statistical issues that need to be addressed to develop a higher throughput assay. We need experiments that provide greater confidence in mosquito choice. In this chapter, the findings suggest that genetic contributions explain little of the inter-individual differences in attractiveness to mosquitoes. Therefore, our study does not provide evidence to support the use of genome-wide association studies (GWAS) for *Anopheles* on a large sample measured for actual attractiveness to mosquitoes to elucidate the human genes and mechanisms responsible for mosquito attraction in the future. However, our results indicate that environmental factors may be important in shaping individual differences in attractiveness to *Anopheles* mosquitoes. The rest of this thesis will focus on the skin microbiome and body odour to better understand the mechanisms underlying human attractiveness to mosquitoes. The findings in this chapter suggest that further research into non-genetic factors is needed to fully understand the mechanisms underlying human attractiveness to mosquitoes.

3) Chapter 3: The skin microbiome and attractiveness to mosquitoes

3.01) Statement of multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper	This chapter uses the microbiome skin swabs collected during the GenoScent project. The UK skin swabs were collected in 2018 by Catherine Oke and Dr Julien Martinez (during my MSc). I co-led setting up fieldwork in the Gambia with Catherine Oke. The majority of the Gambian skin swabs were collected by a field team in The Gambia and shipped back to the UK due to COVID-19 disruption. I conceived the microbial experimental approach. I conducted the DNA extractions at LSHTM. UK 16S rRNA sequencing was performed by Polo GGB, funded through an Infravec2 grant I was awarded (grant number 5254). The UK data was published as Showering *et al*., 2022 (BMC Microbiology, 22). I carried out 16S rRNA sequencing of Gambian samples at LSHTM. I conducted bioinformatics and statistical analysis of UK and Gambian microbiome data. Dr Julien Martinez, Dr Ernest Diez Benavente and Dr Robert Butcher provided feedback on the analysis.

Student signature:

3.02) Introduction

In recent years, there has been increasing interest in the human skin microbiome, particularly in relation to its potential implications on our health [112]. The skin microbiome, composed of bacteria, fungi and viruses that reside on our skin, varies in composition between body sites and individuals. This variability is attributable to multiple factors, including the differential distribution of sweat glands, genetic disparities and environmental influences [112].

Bacterial metabolism releases volatile compounds, hereafter referred to as volatiles, from the skin [95]. These volatiles contribute to the unique odour profile that is characteristic of each individual. These bacteria volatiles are thought to significantly affect how attractive a person is to mosquitoes, guiding them to locate a host [23,123,203]. The differences observed in the human volatile profile between people are thought to explain differences in human attractiveness to mosquitoes [38,121]. The interplay between humans, the bacteria living on their skin and mosquitoes can be conceptualised as a tri-trophic relationship. Humans act as hosts for bacteria and mosquitoes, while the bacteria on human skin produce volatiles that attract or repel mosquitoes. Mosquitoes, in turn, act as vectors for transmitting diseases between human hosts. Verhulst et al. (2011) showed that individuals that were highly-attractive and poorly-attractive to mosquitoes differed in their skin microbiome composition. Additionally, cultured skin microbes have been observed to have differential attractiveness to *An. gambiae [23]*. Specifically, *Staphylococcus epidermis*, *Bacillus subtillis*, *Brevibacterium epidermidis* and *Corynebacterium minutissimum* were shown to be attractive, while *Pseudomonas aeruginosa* was shown not to be attractive [23]. Further understanding of the variability in microbiome composition between attractiveness groups could lead to novel mosquito control strategies.

Quorum sensing represents a complex communication mechanism to coordinate behaviour based on the density of their population. This process relies on generating, emitting, and detecting specific chemical signals. Notably, some of these chemicals are volatile and can contribute to human odour profiles [204]. Zhang et al (2015) compared wild-type *Staphylococcus epidermis* cultivated in tryptic soy broth against a variant lacking the agr gene, which is essential for quorum sensing [204]. They showed a blood-feeder treated with wild type *Staphylococcus epidermis* attracted 72% of *Aedes aegypti* over the blood-feeder treated with the agr- variant [204]. This indicates that mosquitoes may be “eavesdropping” on communication between microbial communication on the skin in their search for a host

[204]. Targeted disruption of quorum sensing could potentially lead to a reduction in human attractiveness to mosquitoes, opening new avenues for vector control.

The skin microbiome metabolises secretions on our skin in sweat, producing attractive microbial volatiles to mosquitoes [122,205,206]. It has been reported that mosquitoes respond differently to the metabolite of different bacteria under controlled laboratory conditions, basing their preference on the unique olfactory cues generated by varying microbial volatiles emitted [205]. Early studies demonstrated that sweat became attractive to *An. gambiae* mosquitoes when cultured with bacteria. Fresh sweat, however, was not attractive to the mosquitoes [127,207]. Lucas-Barbosa et al. (2023) mimicked the skin microbiome composition of highly and poorly attractive people by testing four bacteria: *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Corynebacterium minutissimum* and *Brevibacterium epidermidis* in varying ratios with *Ae. aegypti [202]*. The bacteria in the highly-attractive ratio (1:6:1:8), selected based on previous sequencing data, resulted in more mosquito landings, implying that this composition was more attractive than the poorly-attractive ratio (3:3:2:8) [202]. The relative proportions of various bacteria on the skin play a critical role in influencing human attractiveness to mosquitoes. *Ae. aegypti* were found to be three times as attracted to a culture of *Staphylococcus epidermidis* on its own as opposed to a more complex blend comprising equal parts of *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Corynebacterium minutissimum* [202]. Further observations revealed that the higher complexity model released fewer volatile compounds overall. The presence of numerous bacterial species could complicate the mosquitoes' identification of the appealing compounds from the individual attractive bacteria such as *Staphylococcus epidermidis*. Moreover, there were notable variations in mosquito response when the same bacteria were cultured without competition. Fewer landings were observed on *Staphylococcus epidermidis* alone than on the more complex blends, indicating that resource sharing in a competitive environment was a crucial determinant of outcomes [202]. These findings demonstrate that microbes have a crucial role in influencing human attractiveness to mosquitoes and, therefore, are the focus of this chapter.

A breakthrough study by Zhang et al (2022) delved into the mechanism of flavivirus infection, the skin microbiome, odour profile and attractiveness to mosquitoes. They demonstrated in mice that flaviviruses, transmitted by mosquitoes, can alter the skin microbiome, influencing mosquito attraction [174]. Removing the gut microbiome did not affect the production of acetophenone or mosquito attraction, whereas reducing the skin microbiome significantly reduced acetophenone levels [174]. This indicates that the skin microbiome is crucial for generating acetophenone altering mosquito attraction. The

Bacillus genus of microbes has been identified as a key producer of acetophenone, and its abundance is notably increased in dengue and Zika virus infected mice [174]. These findings underscore the significant role of the skin microbiome in enhancing the attractiveness of hosts and facilitating the transmission of arboviral pathogens.

Despite progress in understanding the role of the skin microbiome in human attractiveness to mosquitoes, there remains a lack of microbiome data from non-western countries, particularly African countries that carry the burden of malaria cases and deaths [5,208]. The existing studies that have compared the microbiome composition between poorly and highly attractive groups to mosquitoes have been focused on western populations [137,209]. Hospodsky *et al.* (2014) compared the skin microbiome of the hand between US and Tanzanian women. They found that US women had more of the skin-associated *Propionibacteriaceae, Staphylococcaceae* and *Streptococceacae.* In contrast, Tanzanian women had higher levels of soil-associated bacteria, specifically *Rhodobacteraceae* and *Nocardioidaceae [210]*. These differences could be attributable to lifestyle factors (cosmetic use, frequency of washing, more contact with the natural environment in Tanzania) or genetic differences, which lead to differences in the skin microbiome composition between the populations. One example of genetic influence on the skin microbiome is the role of a single nucleotide polymorphism (SNP) in the ABCC11 gene required for the transport of lipophilic substances, components found in apocrine sweat and earwax, which results in odour and wet earwax. This SNP has been found to affect axillary odour production, reducing overall odour and certain volatiles in individuals carrying the SNP [211]. Interestingly, the SNP prevalence varies across ethnicities, with 80-95 % of East Asians carrying the SNP compared to just 0-3 % of Europeans or Africans. The genetic variation has been linked to changes in the relative abundance of skin microbes [211].

Geography and lifestyle factors can significantly influence the skin microbiome. Urban environments often see increased use of chemicals, including detergents and cosmetics, reduced exposure to the natural environment, distinct lifestyle practices and improved housing conditions, all of which influence the skin microbiome [154,212–214]. Urbanisation has been identified as a significant factor impacting microbial diversity [215]. Notably, while urban and rural areas have comparable microbial richness, there is more intragroup variation in rural populations [154]. The observed variation could be due to occupational differences, with rural individuals typically working as farmers, in contrast to urban individuals who frequently work in office-based jobs [154]. Such factors may explain why the Tanzanian women had more environment-associated microbes on their skin than the US women [210]. The Tanzanian women, on average, resided in more rural settings and

had higher environmental exposure than the US individuals participating in the study, who spent more time indoors, had enhanced hygiene practices and had less regular contact with soil [210]. The interpopulation differences in the skin microbiome highlight the need for further exploration in this area, particularly to further understand how natural variation in the microbiome may be related to health. Regarding attractiveness to mosquitoes, there is potential to identify additional microbes that are differentially abundant in attractive versus unattractive humans, which may have potential as attractants or repellents. In this chapter, the skin microbiome of an African cohort from, The Gambia, as well as a UK cohort, will be investigated. The Gambian cohort is naturally exposed to *Anopheles spp* mosquitoes and *Plasmodium spp* parasites. Exposure to *Anopheles* and *Plasmodium* may have led to more selective pressure on the Gambian populations. Understanding the relationship between these factors and microbial composition could be particularly important for identifying novel strategies to detect or prevent mosquito-borne diseases, such as malaria, in high-risk areas.

Twin studies have yielded some insights into the role of human genetics in shaping the skin microbiome. A cohort of Korean twins showed that genetics strongly contributed to the composition of the skin microbiome, with the abundance of some bacterial genera on the skin, including *Corynebacterium* and *Brevibacterium*, being highly heritable [114]. A study of the skin microbiome of children found higher microbial community similarity between siblings living together than between pairs of children that did not reside in the same household [216]. This finding could be attributable to genetic factors influencing the composition of the skin microbiome or shared environmental conditions within the households. In a recent GWAS study, Jones *et al.* (2017) found 15 independent loci associated with human attractiveness to mosquitoes [199]. However, the identified genetic factors only explained 9.1 % of the overall variation in attractiveness, indicating that they contribute modestly to the total differences in human attractiveness to mosquitoes between people [199]. Based on my results in Chapter 2, approximately 90 % of mosquitoes' attractiveness is attributable to non-genetic factors such as the environment and skin microbiome. A genetic variant can either directly increase an individual's attractiveness to mosquitoes or alter gene expression, which changes the skin microbiome and increases attraction. Alternatively, a genetic variation might act directly on the skin microbiome, increasing attractiveness to mosquitoes. Host genetics, the skin microbiome and human attractiveness should be explored further. As our study population consists of twins, similarities in alpha diversity between pairs will be compared between monozygotic (MZ), dizygotic (DZ) and unrelated pairs. If the skin microbiome is influenced by genetics, hypothesised that MZ twins will have more similar alpha diversity than DZ, and DZ will have more similar alpha diversity than unrelated pairs.

This chapter aims to examine the association between the skin microbiome and human attractiveness to mosquitoes. To determine if the skin microbiome has a role in attractiveness, differences in alpha and beta diversity will be analysed, and differentially abundant amplicon sequence variants (ASVs) between the attractive and unattractive groups to mosquitoes will be investigated.

3.03) Aim and objectives

The aim of this chapter was to investigate the relationship between the skin microbiome and human attractiveness to *Anopheles coluzzii* mosquitoes. This was achieved through the following objectives:

1) To collect skin microbiome samples (swabs) from the feet of two cohorts of twins (in the UK and The Gambia) and extract microbial DNA for 16S rRNA amplicon sequencing.

2) To explore both cohorts' 10 most abundant taxa in unattractive and attractive groups.

3) To examine if there are differences in microbiome community (alpha and beta diversity) between unattractive and attractive groups of participants.

4) To identify differentially abundant ASVs between the unattractive and attractive groups of participants.

5) To estimate if alpha diversity is more similar in MZ than DZ or unrelated pairs in both cohorts.

3.04) Methods

3.04.01) Sample Collection

There are multiple methods for collecting skin microbiome samples, including swabs, biopsies, scrapes and tape stripping [152,217]. Swabbing is usually preferred because it is less invasive than other methods. For example, biopsies require a skin sample to be removed, and the procedure is limited in where on the body it can be performed [218]. Therefore, we chose to use pre-moistened swabs to collect skin microbiome samples from our participants. The buffer used for sample collection and storage was based on Castelino et al. (2017)'s method [219]. 2.3 g/L of Trizma base was added to 4.88 g/L Trizma HCL, 1 mM EDTA and 0.5 % Tween20 to sterilised water and syringe filtered. 800 µl of buffer was aliquoted into each 2 ml microcentrifuge tube, and UV sterilised for 20 minutes, then stored at 4 ^{o}C until required.

The location of swabbing is critical, as the skin microbiome varies by body site depending on site characteristics [150]. Microbiome samples were collected from the participants' feet in an aseptic manner from two locations, as shown in Figure 42, as they are attractive to *Anopheles* [130]. A sterile swab (Sample collection swab Catch-All, Epicentre, Epicentre Biotechnologies, Madison WI) was pre-moistened by dipping in the buffer and rubbed with relatively firm pressure for 60 seconds using both sides of the swab on the sole of the foot, below the ball.

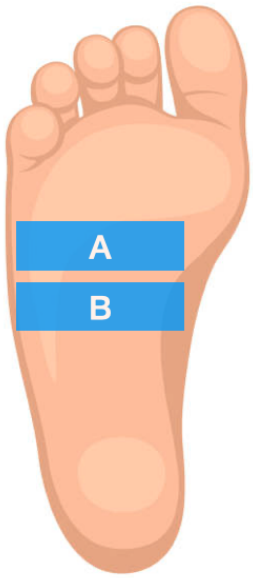

Figure 42. Sampling sites for collection of skin microbiome on the sole of the foot. Site A on the ball of the foot, and site B below. Created with BioRender.com.

Storage of microbiome samples can affect estimates of alpha and beta diversity (diversity within and between samples) [220]. Microbial samples should be rapidly stored at -80 °C to prevent degradation, and freeze-thaw cycles should be avoided as they result in degradation, with the rare microbes most affected [221]. Therefore, swabs in the tubes containing the buffer were stored at -80 °C as soon as possible after collection. In The Gambia, there was temporary storage at -20 °C for up to two weeks. After this, swabs were transferred on ice to -80 °C at MRC Unit, The Gambia. Once all swabs were at the MRC unit in The Gambia, they were transported by cold transfer back to LSHTM London, where they were stored at -80 °C until DNA extraction.

The main steps from sample collection to pair-ended demultiplexed amplicon sequencing files are shown in Figure 43.

Figure 43. Summary of the main steps in skin microbiome research when performing amplicon sequencing.

3.04.02) DNA extraction

The DNA extractions for UK samples were performed using the DNeasy® PowerSoil® Pro Kit (Qiagen, Hilden, Germany), a commercial kit from Qiagen, with slight modifications to increase the DNA yield based on the findings of preliminary experiments to optimise DNA yield. The samples were subjected to additional mechanical cell lysis steps at the beginning by vortexing horizontally for 10 minutes and centrifuged before removing the swab. The resulting pellet was resuspended in 800 µl of buffer CD1 from the DNeasy® PowerSoil® Pro Kit, incubated at 65 °C for 10 minutes, and DNA was extracted using PowerBead Pro tubes as per the manufacturer's protocol. DNA concentrations were quantified using the Qubit® 2.0 Fluorometer with the QubitTM dsDNA HS Assay Kit [222].

For the Gambian and skin microbiome transfer (SMT) samples, the QIAamp DNA microbiome kit (Qiagen, Hilden, Germany) was modified to enrich bacterial DNA yield through efficient mechanical and chemical lysis. Similarly to the UK samples, these were vortexed with the swabs in the tubes horizontally for 10 minutes, centrifuged, and the pellets from two swabs collected separately were combined. The kit instructions were followed,

except for avoiding the addition of benzonase. DNA concentrations were quantified using the Qubit® 3.0 Fluorometer with the QubitTM dsDNA HS Assay Kit [222].

3.04.03) Library preparation

The optimal 16S rRNA gene region to amplify for species identification depends on the research questions and expected bacteria composition. For instance, V1-V3 primers are better for accurately classifying common skin bacteria than V4 [223]. However, V3/4 are the standard primers used in most analyses and were recommended for the sequencing run, which was run by Polo Genomics Genetics and Biology (Polo GGB, Siena, Italy). V3/4 primers, therefore, were used throughout to keep the primers consistent. PCR conditions and the number of cycles were optimised before processing the samples. Gradient PCR was used to select the optimal annealing temperature. Cycles were varied between 20-40, and the optimal number was selected.

UK samples

The UK microbial DNA was sent to Polo GGB for amplicon sequencing. Polo GGB conducted the library preparation to amplify the 16S V3/V4 region using specific primers with overhang adapters attached. The 16S V3/V4 primers were forward 16S_V3V4-314F: CCTACGGGNGGCWGCAG and reverse 16S_V3V4-805R: GACTACHVGGGTATCTAATCC [224]. PCR clean-up used AMPure XP beads to purify the 16S amplicons and remove the free primer and primer dimers. A second PCR step attached dual indices and Illumina sequencing adapters using Nextera XT Index Kit. The AMPure XP beads were used to clean up the final library before validation and quantification. The resulting libraries were validated using a Fragment Analyzer (High Sensitivity Small Fragment Analysis Kit) to check size distribution. The concentration of library samples was defined based on the Qubit® 3.0 Fluorometer quantification and average library size. Indexed DNA libraries were normalised to 4 nM and then pooled in equal volumes.

Gambian samples

16S Library preparation:

For Gambian and SMT samples (chapter 5), 16S PCR was conducted to amplify the V3/4 hypervariable region in-house at LSHTM. 16S V3/4 314F/805R primers [224] with Illumina adapters allow for the indexing shown in Table 8.

Table 8. The 16S V3/4 PCR primers with Illumina adapters (blue) and locus-specific (red) that were used in the UK and Gambian cohorts

Primer	**Sequence 5' - 3'**
16S Amplicon PCR Forward Primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S Amplicon PCR Reverse Primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

For amplicon PCR, microbial DNA was amplified using Kapa Hifi HotStart ReadyMix (2X) (Kapa Biosystems) in a reaction volume of 25 µL. This included 12.5 µL of the 2X ready mix, 5 µL of DNA template, 0.5 µL each of forward and reverse primers (10 µM), and 6.5 µL of H_2O. The PCR program consisted of an initial denaturation step at 95 °C for 3 mins, followed by 30 cycles of denaturation at 98 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute. The final extension step was carried out at 72 °C for 5 minutes. Negative PCR controls (water) were included to check for contamination. Gel electrophoresis was performed to confirm the amplification of the target fragment, which was 463 bp in size. PCR products were purified using AMPure XP beads (AmpliClean™) per the Illumina 16S Metagenomic Sequencing Library Preparation protocol [225].

The Nextera XT Index Kit v2 Illumina sequencing indexes (Illumina, Inc) and Kapa Hifi HotStart ReadyMix (2X) were used for the Index PCR to add the Nextera XT barcodes. The amplification program consisted of initial denaturation at 95 °C for 3 mins, followed by eight cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 5 mins. AMPure XP beads were used to purify the indexed PCR product.

Quantification and normalisation:

For quantification and normalisation, the indexed PCR product was quantified using Qubit 3 or 4 Fluorometer (Invitrogen, Massachusetts, United States). Samples were normalised to 4 nM using Tris buffer. The samples were pooled and diluted to a final concentration of 4

pM for sequencing. Negative controls were included throughout the process to monitor for any contamination.

3.04.04) 16S Sequencing

In this study, 16S amplicon sequencing was used to examine differences in the skin microbiome between unattractive and attractive groups to mosquitoes. The UK 16S sequencing run was completed by Polo GGB and all other 16S runs were performed in-house at LSHTM. A summary of the runs and the corresponding MiSeq kit used is shown in Table 9. The method followed the Illumina 16S Metagenomic Sequencing Library Preparation protocol [225], except for the PhiX concentration, detailed in Table 9. A 20 % PhiX control was used in later runs due to the low 16S diversity in microbiome samples. The UK samples were sequenced using a MiSeq V2 kit, resulting in a 2x250 base pair paired-end run. The Gambian and *Aedes* SMT samples were sequenced using a MiSeq V3 kit, resulting in a 2x300 base pair paired-end run. The MiSeq then demultiplexed the reads into separate folders for each sample.

Table 9. Details of the sequencing run, including the MiSeq kit used and the percentage of PhiX mixed with the DNA library.

Run Nane	MiSeq Kit	PhiX %
UK	MiSeq V2, 2x250	12.5
Gambia Run 1	MiSeq V3, 2x300	10.0
Gambia Run 2	MiSeq V3, 2x300	20.0
SMT	MiSeq V3, 2x300	20.0

3.04.05) Genetics

Similarities in alpha diversity within each pair were compared between MZ twin pairs, DZ twin pairs and unrelated pairs. The data was visualised using a boxplot. To detect any statistically significant differences in the means across the groups, an Analysis of Variance (ANOVA) was used. Where differences in the means were observed, Tukey's Honestly Significant Difference (Tukey HSD) tests were conducted on the pairs MZ vs DZ, MZ vs unrelated and DZ vs unrelated.

3.04.06) Microbiome controls

Incorporating positive controls, such as mock communities, can improve standardisation across sequencing runs and centres [226]. Low biomass samples, such as skin, pose a higher contamination risk from the laboratory environment, extraction kits and other reagents [227]. It is, therefore, necessary to identify negative controls to identify and remove potential contamination [227]. Hence, positive and negative controls were included in all sequencing runs. The controls included in the UK sequencing and the purpose of each are detailed in Table 10. For all other projects, multiple negative controls were used, including the DNA control D6305/6 and microbial standard D6300 from ZymoBIOMICS.

Table 10. The positive and negative controls that were included in the 16S amplicon sequencing run. Positive controls, sourced from ZymoBIOMICS, consist of genomic DNA extracted from a pure culture. Negative controls are blank swabs processed in the same way as samples.

Control Name	**Type of control**	**Purpose**
Negative	Negative control (blank swab)	To identify contamination
Negative 1	Negative control (blank swab)	To identify contamination
D6300 ZymoBIOMICS Microbial Community Standard	Positive control consisting of 8 bacteria (3 Gram-negative and 5 Gram-positive) and 2 yeasts	To identify if the extraction process introduces bias
D6310 ZymoBIOMICS Microbial Community Standard II	Positive control consisting of 8 bacteria (3 Gram-negative and 5 Gram-positive) and 2 yeasts	To identify if the extraction process is biased
D6306 ZymoBIOMICS DNA Standard	DNA control consisting of 8 bacteria (3 Gram-negative and 5 Gram-positive) and 2 yeast	To identify if the sequencing is biased
D6311 ZymoBIOMICS DNA Standard II	DNA control consists of genomic DNA of eight bacteria and two yeasts	To identify if the sequencing is biased

3.04.07) Microbiome statistical analyses

Bioinformatics

There are many bioinformatics options, QIIME2 and R pipelines are popular choices. In this thesis, a pipeline in R Statistical Software v4.1.0 using DADA2 was chosen, as summarised in Figure 44. This pipeline was used to trim off the primers and adapters, quality filter, denoise (model and corrects for Illumina amplicon errors), merge the denoised reads, and remove chimaeras and singletons [228]. ASVs present in less than 10 % of samples were removed from the samples but not the controls [228]. The Silva database was used to assign taxonomy [229], and a phyloseq object was created [230].

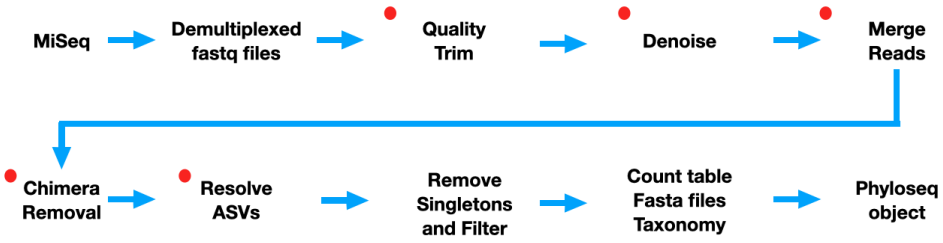

Figure 44. A summary of the bioinformatics steps involved in getting from demultiplexed sequencing reads from the MiSeq run to a phyloseq object that can be used for statistics in R. The DADA2 steps are labelled with red dots.

The main DADA2 steps are:

1) Trim reads

The amplicon length is 464 bp. 17 bases were removed, the length of the forward primer from the 5′ end of the forward reads, and 21 bases, the length of the reverse primer from the 5′ end of the reverse. All reads were truncated to 240 base pairs. The maxEE (the maximum number of expected errors per read) was set to 2. The final overlap should be more than 15 bases to allow merging. It is calculated as:

$$Final\ overlap\ =\ (length\ forward\ read)\ +\ (length\ reverse\ read)\ -\ (length\ of\ amplicon)\ -\ (length\ forward\ read\ -\ forward\ truncation\ length)\ -\ (length\ of\ amplicon)\ -\ (length\ reverse\ \ read\ -\ reverse\ \ truncation\ length)$$

For this run, the overlap is therefore:

$$Final\ overlap\ =\ 250 + 250 - 464 - (250 - 240) - (250 - 240)\ =\ 22\ bases$$

2) Denoising forward and reverse reads

The core DADA2 was applied to the truncated data which models and corrects for Illumina amplicon errors. DADA2 uses a parametric error model which learns error models from the filtered data by alternating estimation of the error rates and inference until they converge. The core method corrects substitutions and indel errors and removes singletons. Pseudo-pooling was used instead of processing samples individually to increase sensitivity to rare sequence variants. Pseudo-pooling processes samples independently after sharing information between samples and approximates pooled sample inferences.

3) Merge forward and reverse reads

Next, the denoised forward and reverse reads were merged by aligning the forward reads with the reverse-complement of the corresponding reverse read and constructing a merged sequence. Merged sequences require a 12-base overlap to be identical in the overlap region. 3488 paired reads were successfully paired out of 3807, meaning most of the reads were successfully merged.

4) Removing chimaeras

Chimaeras are artefacts formed when two sequences incorrectly join together. DADA2 uses the “consensus” chimaera identification method to remove them. Chimeric sequences are detected if they can be constructed by combining segments from two more abundant sequences. Removing the chimaeras allows for a more accurate analysis of the microbial community.

Additionally, singletons were filtered out. Taxonomy was then assigned to the reads using a silva database [229], silva_nr99_v138. The metadata, containing the metadata for each sample, was assigned with the feature table of ASV abundances and the taxonomy table from the above analysis to create a phyloseq object [230].

Statistics

Exploratory analysis of abundance was performed by comparing the composition of microbiome samples collected from the UK cohort and control samples, as summarised in Figure 45. The differences in the top 10 genera were explored between unattractive and attractive groups from the cohort. Additionally, alpha diversity was investigated using Shannon diversity. For beta diversity, sPLS-DA was used to explore global differences and DESeq2 and corncob to identify differentially abundant ASVs between the unattractive and attractive groups.

Figure 45. Main statistical analysis steps for microbial data. Exploratory analysis, alpha diversity (Shannon) investigation, beta diversity sPLS-DA for exploring discrimination between unattractive and attractive groups, and DESeq2 / corncob for differential abundance testing.

There are several methods for differential abundance testing in microbiome data. Classical non-parametric tests like the Wilcoxon rank-sum test can be used to compare medians between groups. DESeq2, a variance stabilising technique, is popular for differential abundance testing. However, it is unsuitable for compositional data as it does not account for the sparsity of the data. ANCOM is another method designed for compositional data, which exploits the fact that inferences regarding the relative abundance of a taxon in the ecosystem can be made using the relative abundance of taxa in the specimen. ALDEx2 is another popular method that analyses compositional data and deals with sparsity in microbiome count data. Corncob is a recently published method that models the zeros and does not assume that taxa are independent of each other. It uses count data to model the relative abundance, which allows the testing of hypotheses for relative abundances using count data. Corncob also adjusts for different sequencing depths, enables valid hypothesis testing with small sample sizes, and has built-in false discovery rate control for testing many taxa.

Overall, the choice of method for differential abundance testing should be based on the type of data and research question being addressed. In this chapter, both DESeq2 and the more appropriate Corncob will be used and the results will be compared.

3.05) UK Results

3.05.01) Pre-processing of the UK cohort

Taxa bar plots of the top 20 most abundant genera between cohort samples, positive and negative controls were compared. The UK cohort comprised 184 microbiome samples (Samples = 178, Positive controls = 4 and Negative controls = 2; Figure 46). The grey bars represent the low abundance taxa that are not included in the top 20. The relative abundances between the controls and samples exhibit significant differences. The samples and negative controls, sourced from blank swabs, share more taxa than the positive controls and samples. Notably, both the samples and negative controls are characterised by a higher number of low-abundance genera in comparison to the positive controls. Many of these consist of over 50 % low-abundance taxa.

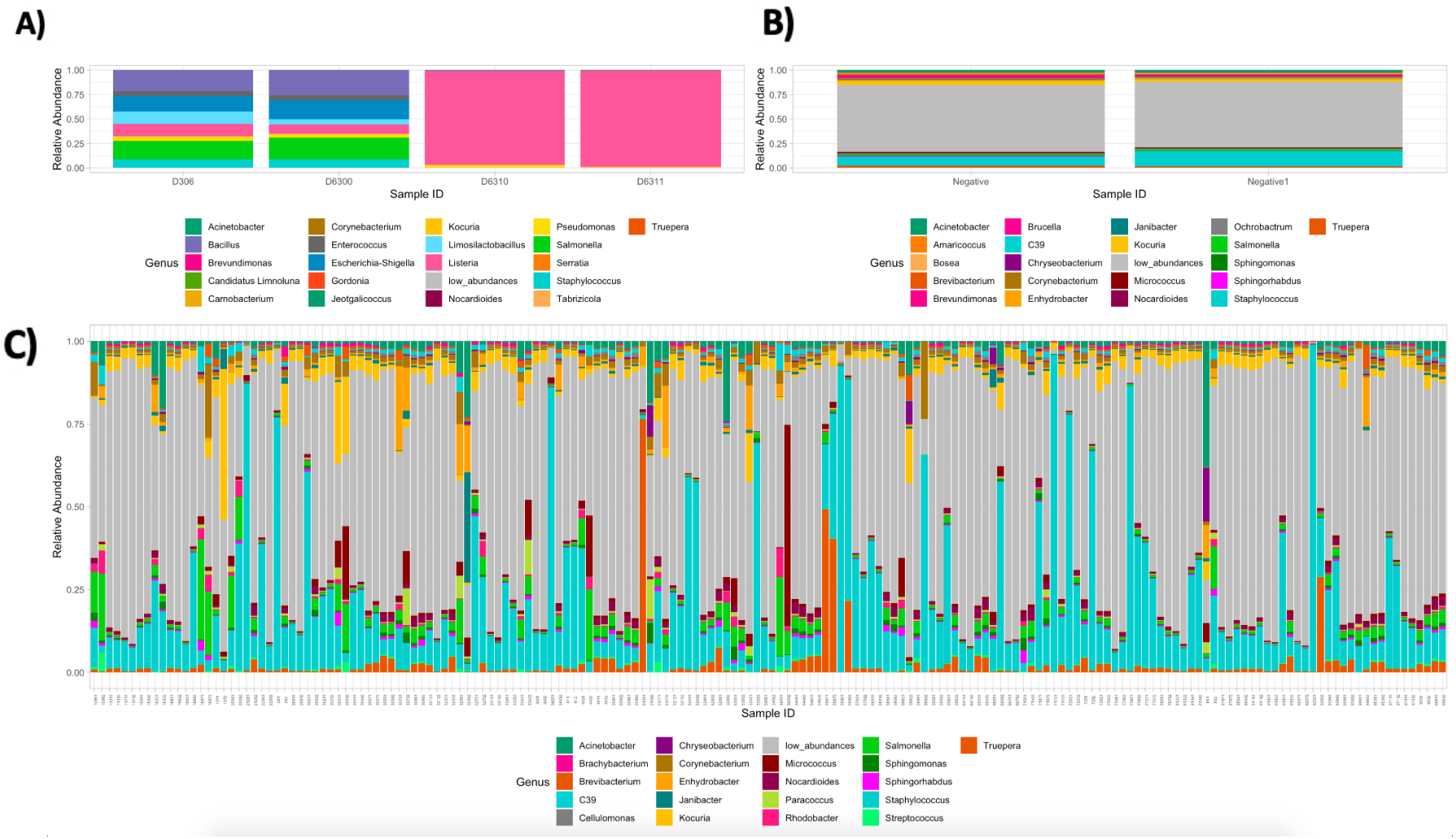

Figure 46. Summary of the taxonomy at the genus level as a taxa barplot of relative abundances for A) positive controls, B) negative controls and C) samples. There are clear differences in taxonomy between the samples and controls. Samples are a subset of the 20 most abundant taxa at the genera level. NA is the less abundant taxa combined. C39 belongs to Rhodocyclaceae family.

3.05.02) Exploring the skin microbiome of the UK cohort

Expected hypothesis (H0):
There is no difference in the abundance of any of the top 10 genera between the attractive and unattractive groups in the UK cohort.

Alternative hypothesis (H1):
There is a statistically significant difference in the abundance of any of the top 10 genera between the attractive and unattractive groups in the UK cohort.

Comparative analysis: abundance of top 10 bacterial taxa in attractive vs unattractive groups

To begin, the top 10 most abundant genera between the unattractive and attractive groups were explored as shown in Figure 47. Out of the 178 microbiome samples that passed filtering, 128 were from unattractive individuals, while 50 were from attractive individuals based on the *Anopheles coluzzii* behavioural data. The median absolute abundance of all genera appeared to be similar between the unattractive (dark blue) and attractive groups (dark green). Wilcoxon tests with Benjamini-Hochberg correction for multiple testing between the groups provided no statistical evidence of a significant difference in the medians between the unattractive and attractive groups for any of the top genera. Most of these genera are known skin microbes, including *Staphylococcus, Micrococcus, and Corynebacterium,* which are common Gram positives [231]. *Kocuria,* a microbe reported on human skin [232], as well as *Acinetobacter,* typically found in moist areas of the body, were also identified [233]. Other microbes, such as *Truepera* have been reported on the skin less often [234]. There is uncertainty surrounding the presence of *Salmonella* and the less abundant genera *C39. Nocardioles* and *Enhydrobacter* have been found to be present on the skin. *Enhydrobacter* is common on body parts in close contact with textiles. It is possible that these could be contaminations from the environment, the extraction kit or the laboratory rather than true skin microbes, as they were also found in the negative controls.

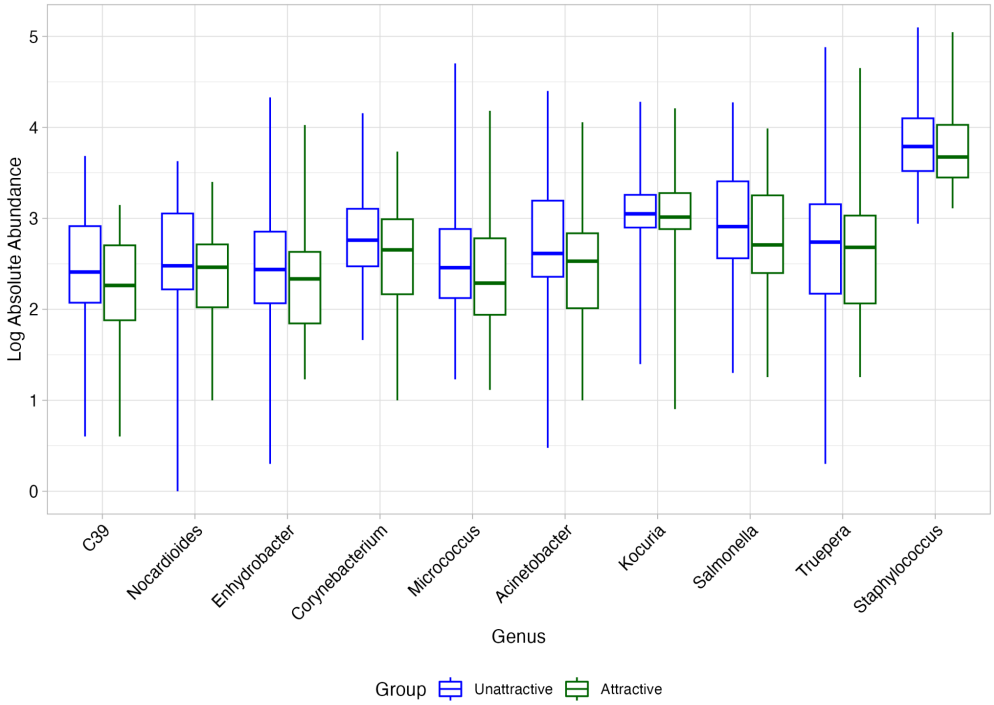

Figure 47. Comparison analysis of the top ten most prevalent bacterial genera between unattractive (dark blue) and attractive (dark green) participants in the UK cohort. The x-axis categorises the bacterial genera, ordered from least to most abundant from left to right. The y-axis illustrates the absolute abundance of each genus on a logarithmic (base 10) scale. Boxplot whiskers include the full range of genus abundances, represented as sequence variant (ASV) counts and displayed as log base 10 absolute abundance. Statistical differences in median abundances between the donor and recipient groups for each genus were assessed using a Wilcoxon test, with adjustments for multiple testing via the Benjamini-Hochberg method. The statistical significance is visualised by asterisks above each boxplot. The absence of an asterisk suggests no significant difference, while a single (*) and double (**) asterisk denote significant (adjusted p-value < 0.05) and highly significant (adjusted p-value < 0.005) differences, respectively.

Comparative boxplot analysis: relative abundance of top 10 bacterial taxa in attractive vs. unattractive groups

The counts to relative abundance were converted to adjust for differences in the number of reads per sample. The correlations observed between the unattractive and attractive groups were investigated to see whether they would remain consistent, as seen with the counts. Based on Wilcoxon tests with Benjamini-Hochberg correction between the groups, there was no statistical evidence of a significant difference in the medians between unattractive and attractive groups for any of the top genera shown in Figure 48. The notable difference was that *Pseudomonas*, another common skin microbe [137], was detected among the top 10 most abundant based on relative abundance but not counts.

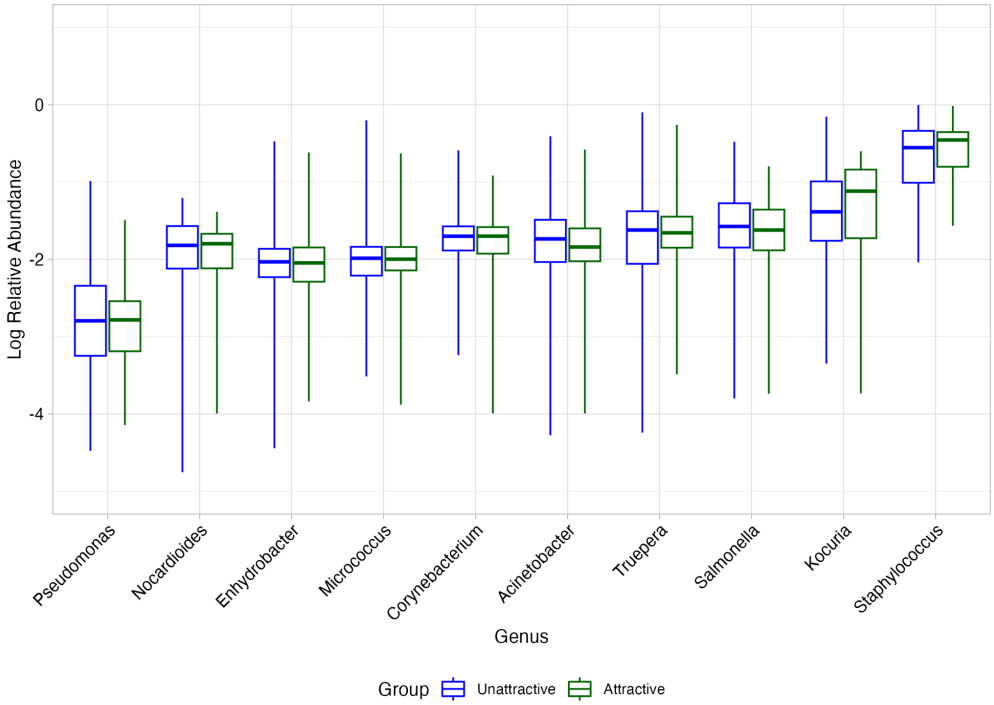

Figure 48. Comparison analysis of the top ten most prevalent bacterial genera relative abundance between unattractive (dark blue) and attractive (dark green) participants in the UK cohort. The x-axis categorises the bacterial genera, ordered from least to most abundant from left to right. The y-axis illustrates the relative abundance of each genus on a logarithmic (base 10) scale. Boxplot whiskers include the full range of genus abundances, represented as sequence variant (ASV) counts and displayed as log base 10 relative abundance. Statistical differences in median abundances between the donor and recipient groups for each genus were assessed using a Wilcoxon test, with adjustments for multiple testing via the Benjamini-Hochberg method. The statistical significance is visualised by asterisks above each boxplot. The absence of an asterisk suggests no significant difference, while a single (*) and double (**) asterisk denote significant (adjusted p-value < 0.05) and highly significant (adjusted p-value < 0.005) differences, respectively.

3.05.03) Contrasting alpha diversity: comparisons between attractive and unattractive groups

Expected hypothesis (H0):
There is no difference in the alpha diversity between the attractive and unattractive groups in the UK cohort.

Alternative hypothesis (H1):
There is a statistically significant difference in the alpha diversity between the attractive and unattractive groups in the UK cohort.

Following this, the global differences in the skin microbiome between the unattractive and attractive groups were explored. Initially, differences in alpha diversity, measured using the Shannon index, were explored, as shown in Figure 49. The Wilcoxon test, used to compare the medians, showed no statistically significant difference in median alpha diversity between the unattractive (4.59) and attractive (4.60) groups (W=3180, p-value = 0.950). The permutation test of equality, used to compare the distributions, also indicated no significant difference in distribution (permutation test of equality p=0.46).

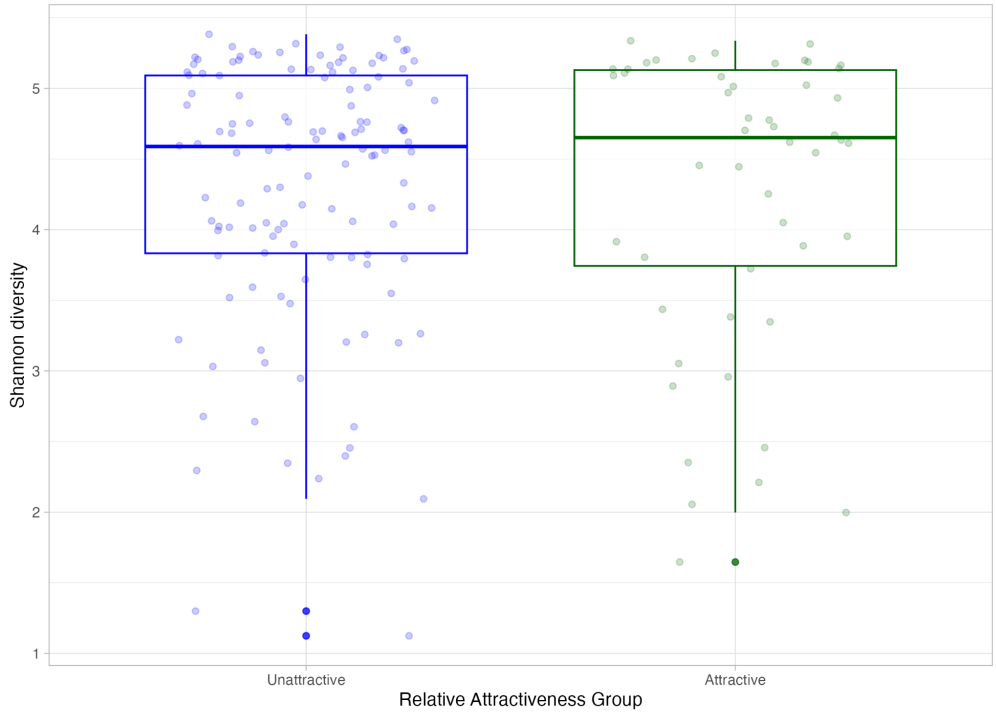

Figure 49. Comparison analysis of Shannon diversity between unattractive and attractive groups prior to transfers. The x-axis categories into two groups: unattractive (dark blue) and attractive (dark green) in the UK cohort. The y-axis represents the Shannon diversity index. The boxplot displays the data distribution for each group, demonstrating the data's median and interquartile range and total range. The individual data points overlaid as scatter represent the individual samples.

3.05.04) Exploratory differential abundance analysis of the UK cohort

Expected hypothesis (H0):
There is no difference in the beta diversity between the attractive and unattractive groups in the UK cohort. There are no differentially abundant taxa between the attractive and unattractive groups.

Alternative hypothesis (H1):
There is a statistically significant difference in the beta diversity between the attractive and unattractive groups in the UK cohort. There are differentially abundant taxa between the attractive and unattractive groups.

Global differences in the microbiome community between unattractive and attractive groups

Sparse partial least squares discriminant analysis (sPLS-DA) was used to compare unattractive and attractive groups based on beta diversity, a measure of variation in species diversity between individuals. Initially, the dataset was filtered by adding an offset of 1, removing low counts (below 0.01 %), and applying a CLR transformation. Cross-validation was performed to determine the optimal number of components and the number of loadings on each component. Components 1 and 2 combined explain 15 % of the differences between attractiveness groups, as shown in Figure 50. The centroids for the groups are separated on the 1st and 2nd components, and the 95 % confidence intervals overlap. Some separation between the groups gives some evidence of a difference in dispersion, but there is no evidence from a Permutational multivariate analysis of variance (PERMANOVA) test (P = 0.29). Additionally, density pots on the outside show separation in the distribution, especially on component 1.

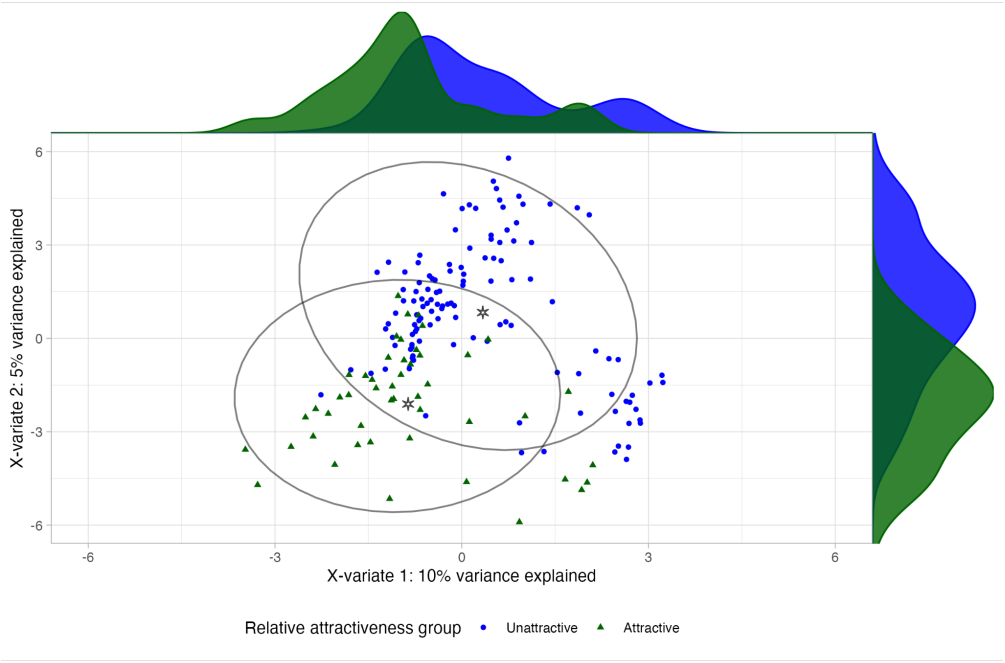

Figure 50. Sample plot visualises differences between the groups from a sparse partial least squares discriminant analysis (sPLS-DA) implemented in the MixOmics package (Rohart et al. 2017). It compares skin microbial composition (beta diversity based on relative amounts of ASVs) between the unattractive (dark blue) and attractive (dark green) in the UK cohort. Individuals are presented as small circles (unattractive) or small triangles (attractive). Microbiome data were pre-processed by filtering, CLR transformed and scaled (centred and standardised). The centroids, depicted as black stars, represent the average microbiome composition in the two-dimensional space on the first and second components of the analysis for each group. The enclosing ellipses (large circles) represent these groups' 95 % confidence intervals, visually estimating the dispersion around the group means. The first component explains 10 % variance, and the second component 5 % variance, summing to a cumulative variance of 15 %. Additionally, density plots are placed above and to the right of the loading plot presenting the distribution of the scores for the first and second components for each group. These provide a view of the spread of the data for each component within the donor and recipient groups.

Differences in individual bacterial taxa between unattractive and attractive groups

The contribution of individual genera of bacteria to differences in microbiome composition between the attractiveness groups was investigated by selecting and ranking taxa by order of importance on the two components of the sPLS-DA model as shown in Figure 51). The strongest correlations ($r > 0.3$ or $r < -0.3$) between relative abundance and global differences in the microbiome between the attractiveness groups were observed for an ASV assigned to the *Staphylococcus* genus on component 1 of Figure 51. The *Staphylococcus* ASVs are more abundant in the attractive group (coloured dark green). The direction

corresponds with the sign of the regression coefficient, which is associated with a particular axis of discrimination between groups in the sample plot (Figure 50). *Staphylococcus* in culture has been reported as attractive to mosquitoes [23]. Among the top 10 bacterial genera selected on the first component contributing to the differences in microbiome composition, additional taxa showed weaker contributions, including *Acinetobacter,* which were more abundant in the unattractive group.

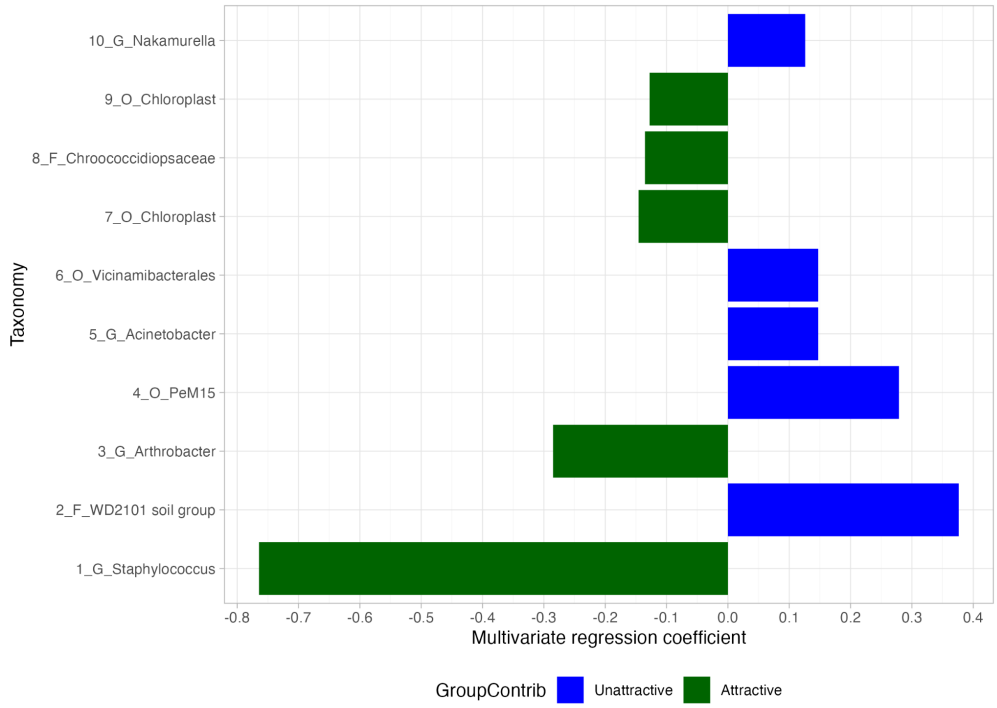

Figure 51. Bacterial genera with the greatest contribution to differences in microbiome composition between unattractive and attractive groups of relative attractiveness in the UK cohort on component 1 of the sPLS-DA. The loading plot represents the bacterial genera contributing the most to differences between the attractiveness group on component 1 (10 ASVs) of the sPLS-DA. Bars represent the loading weights or correlation coefficients of each bacterial genus to the components of the sPLS-DA. The direction of the bars (left or right) relates to the direction of the loadings in Figure 50. Dark blue and green bars indicate a higher abundance in the unattractive and attractive groups, respectively.

The loading plot for the second component of the sPLS-DA is shown in Figure 52. 50 ASVs were selected during cross-validation on this component and are therefore included in the plot. Two *Staphylococcus* ASVs explain most of the variation on this component. surprisingly, these *Staphylococcus* ASVs are higher in abundance in the unattractive group compared to the attractive, which is the opposite trend to that in Figure 50. Additional taxa that are contributing to the difference include *Corynebacteria* and *Streptococcus,* which are more abundant in the attractive group and *Pseudomonas,* which is more abundant in the unattractive group.

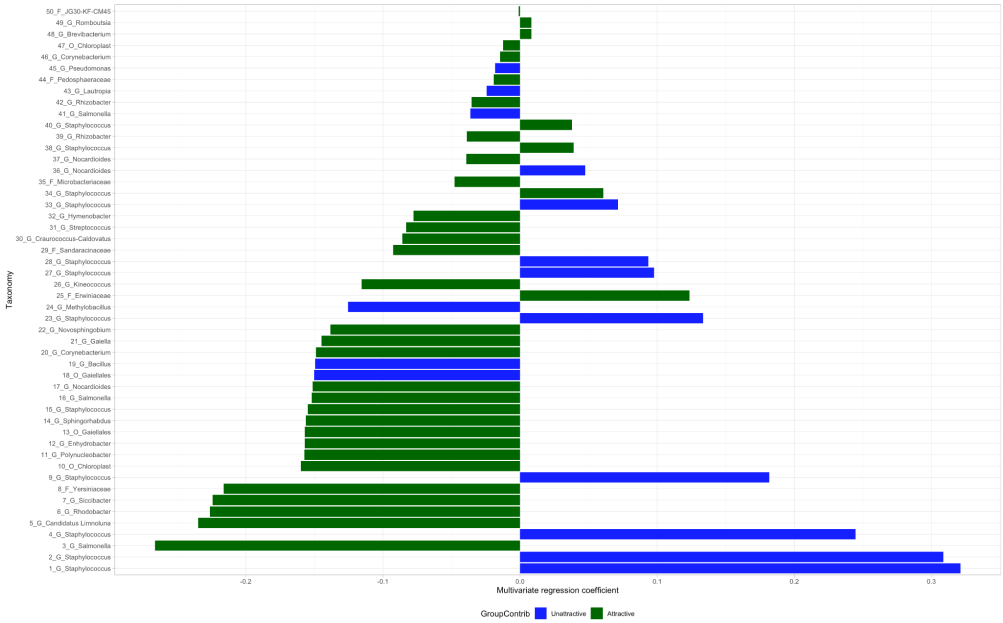

Figure 52. Bacterial genera with the greatest contribution to differences in microbiome composition between unattractive and attractive groups of relative attractiveness in the UK cohort on component 2 of the sPLS-DA. The loading plot represents the bacterial genera contributing the most to differences between the attractiveness group on component 2 (50 ASVs) of the sPLS-DA. Bars represent the loading weights or correlation coefficients of each bacterial genus to the components of the sPLS-DA. The direction of the bars (left or right) relates to the direction of the loadings in Figure 50. Dark blue and green bars indicate a higher abundance in the unattractive and attractive groups, respectively.

Evaluating model performance: ROC curve analysis of attractiveness classification

The receiver operating characteristic (ROC) curve was used to evaluate the sPLS-DA model's performance. The AUC estimates indicate that the model performs well at discriminating the unattractive and attractive groups, with better performance observed with four components. The ROC showed with the first component of the sPLS-DA model Area under the ROC Curve (AUC) is 0.772, whereas the model with all four components of the sPLS-DA selected by cross-validation increased the AUC to 0.984, as shown in Figure 53. These results indicate that the sPLS-DA model of skin microbiome composition can discriminate between unattractive and attractive participants to mosquitoes.

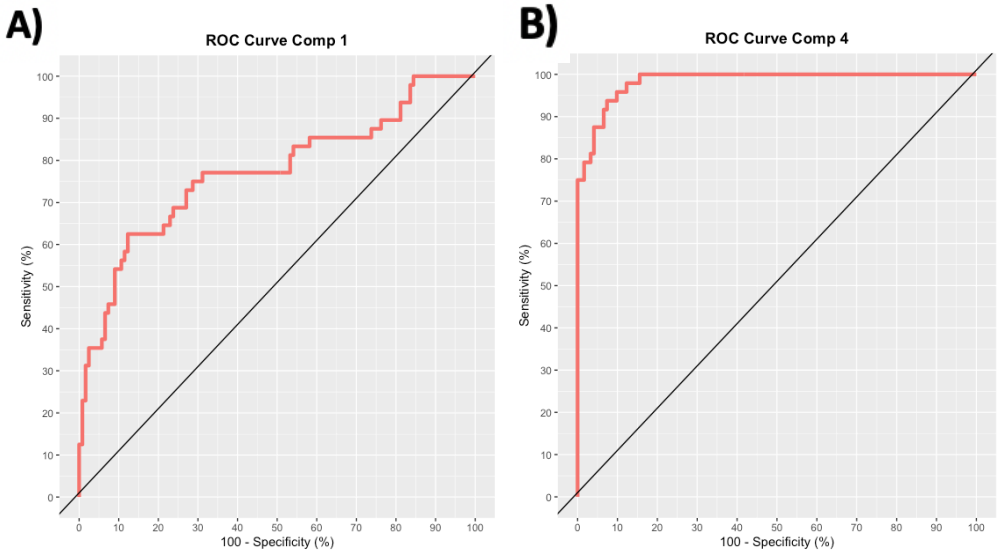

Figure 53. ROC curve for UK sPLS-DA for microbiome composition and category of attractiveness (unattractive vs attractive relative attractiveness) to mosquito on A) The first component and B) All four components selected with tuning.

3.05.05) Differentially abundant individual bacterial taxa between unattractive and attractive groups: DESeq2

The differential abundance of bacterial genera between attractive and unattractive groups were then tested using DESeq2 [235]. 57 ASVs were identified with abundances significantly different between the unattractive and attractive groups, as shown in Figure 54. Three *Staphylococcus* ASVs and a *Sphingomonas* ASV are in higher abundance in the attractive group compared to the unattractive group. The *Staphylococcus* ASVs identified on the first component of the sPLS-DA and *Sphinomonas* ASV identified on the second component have the same trend with higher abundance in the attractive group than the unattractive. The other ASVs identified are in higher abundance in the unattractive group than the attractive group, including *Staphylococcus, Brevibacterium, Acinetobacter,* and *Pseudomonas* ASVs. Several additional *Staphylococcus* ASVs were identified on the second component, most of which had a higher abundance in the unattractive group than in the attractive group. *Acinetobacter* and *Pseudomonas* were also identified in the exploratory multivariate analysis and found to have a higher abundance in the unattractive group than in the attractive, while *Brevibacterium* was shown to have a higher abundance in the attractive group than the unattractive on component 2 of the sPLS-DA. In summary, the DESeq2 analysis identified significant differences in the abundance of 57 ASVs between the attractive and unattractive groups without p-value adjustment, suggesting a distinct microbial profile for each group.

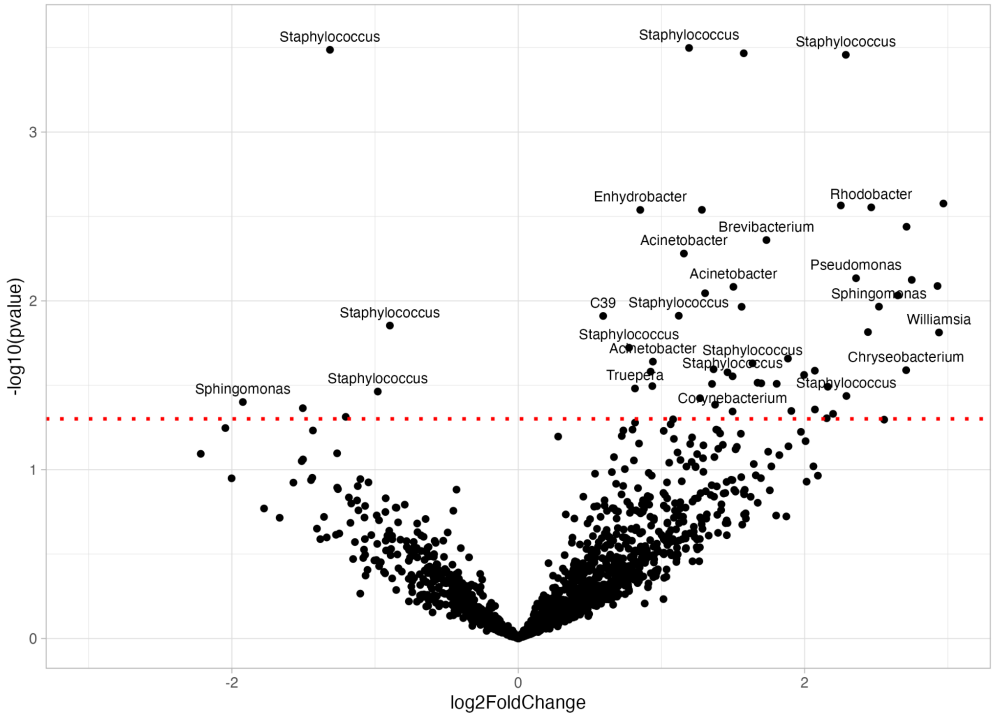

Figure 54. DESeq2 differential abundance of ASVs between unattractive and attractive groups. Volcano plot of ASVs (black dots) showing differential abundance between the relative attractiveness groups. DESeq2 was used to calculate log 2-fold changes, i.e. if bacterial ASVs are more or less abundant in the unattractive compared to the attractive group. The x-axis represents log 2-fold change abundance in the unattractive compared to attractive groups, with the biggest changes furthest from the centre. Y axis indicates the negative log-10 transform of the nominal p-value, i.e. increasing significance away from the origin. The red line represents P = 0.05 for exploratory purposes. There are 57 ASVs above the red line without adjustment for multiple testing.

A more stringent DESeq2 test was then applied, only selecting ASVs that were differentially abundant between the attractive and unattractive group after accounting for multiple testing using Benjamini-Hochberg adjustment. No ASVs remained significantly different when accounting for multiple testing.

3.05.06) Differentially abundant individual bacterial taxa between unattractive and attractive groups: Corncob

Although the DESeq2 analysis provided valuable insights into the differentially abundant bacteria, the investigation was expanded using beta-binomial regression in corncob, a method specifically designed for microbiome data [236]. Corncob applies parametric Wald tests to examine differential abundance hypotheses. The relative attractiveness group was coded as 0 for the unattractive group and 1 for the attractive group. This approach identified 15 ASVs as differentially abundant using a cut-off of value of p=0.05, as shown in Figure 55. Six ASVs were differentially enriched in the attractive group compared to the unattractive group, including the genera *Staphylococcus* and *Kocuria*. Conversely, nine ASVs were differentially depleted in the attractive group compared to the unattractive group, including the *Acinetobacter* and *Pseudomonas* genera. On comparing these results with those obtained with DESeq2, the trends in *Staphylococcus* and *Acinetobacter* ASVs were consistent in both analyses. However, they did not reach significance after multi-test adjustment with DESeq2.

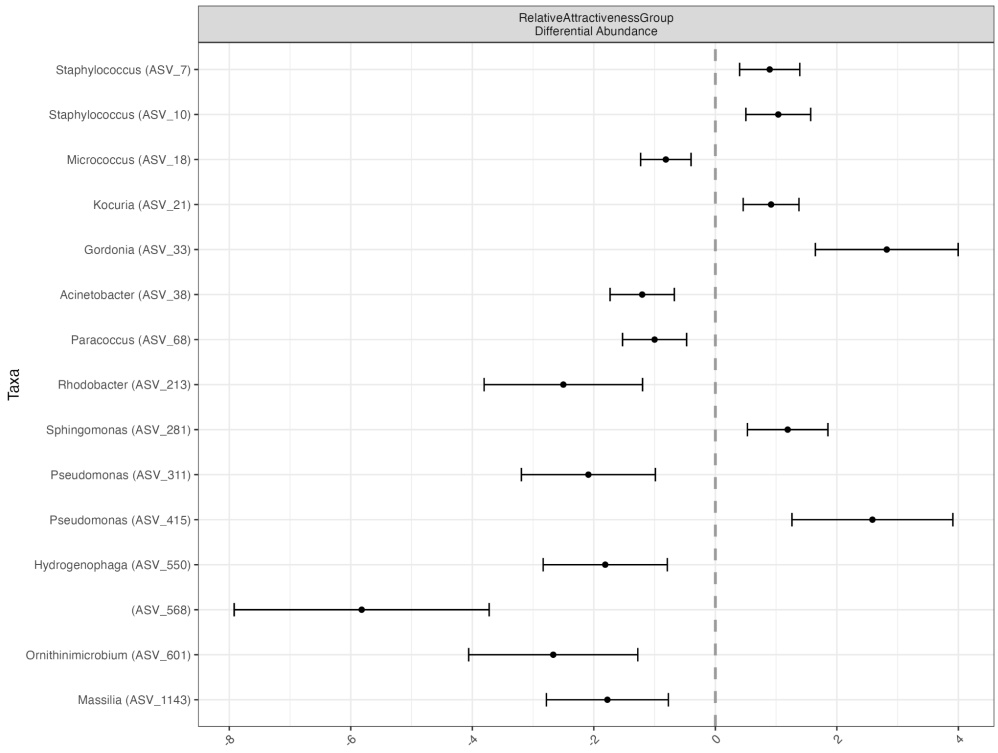

Figure 55. ASVs that were significantly differential abundant at an FDR cut-off of 0.05 between unattractive and attractive relative attractiveness groups in the UK cohort.

Following the beta-binomial regression analysis in corncob, The detailed statistics for every differentially abundant ASV, with genus-level taxonomy, are outlined in Table 11. This table provides an overview of the parametric Wald test results for each ASV identified in Figure 55. The parametric Wald test, a form of hypothesis testing, assumes certain data distribution properties and utilises these to calculate the probability of obtaining the observed data under the null hypothesis. Here, the null hypothesis assumes no difference in abundance for each ASV between the attractive and unattractive groups. For each ASV, the estimate represents the effect size and the estimated difference in abundance of the ASV between the attractive and unattractive groups. Each of the ASVs present in Table 11 met the significance threshold of p=0.05.

Table 11. Results from corncob analysis for differentially abundant ASVs with genus-level taxonomy.

Estimate	**Standard Error**	**T value**	**False discovery rate p-value**	**ASV name**	**Genus**
-5.82	1.07	-5.44	<0.001	568	*NA*
2.82	0.60	4.70	<0.001	33	*Gordonia*
-1.20	0.27	-4.46	<0.001	38	*Acinetobacter*
0.92	0.23	3.92	<0.001	21	*Kocuria*
-0.81	0.21	-3.85	<0.001	18	*Micrococcus*
2.59	0.68	3.83	<0.001	415	*Pseudomonas*
1.04	0.27	3.80	<0.001	10	*Staphylococcus*
-2.50	0.67	-3.76	<0.001	213	*Rhodobacter*
-2.67	0.71	-3.76	<0.001	601	*Ornithinimicrobium*
-1.00	0.27	-3.72	<0.001	68	*Paracoccus*
-2.09	0.56	-3.72	<0.001	311	*Pseudomonas*
0.89	0.25	3.54	0.001	7	*Staphylococcus*
1.19	0.34	3.52	0.001	281	*Sphingomonas*
-1.81	0.52	-3.48	0.001	550	*Hydrogenophaga*
-1.78	0.51	-3.47	0.001	1143	*Massilia*

3.05.07) Assessing genetic factors: a comparative analysis of alpha diversity among MZ, DZ, and unrelated pairs

Expected hypothesis (H0):
There is no difference in the alpha diversity of the pair between the MZ, DZ and unrelated pairs in the UK cohort.

Alternative hypothesis (H1):
There is a difference in the alpha diversity of the pair between the MZ, DZ and unrelated pairs in the UK cohort. MZ is more similar than DZ, and DZ is more similar than unrelated pairs.

The difference in alpha diversity between MZ twin pairs (N=39), DZ twin pairs (N=50) and unrelated pairs (N=31204) were then compared. If genetic factors control alpha diversity, then it is expected that MZ twin pairs would be more similar than DZ twin pairs and unrelated pairs of individuals. Figure 56 shows a boxplot and density plot that compares the distribution of alpha diversity differences between MZ, DZ and unrelated pairs. An ANOVA comparing the means of the three groups provided strong evidence of a difference (F value = 10.3, P value = <0.001). A Tukey's HSD test was then performed on the pairs. The test showed strong evidence of a difference in means between MZ-unrelated pairs (Difference = 0.49, 95 % CI = 0.21-0.76, p-value = 0.0001). However, there was no evidence of a difference between MZ-DZ pairs (Difference = 0.29, 95 % CI = -0.08-0.66, p-value = 0.155) or DZ-unrelated pairs (Difference = 0.20, 95 % CI = -0.05-0.44, p-value = 0.142). The boxplots show the distribution of the Shannon index (alpha diversity) per pair between MZ, DZ and unrelated pairs. The unrelated group has a much larger sample size and some extreme outliers. Quantifying the influence of genetics on Shannon diversity, Q scores were computed for MZ twins: Q = 0.24 (9% CI: 0.155 - 0.319) and DZ twins: Q = 0.36 (95 % CI: 0.261 - 0.451). These scores provide some evidence that genetic factors are contributing to differences in Shannon diversity.

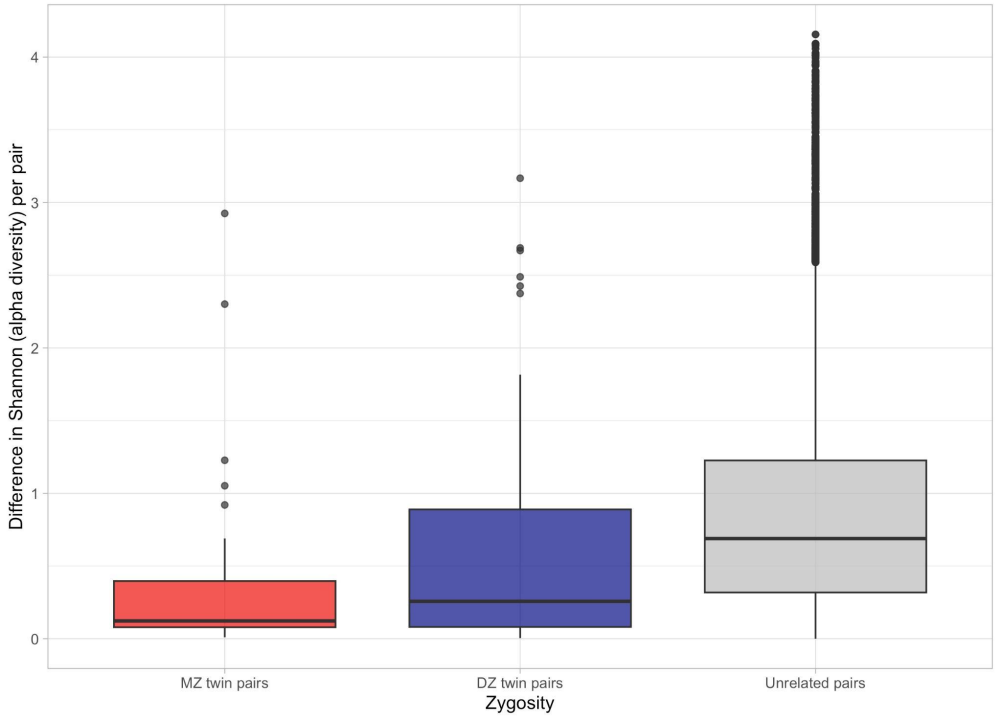

Figure 56. Boxplot comparing similarities in the Shannon diversity per pair between MZ (red), DZ (blue) and unrelated (grey) pairs in the UK cohort.

3.05.08) Summary

After conducting a comprehensive analysis of the UK cohort, which explored the impact of the skin microbiome on human attractiveness to mosquitoes, several key findings emerged. They include compositional differences between the attractive and unattractive groups, the identification of differentially abundant ASVs in the attractive and unattractive groups, and an exploration of the genetic influence on alpha diversity. Next, the skin microbiome and human attractiveness to mosquitoes was investigated in another geographical setting, in the Gambian cohort. Here, the interplay between the skin microbiome and human attractiveness to mosquitoes was explored in a population that is naturally exposed to *Anopheles* mosquitoes and *Plasmodium* parasites.

3.06) Gambian Results

3.06.01) Pre-processing of the Gambian cohort

The Gambian analysis began by comparing taxa bar plots of the top 20 most abundant genera between samples, positive and negative controls, as shown in Figure 57. The Gambian cohort consisted of 182 microbiome samples, with 173 samples, 2 positive controls and 7 negative controls. The low abundance taxa, which are not in the top 20, are represented as grey bars in the plot. There are clear differences in the relative abundances of genera between the control and sample groups. Moreover, the proportion of low abundant taxa within the samples shows considerable variation. In some samples, over 50 % of the reads are not the top 20 most abundant taxa.

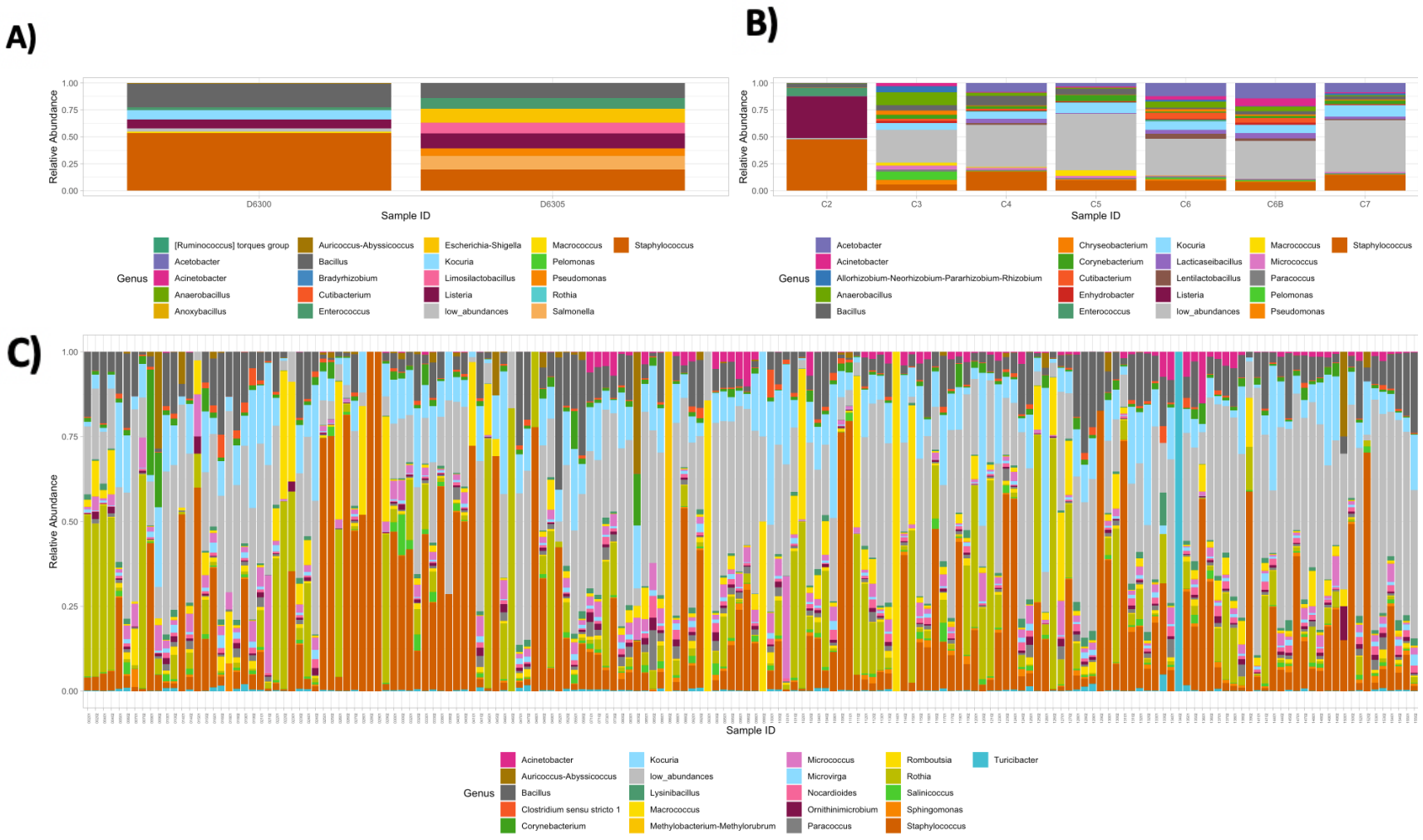

Figure 57. Summarises the taxonomy at the genus level as a taxa barplot of relative abundances for A) positive controls, B) negative controls and C) samples. There are clear differences in taxonomy between the samples and controls. Samples are a subset of the 20 most abundant taxa at the genera level, NA is the less abundant taxa combined.

3.06.02) Exploring the skin microbiome of the Gambian cohort

Expected hypothesis (H0):
There is no difference in the abundance of any of the top 10 genera between the attractive and unattractive groups in the Gambian cohort.

Alternative hypothesis (H1):
There is a statistically significant difference in the abundance of any of the top 10 genera between the attractive and unattractive groups in the Gambian cohort.

Comparative boxplot analysis: abundance of top 10 bacterial taxa in attractive vs. unattractive groups

First, the top 10 most abundant genera between the unattractive and attractive groups were explored, as shown in Figure 58. Out of the 171 microbiome samples that passed filtering, attractiveness data were available for 138 (behavioural assays were only run on twin pairs for which we had samples for both twins). Of these, 98 were categorised as unattractive, while 40 were attractive according to the *Anopheles coluzzii* behavioural data. The median absolute abundance of all genera was similar between the unattractive (dark purple) and attractive groups (dark orange). Wilcoxon tests with Benjamini-Hochberg correction between the groups provided some statistical evidence of a significant difference in the medians of *Micrococcus* (W=2591.5, Adjusted p-value=0.03) and *Bacillus* (W=2545, p-value=0.03) between the unattractive and attractive groups. However, there was no evidence of a difference in the other top genera based on Wilcoxon tests conducted between the attractiveness groups. *Staphylococcus, Micrococcus, Corynebacterium, Kocuria* and *Acinetobacter,* which are known skin genera, were identified among the top 10 most abundant genera in the UK cohort. In contrast, *Parococcus, Salinicoccus, Macrococcus, Bacillus* and *Rothia* were not part of the most abundant genera in the UK cohort. Previous studies have reported the presence of *Parococcus* on human skin [237]. *Macrococcus,* although closely related to *Staphylococcus,* is not frequently reported in skin studies. *Salinicoccus,* a soil microbe, is likely a contaminant from the environment. *Bacillus* is commonly isolated from the skin [238], while *Rothia,* usually found in the respiratory flora, has been reported in the oral microbiome and on the face [239].

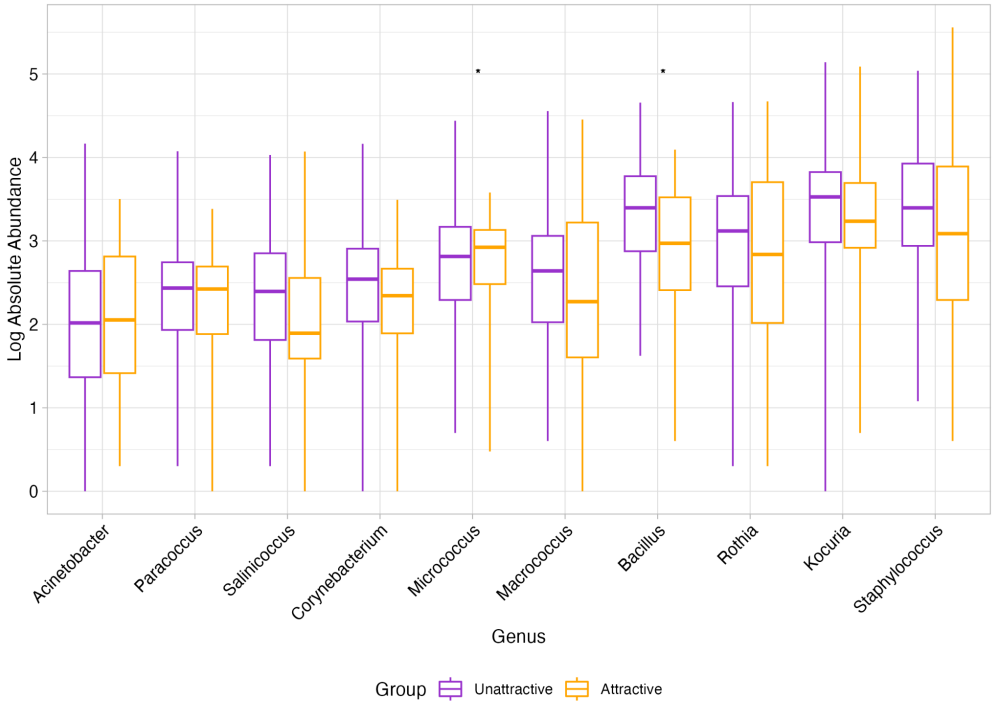

Figure 58. Comparison analysis of the top ten most prevalent bacterial genera between unattractive (dark purple) and attractive (dark orange) participants in the Gambian cohort. The x-axis categorises the bacterial genera, ordered from least to most abundant from left to right. The y-axis illustrates the absolute abundance of each genus on a logarithmic (base 10) scale. Boxplot whiskers include the full range of genus abundances, represented as sequence variant (ASV) counts and displayed as log base 10 absolute abundance. Statistical differences in median abundances between the donor and recipient groups for each genus were assessed using a Wilcoxon test, with adjustments for multiple testing via the Benjamini-Hochberg method. The statistical significance is visualised by asterisks above each boxplot. The absence of an asterisk suggests no significant difference, while a single (*) and double (**) asterisk denote significant (adjusted p-value < 0.05) and highly significant (adjusted p-value < 0.005) differences, respectively.

Comparative boxplot analysis: relative abundance of top 10 bacterial taxa in attractive vs. unattractive groups

To adjust for differences in the number of reads per sample, the counts were transformed into relative abundance, as shown in Figure 59. Then, the correlations observed between the unattractive and attractive groups were investigated to see if they remained consistent, as seen with the counts. Based on Wilcoxon tests with Benjamini-Hochberg correction between the groups, there was strong statistical evidence of a difference in the medians between the attractiveness groups for *Macrococcus* (W=2741.5, Adjusted p-value=0.002). However, there is no evidence of a difference in the other top genera based on the Wilcoxon tests conducted between groups. Interestingly, *Auricoccus-Abyssicoccus* occurred in the top 10 for relative abundance but not for absolute abundance.

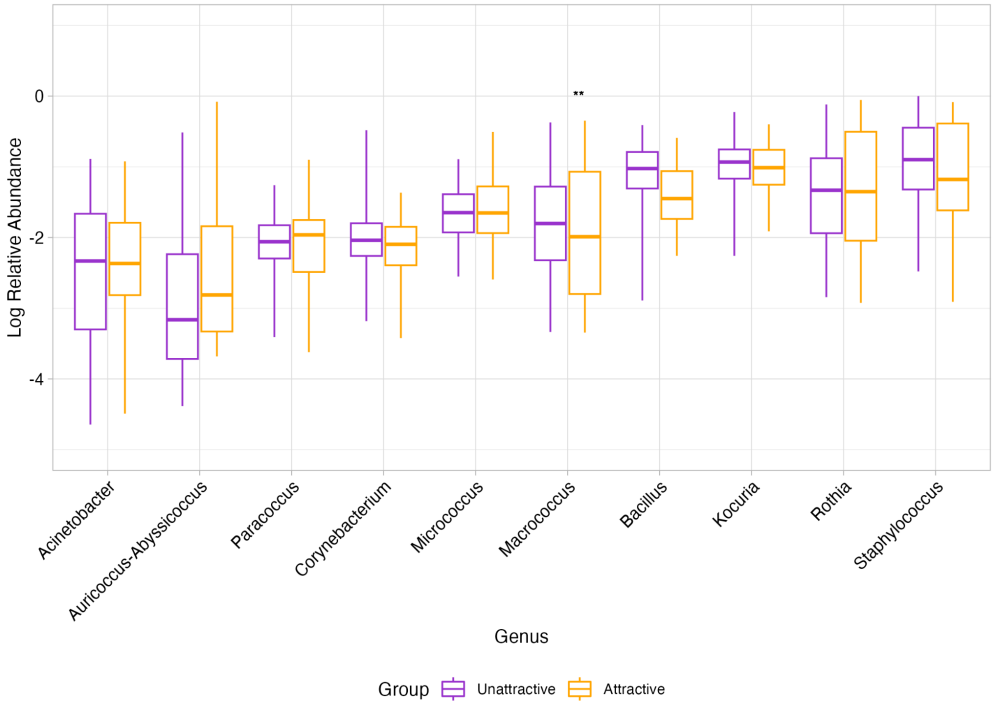

Figure 59. Comparison analysis of the top ten most prevalent bacterial genera relative abundance between unattractive (dark purple) and attractive (dark orange) participants in the Gambian cohort. The x-axis categorises the bacterial genera, ordered from least to most abundant from left to right. The y-axis illustrates the relative abundance of each genus on a logarithmic (base 10) scale. Boxplot whiskers include the full range of genus abundances, represented as sequence variant (ASV) counts and displayed as log base 10 relative abundance. Statistical differences in median abundances between the donor and recipient groups for each genus were assessed using a Wilcoxon test, with adjustments for multiple testing via the Benjamini-Hochberg method. The statistical significance is visualised by asterisks above each boxplot. The absence of an asterisk suggests no significant difference, while a single (*) and double (**) asterisk denote significant (adjusted p-value < 0.05) and highly significant (adjusted p-value < 0.005) differences, respectively.

3.06.03) Contrasting alpha diversity: comparisons between attractive and unattractive groups

Expected hypothesis (H0):
There is no difference in the alpha diversity between the attractive and unattractive groups in the Gambian cohort.

Alternative hypothesis (H1):
There is a statistically significant difference in the alpha diversity between the attractive and unattractive groups in the Gambian cohort.

Next, global differences in the skin microbiome between the unattractive and attractive groups were explored, starting with differences in alpha diversity, measured using the Shannon index, as shown in Figure 60. The Wilcoxon test, used to compare the medians, showed no statistically significant differences in median alpha diversity between the unattractive (4.38) and attractive (4.50) groups ($W = 1874$, p-value = 0.51). The permutation test of equality, used to compare the distributions, also indicated no significant difference in distribution (permutation test of equality $p=0.68$).

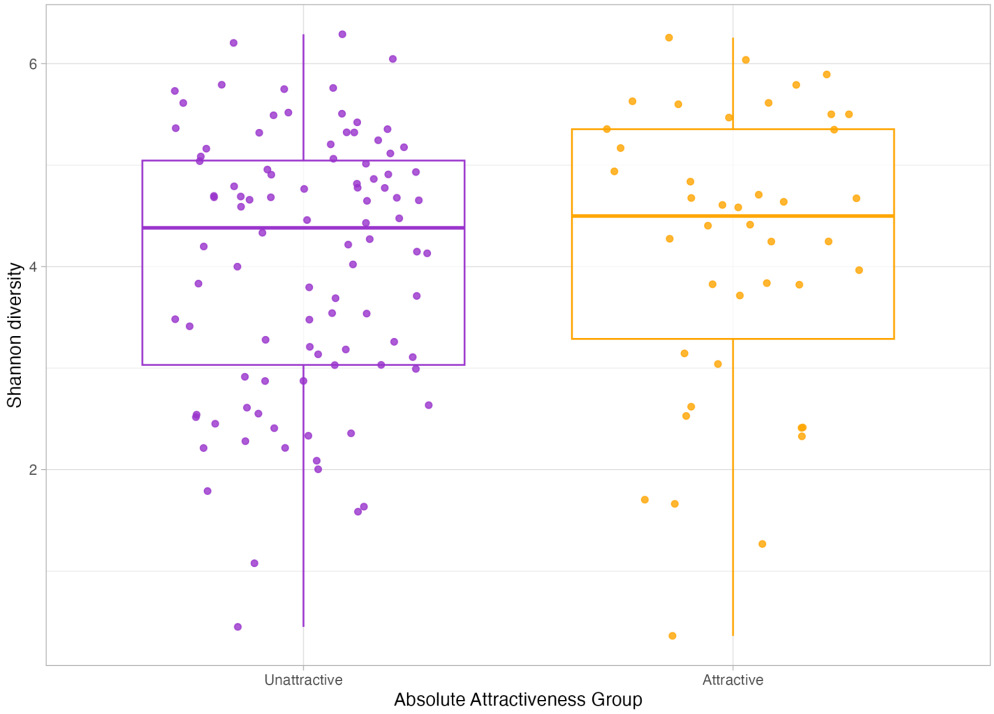

Figure 60. Comparison analysis of Shannon diversity between unattractive and attractive groups prior to transfers. The x-axis categories fall into two groups: unattractive (dark purple) and attractive (dark orange) in the Gambian cohort. The y-axis represents the Shannon diversity index. The boxplot displays the data distribution for each group, demonstrating the median and interquartile range and total range of the data. The individual data points overlaid as scatter represent the individual samples.

3.06.04) Exploratory differential abundance analysis of the Gambian cohort

Expected hypothesis (H0):
There is no difference in the beta diversity between the attractive and unattractive groups in the Gambian cohort. There are no differentially abundant taxa between the attractive and unattractive groups.

Alternative hypothesis (H1):
There is a statistically significant difference in the beta diversity between the attractive and unattractive groups in the Gambian cohort. There are differentially abundant taxa between the attractive and unattractive groups.

Global differences in the microbiome community between unattractive and attractive groups

SPLS-DA was used to compare unattractive and attractive groups based on beta diversity. Initially, the dataset was filtered by adding an offset of 1, removing low counts (below 0.01 %), and applying a CLR transformation. Consequently, 134 samples remained, with 95 classified as unattractive and 39 as attractive. Then, across-validation was performed to determine the optimal number of components and the number of loadings on each component. Components 1 and 2 combined explain 10 % of the differences between attractiveness groups, as shown in Figure 61. The centroids for the groups are separated on the 1st and 2nd components, and the 95 % confidence intervals overlap. Some separation between the groups gives some evidence of a difference in dispersion, but there is no evidence from a Permutational multivariate analysis of variance (PERMANOVA) test ($P = 0.64$). Additionally, density pots on the outside show differences in the distribution, especially on both components.

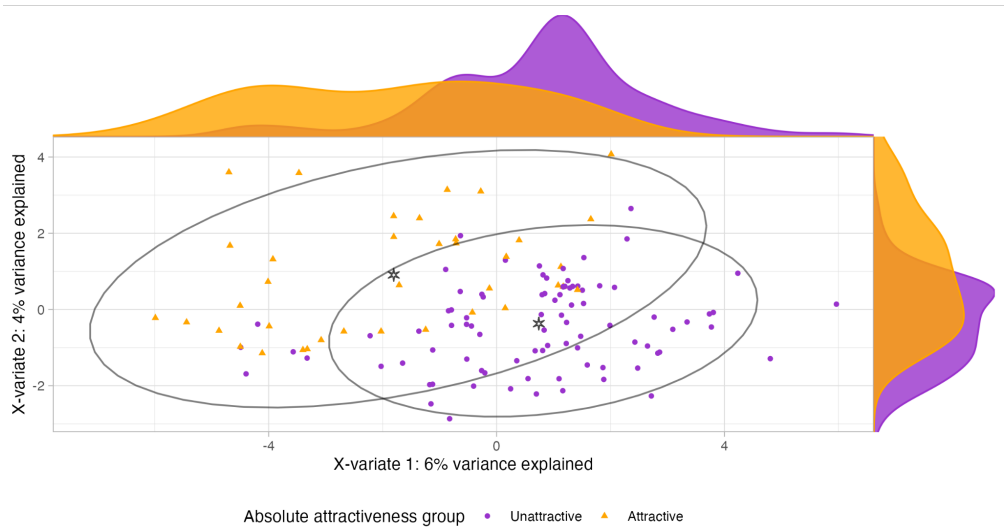

Figure 61. Sample plot visualises differences between the groups from a sparse partial least squares discriminant analysis (sPLS-DA) implemented in the MixOmics package (Rohart et al. 2017). It compares skin microbial composition (beta diversity based on relative amounts of ASVs) between the unattractive (dark purple) and attractive (dark orange) in the Gambian cohort. Individuals are presented as small circles (unattractive) or small triangles (attractive). Microbiome data were pre-processed by filtering, CLR transformed and scaled (centred and standardised). The centroids, depicted as black stars, represent the average microbiome composition in the two-dimensional space on the first and second components of the analysis for each group. The enclosing ellipses (large circles) represent these groups' 95 % confidence intervals, visually estimating the dispersion around the group means. The first component explains 6 % variance, and the second component 4 % variance, summing to a cumulative variance of 10 %. Additionally, density plots are placed above and to the right of the loading plot presenting the distribution of the scores for the first and second components for each group. These provide a view of the spread of the data for each component within the donor and recipient groups.

Differences in individual bacterial taxa between unattractive and attractive groups

The contribution of individual genera of bacteria to differences in microbiome composition between the attractiveness groups was investigated by selecting and ranking ASVs by order of importance on the two components of the sPLS-DA model. Loading plots provided a summary of the significance of genera in the multivariate model, ranked in decreasing order of importance from the bottom to top, which were associated with the differences between the attractiveness groups. The strongest correlations ($r > 0.3$ or $r < -0.3$) between relative abundance and global differences in the microbiome between the attractiveness groups were observed for *Nonomuraea, Staphylococcus, Clostridium* and *Streptococcus* on component 1 as shown in Figure 62. *Nonomuraea, Staphylococcus* and *Clostridium* were more abundant in the unattractive group (coloured light orange). The direction corresponds

with the sign of the regression coefficient, which is associated with a particular axis of discrimination between groups in the sample plot (Figure 61).

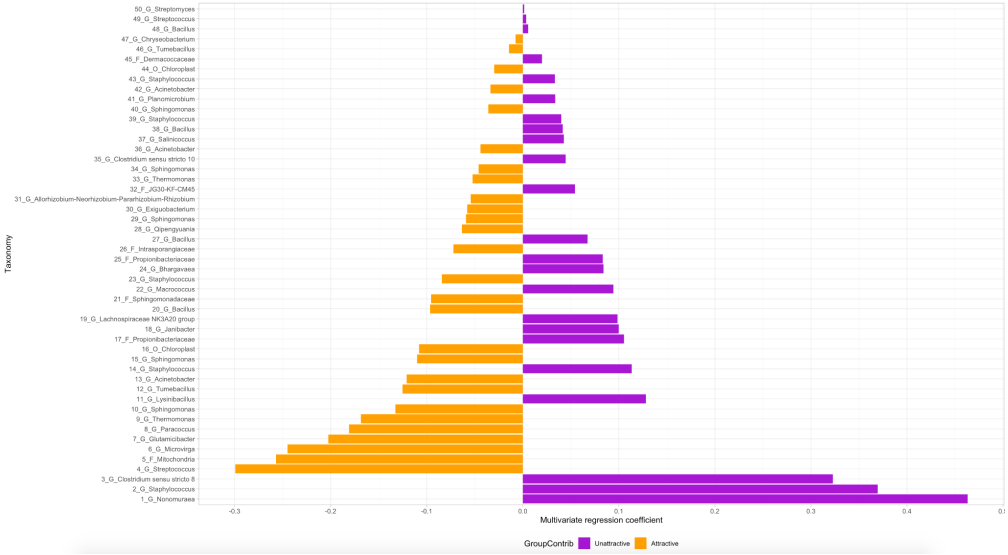

Figure 62. Bacterial genera with the greatest contribution to differences in microbiome composition between unattractive and attractive groups of relative attractiveness in the Gambian cohort on component 1 of the sPLS-DA. The loading plot represents the bacterial genera contributing the most to differences between the attractiveness group on component 1 (50 ASVs) of the sPLS-DA. Bars represent the loading weights or correlation coefficients of each genus to the components of the sPLS-DA. The direction of the bars (left or right) relates to the direction of the loadings in Figure 61. Dark purple and dark orange bars indicate a higher abundance in the unattractive and attractive groups, respectively.

The loading plot for the second component of the sPLS-DA is shown in Figure 63. Ten ASVs were selected during cross-validation on this component and are therefore included in the plot. A *Parococcus* ASVs explains most of the variation on this component. These bacteria are more abundant in the unattractive group than in the attractive.

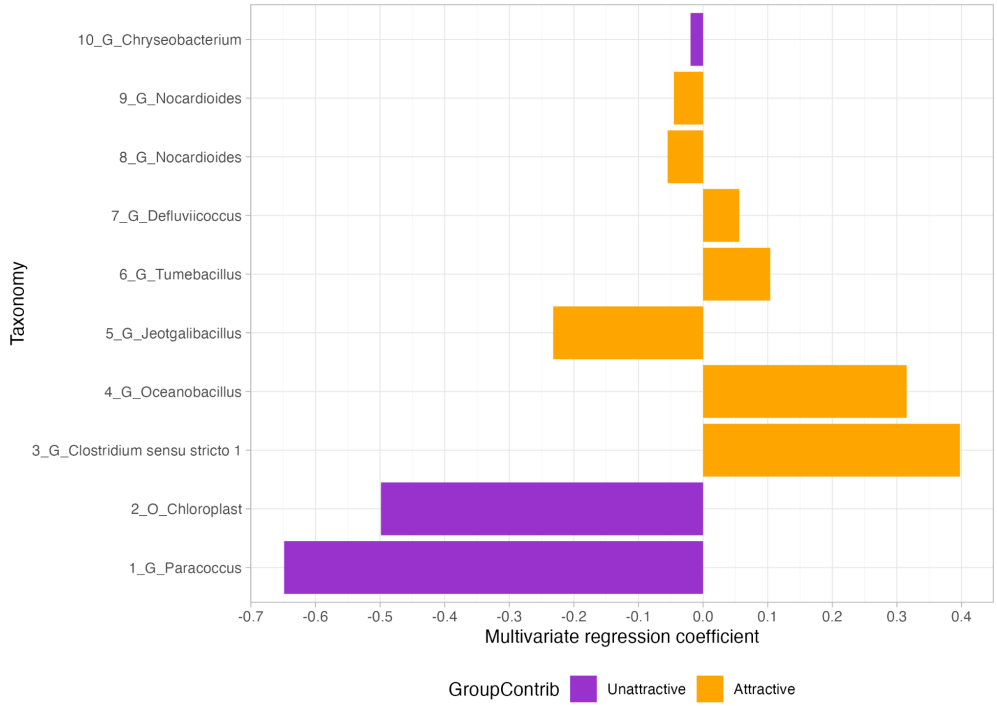

Figure 63. Bacterial genera with the greatest contribution to differences in microbiome composition between unattractive and attractive groups of relative attractiveness in the Gambian cohort on component 2 of the sPLS-DA. The loading plot represents the bacterial genera contributing the most to differences between the attractiveness group on component 2 (10 ASVs) of the sPLS-DA. Bars represent the loading weights or correlation coefficients of each bacterial genus to the components of the sPLS-DA. The direction of the bars (left or right) relates to the direction of the loadings in Figure 61. Dark purple and dark orange bars indicate a higher abundance in the unattractive and attractive groups, respectively.

Evaluating model performance: ROC curve analysis of attractiveness classification

The ROC curve was used to evaluate the performance of the sPLS-DA. The ROC shown with the first component from the sPLS-DA AUC was 0.802. Whereas the model with both components of the sPLS-DA selected by cross-validation increased the AUC to 0.920, as shown in Figure 64. These results indicate that the sPLS-DA model of skin microbiome composition can discriminate between unattractive and attractive participants to mosquitoes.

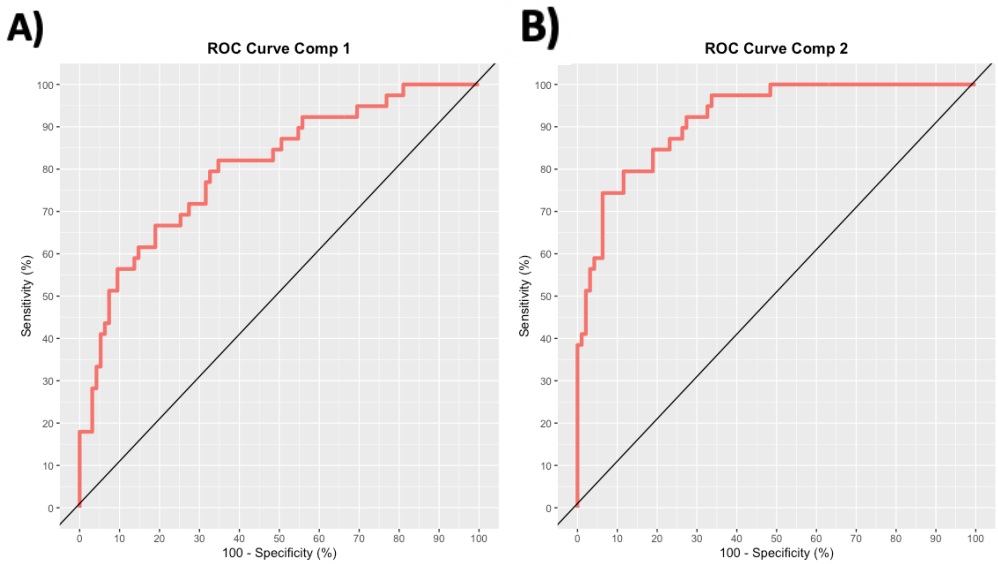

Figure 64. ROC curve for sPLS-DA for microbiome composition of Gambia cohort and category of attractiveness (unattractive vs attractive absolute attractiveness) to mosquitoes on A) the first component and B) both components (selected by cross-validation).

3.06.05) Differentially abundant individual bacterial taxa between unattractive and attractive groups: DESeq2

The differential abundance of bacterial genera between attractiveness groups were then tested with DESeq2. 121 ASVs with abundances significantly different between the unattractive and attractive groups were identified, as shown in Figure 65. *Staphylococcus, Corynebacterium* and *Brevundimonas* ASVs are amongst those higher in abundance in the attractive group compared to the unattractive group. More ASVs were identified as higher in abundance in the unattractive group than in the attractive group, including *Staphylococcus, Bacillus, Kocuria* and *Micrococcus* ASVs.

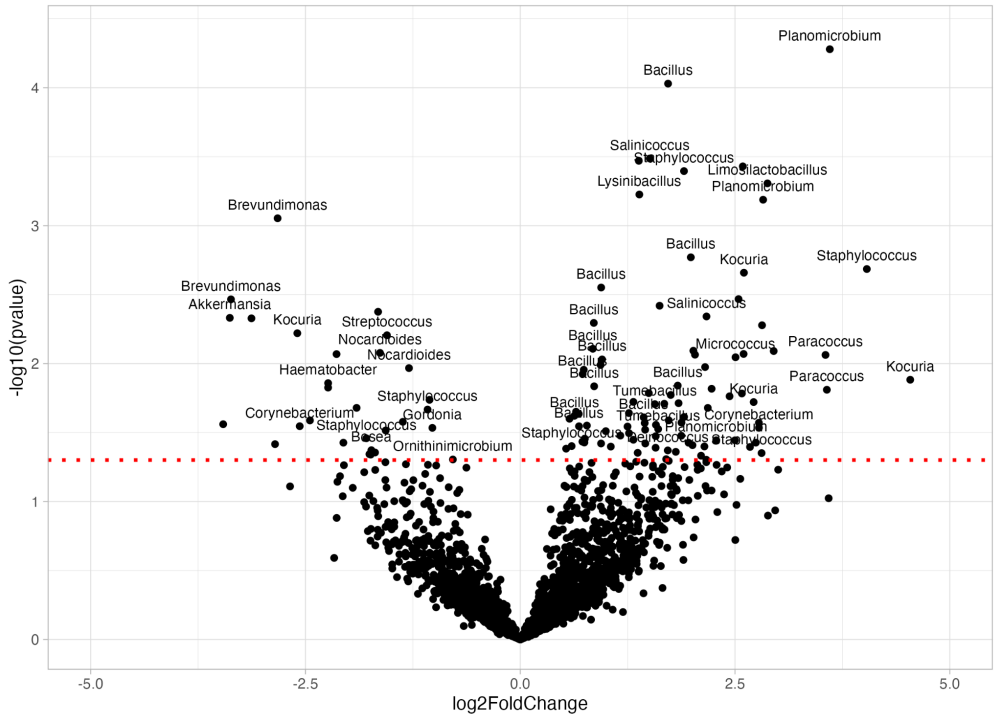

Figure 65. DESeq2 differential abundance of ASVs between unattractive and attractive groups. Volcano plot of ASVs (black dots) showing differential abundance between the relative attractiveness groups. DESeq2 was used to calculate log 2-fold changes, i.e. if bacterial ASVs are more or less abundant in the unattractive compared to the attractive group. The x-axis represents log 2-fold change abundance in the unattractive compared to attractive groups, with the biggest changes furthest from the centre. Y axis indicates the negative log-10 transform of the nominal p-value, i.e. increasing significance away from the origin. The red line represents P = 0.05 for exploratory purposes. There are 121 ASVs above the red line without adjustment for multiple testing.

Next, a more stringent DESeq2 test was applied, only selecting ASVs that were differentially abundant between the attractive and unattractive group after accounting for multiple testing using Benjamini and Hochberg adjustment. No ASVs remained significantly different when accounting for multiple testing.

3.06.06) Differentially abundant individual bacterial taxa between unattractive and attractive groups: corncob

Beta-binomial regression models in corncob were then applied to test for ASVs differentially abundant between the unattractive and attractive groups [236]. Differential abundance was modelled as a function of the attractiveness group. The parametric Wald test was used to test differentially abundant hypotheses. The model identified no ASVs that were differentially abundant with a cut-off of p=0.1.

3.06.07) Assessing genetic factors: a comparative analysis of alpha diversity among MZ, DZ and unrelated pairs

Expected hypothesis (H0):
There is no difference in the alpha diversity of the pair between the MZ, DZ and unrelated pairs in the Gambian cohort.

Alternative hypothesis (H1):
There is a difference in the alpha diversity of the pair between the MZ, DZ and unrelated pairs in the Gambian cohort. MZ twins more similar than DZ and DZ twins are more similar than unrelated pairs.

The difference in alpha diversity between MZ twin pairs (N=34), DZ twin pairs (N=27) and unrelated pairs (N=20190) were compared. The reduction in the number of twins was due to the usage of the decontaminated, filtered phyloseq object to export the Shannon data, resulting in fewer pairs with the necessary data available. If genetic factors control alpha diversity, MZ twins would be expected to be more similar to each other than DZ twins, and both would be more similar than unrelated individuals. Figure 66 shows a boxplot comparing the distribution of differences in alpha diversity between MZ, DZ and unrelated pairs. An ANOVA comparing the means of the three groups gave no evidence of a difference (F value = 2.21, P value = 0.11). The boxplots show similarities in the distribution of the difference in Shannon (alpha diversity) between MZ/DZ twins and unrelated pairs. Notably, the unrelated

pairs have a much larger sample size and some extreme outliers. Quantifying the influence of genetics on Shannon diversity, Q scores for MZ twins were computed: Q = 0.37 (95 % CI: 0.264 - 0.484) and DZ twins: Q = 0.27 (95 % CI: 0.176 - 0.372), which gives no evidence that genetics factors are contributing to differences in Shannon diversity.

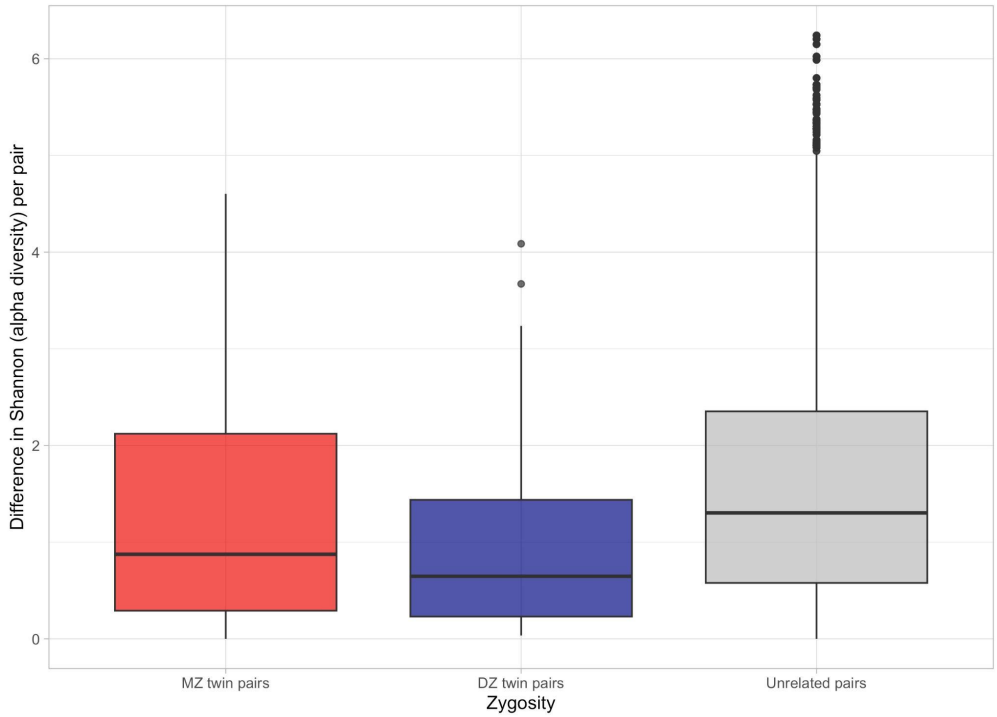

Figure 66. Boxplot comparing the Shannon diversity per pair between MZ (red), DZ (blue) and unrelated (grey) twins in the Gambia cohort.

3.07) Discussion

This chapter explores the role of the skin microbiome on human attractiveness to mosquitoes. The findings indicate that the skin microbiome has a role in determining attractiveness to *Anopheles coluzzii* mosquitoes. Specifically, the analysis revealed differences in microbiome composition and identified some evidence of specific bacterial ASVs being differentially abundant between unattractive and attractive groups to mosquitoes.

In the differential abundance analysis, microbiome composition was shown to account for 15 % of the variance in attractiveness in the UK cohort and 10 % in the Gambia cohort. This finding aligns with Verhulst et al. (2011), who reported that 47 % of the variance in attractiveness to mosquitoes was attributable to differences in the relative abundances of bacterial genera in the microbiome profiles. They compared highly attractive (N=9) and poorly attractive (N=7) groups that had been determined to have significantly different mean attractiveness based on behavioural data from six replicates in a dual-choice olfactometer [137]. Unlike Verhulst's method of focusing on the extremes, in this thesis, all subjects were divided into attractive and unattractive groups (Chapter 2) to increase power. The results of this study corroborate the existence of a compositional difference between attractive and unattractive groups. The ROC analysis further revealed that the overall microbiome composition could be a reliable predictor for determining whether an individual is attractive or unattractive to mosquitoes. The ROC AUC values obtained from our study are consistent across both cohorts. However, it is important to note that the sample does not represent the entire population. These findings should be validated through in-depth experimental studies for a more conclusive understanding. Such studies might involve analysing the skin microbiome, predicting group classifications based on the microbiome composition, and assessing mosquitoes' attractiveness to validate the study's exploratory results.

Using the corncob method, 15 bacterial ASVs were identified that differed significantly in abundance between attractive and unattractive groups in the UK cohort. Of these, 14 were assigned genus-level taxonomy. However, the findings were not consistent across methods (DESeq2 and corncob) or cohorts (UK and Gambia). Certain ASVs, including *Staphylococcus* and *Kocuria,* were more abundant in the attractive group in the UK cohort. In contrast, *Micrococcus* and *Acinetobacter*, among others, were less abundant in the attractive group. In a previous study, Verhulst et al. (2011) found that a *Pseudomonas* ASV was significantly associated with the poorly-attractive group to *An. gambiae*. Verhulst et al.

also identified other significant genera, *Acidobacteria gp3, Delftia* and *Leptotrichia*, with the highly-attractive group, and *Variovorax*, with the poorly-attractive group. These genera were not identified as being differentially abundant in this study. These contrasting results could be due to variations in the study population, measurement or grouping of attractiveness to mosquitoes, 16S primers used, sequencing platform or analysis approach. In the Gambia cohort, no significant differences were found using corncob, possibly due to imprecision in the attractiveness assessment as described in Chapter 2. No ASVs were consistently identified as significantly differentially abundant in both cohorts, limiting confidence in our results. The differential abundance analysis indicated bacteria that may be important in human attractiveness to mosquitoes. Future investigations should ensure there is more precision in the attractiveness groups, use long-read sequencing to get species-level taxonomy and incorporate culturing to test microbes of interest behaviourally with mosquitoes to validate molecular microbiology results from differential abundance analysis functionally.

In this study, *Staphylococcus* was the most important in the multidimensional analysis for the UK cohort. *Staphylococcus* ASVs were higher in the attractive than the unattractive group in both cohorts and with multiple methods, aligning with previous findings of *Staphylococcus epidermis* cultures in stationary phase attracting *Anopheles* mosquitoes (Verhulst et al. 2009). *Staphylococcus* are known to produce volatile fatty acids, including isovaleric acid, which is associated with a foot-like odour, as well as propionic, butyric, caproic, and acetic acids [110,240]. Verhulst et al. (2009) noted *that Staphylococcus epidermis* produces different volatiles than bacteria-free blood agar [136]. The significant influence of *Staphylococcus* ASVs in the findings and evidence from the literature indicates the importance of *Staphylococcus* in attracting mosquitoes and contributing to the inter-individual variation in human attractiveness to mosquitoes. From the analysis, there is suggestive evidence that some *Staphylococcus* ASVs are associated with high attractiveness to *An. coluzzii*. However, some *Staphylococcus* ASVs showed higher abundance in the unattractive group in both the UK and Gambian cohorts, according to the DEseq2 results. This suggests the possibility of different species or strains within the same genus having opposite effects on mosquitoes. A previous study demonstrated this by culturing microbes from the skin, selecting single isolates belonging to different species and testing them in a two choice olfactometer with *Aedes albopictus*. It showed that *Staphylococcus saprophyticus* was attractive and *Staphylococcus hominis* was repellent compared to a control (liquid LB agar) [241]. While *Staphylococcus epidermis* has been shown to reduce trap catches during the exponential phase compared to control plates but increase them during the stationary phase due to qualitative differences in volatile profiles

between the growth phases [23]. Functional differences exist between strains of bacteria within a genus [242]. Inconsistencies in the direction of the observed trend suggest the presence of strain-specific differences in volatile profiles within the *Staphylococcus* genus. It would be interesting to investigate many strains of *Staphylococcus* from human skin using a two-choice olfactometer, building on Michalet et al (2019)'s work which showed differences in attractiveness between *Staphylococcus saprophyticus* and *Staphylococcus hominis*. A higher throughput assay would be preferred, but as shown in Chapter 2, variability between replicates means several replicates are required. Nayani et al. 2023 demonstrated variation in the volatile profile of *Staphylococcus* strains found on cow skin and their impact on attracting blood-feeding stable flies [89]. Overall, it is likely that some strains of *Staphylococcus* produce an attractive volatile profile, while others might generate a less attractive volatile profile to the mosquitoes.

In addition, two ASVs belonging to the *Pseudomonas* genus were found to be differentially abundant in the UK cohort. One ASV has a higher abundance in the attractive group than the unattractive whilst the other had a higher abundance in the unattractive than the attractive using corncob. Verhulst *et al.* (2011) found that *Pseudomonas* were significantly more abundant in poorly attractive than highly attractive people and using PLS that a *Pseudomonas* spp is correlated with the poorly attractive group, although small sample sizes for the groups [137]. It has also been shown in culture that *Pseudomonas aeruginosa* is less attractive than *Bacillus subtilis*, *Brevibacterium epidermidis*, *Corynebacterium minutissimum*, and *Staphylococcus epidermidis* during the stationary phase of growth [23]. *Pseudomonas aeruginosa* had a similar number of landings to a control plate of medium alone, indicating it is unattractive [23]. The inconsistency in the trend of *Pseudomonas* ASVs being higher in attractive or unattractive shown in this study could be due to differences in species or strain levels of *Pseudomonas* having different metabolic profiles as described above, that lead to differences in mosquito behaviour, or it could be due to differences in the overall composition. *Pseudomonas aeruginosa* is present in the environment as well as on human skin [243], which may result in more diversity due to the variety of ecological niches and environments it is able to grow in compared to skin that has similar temperature, pH and nutrients availability.

Moreover, ASVs belonging to the genus *Acinetobacter*, *Micrococcus* and *Paracoccus* were found that were differentially abundant, with higher abundance in the unattractive group compared to the attractive group in multiple analyses presented in this chapter. This suggests these bacteria may produce either unattractive or repellent compounds to *An. coluzzii*, or they may mask the attractive odour from other bacteria, as previously suggested

in the literature [137]. Other mechanisms that have been suggested include bacteria converting attractive compounds produced by other bacteria to repellent compounds and signalling to other bacteria to prevent them from emitting attractive compounds [137]. Bacteria may produce attractive VOCs when cultured individually, but in a blend, they may interact antagonistically and no longer be highly attractive [202]. The findings highlight the potential importance of *Acinetobacter*, *Micrococcus* and *Paracoccus* abundance in differentiating unattractive and attractive participants. Furthermore, differentially abundant ASVs were identified that could be assigned genus taxonomy, including *Kocuria,* which was more abundant in the attractive group compared to the unattractive in the UK cohort using corncob. Most previous studies have focussed on *Bacillus*, *Brevundimonas*, *Corynebacterium* and *Staphylococcus* species as potential mosquito attractants [23,202]. *Kocuria* has not been discussed in previous studies focusing on identifying attractive bacteria that produce attractive VOCs. These bacteria may produce VOCs that can be used in synthetic blends and warrant further investigation and could be tested in lures previously created based on *Bacillus*, *Brevundimonas*, *Corynebacterium* and *Staphylococcus* headspace.

Differences in the top 10 genera between the UK and Gambian cohorts were observed in this study. *Staphylococci* were the most abundant genera in both cohorts, while *Corynebacteria* also featured among the top 10. *Pseudomonas* ranked among the top 10 based on relative attractiveness within the UK cohort, whereas *Bacillus* appeared in the top 10 most abundant in the Gambian cohort. *Brevibacteria*, however, did not make it into the top 10 in either cohort. The differences observed between the populations were exploratory based on 16S data. In the Gambian cohort, more soil bacteria were expected due to the evident soil contamination on many swabs in this cohort. However, all of the top 10 genera were probable skin microbes previously reported on the skin. *Lynsinibacillus* and *Microvirgia* were among the top 20 genera in the Gambian cohort and are likely environmental contaminants from soil or plants [244,245]. This finding is consistent with similar findings by Hospodsky *et al.* (2014) that showed more environmental contamination on Tanzanian hands than on US students' hands [210]. Environmental samples could have been collected to address this. Furthermore, the Gambian cohort showed fewer significantly different ASVs between attractiveness groups identified with corncob than the UK cohort, although there were more when using DEseq2 without p-value adjustment. Such variation could be attributable to ethnic and lifestyle differences, genetic factors or the disparities in the mosquito behavioural assay or DNA extraction kit used between the cohorts. The environment in which individuals live plays a crucial role due to environmental and cultural differences [216]. The participants from Farafenni, a rural village in The Gambia, had mud

floors in their homes, contrasting with the UK cohort who had less environmental exposure in their homes.

Moreover, alpha diversity, as measured using Shannon Index, was not significantly different between the attractiveness groups. This finding contrasts with the study by Verhulst *et al.* (2011), which demonstrated higher microbial diversity in people that are less attractive to mosquitoes [137]. However, it is important to note that their data showed that increasing the threshold for sampling depth gave weaker evidence of a difference between the groups [137]. The findings presented in this chapter from both the UK and Gambian cohorts indicate that there is no difference in species diversity between attractive and unattractive groups to *Anopheles* mosquitoes. Additionally, a genetic influence on alpha diversity was evident in the UK cohort, but this was absent in the Gambian group, presenting inconclusive evidence. The UK result supports previous literature that has suggested the skin microbiome is highly heritable [114]. While further research into this aspect could provide insights, this evidence does not support it being a priority. Genetics might directly influence gene variants affecting attractiveness or via the microbiome. However, this chapter concludes that there is no discernible difference in alpha diversity among the different attractiveness groups.

This study has several limitations. Firstly, there were limitations to the precision in human attractiveness to mosquitoes measured in Chapter 2 due to low numbers of replicates. Potential contaminant reads were encountered due to the low bacterial yield in skin microbiome samples. To address this, positive and negative controls were included in each sequencing run and the decontam method was used to filter out potential contaminants. Another limitation is that the amount of bacteria present on the participant's skin was not quantified. A tool such as BactQuant could have been used for this purpose [246]. The primer pair was kept consistent, but it should be noted that 16S primers are known to amplify certain bacteria more than others, resulting in some biases. The selected primers amplified the V3/4 region, not the whole 16S, meaning taxonomy could only be assigned confidently to the genus level. Additionally, fungi were not investigated in this study, despite the foot mycobiome being more diverse than on other body sites and known to include such genera as *Malassezia*, *Aspergillus*, *Cryptococcus*, *Rhodotorula*, and *Epicoccum* [247]. The mycobiome may explain some of the differences seen between attractiveness groups. Although the sample size in this study is larger than in previous studies, it still had limited power to detect differences between the groups. Multiple approaches were used to improve confidence in the results obtained. Given that age and sex may be associated with attractiveness to mosquitoes, these findings may only apply to these particular demographics. Including more metadata for potential confounders of attractiveness to

mosquitoes and the microbiome, such as BMI in the Gambian cohort and diet in both cohorts, would have allowed for the investigation of these factors. These limitations should be considered when interpreting these results and generalising them to broader populations.

In conclusion, this chapter explored the role of the skin microbiome in determining human attractiveness to *Anopheles coluzzii* mosquitoes. There are differences in the overall microbiome composition between attractive and unattractive groups. Further experiments are needed to confirm if the skin microbiome can predict human attractiveness to mosquitoes. It would be interesting to further validate the ratios of abundant microbes that make a person attractive or unattractive to mosquitoes beyond Lucas-Barbosa et al. (2023) culturing work. This study identified differentially abundant bacterial taxa between the unattractive and attractive groups, providing evidence that the skin microbiome has a role in determining human attractiveness to mosquitoes. The findings indicate that *Staphylococcus* was found to be more abundant in the attractive group, consistent with previous reports, while *Acinetobacter* and *Pseudomonas* were less abundant in the attractive group. However, differential abundance results were generally not consistent across methods or cohorts. Further experimental and longitudinal work is needed to overcome some of the potential confounding effects and validate the functional roles of the candidate bacteria identified. Fungi may be a missing link explaining some differences between attractive and unattractive groups and warrant further investigation. Consideration of the competitive nature of the skin microbiome is crucial for a deeper insight into its role in mosquito attraction. Following the recommendations of Lucas-Barbosa et al. (2023), co-culture experiments could be conducted to study microbial interactions and their impact on various mosquito species. Overall, this chapter sheds light on the complex interplay between skin microbiota and mosquito attraction. The findings could lead to the development of novel mosquito control strategies in the future. This study emphasises the importance of investigating differences in the microbiome at species or strain level in future studies to gain a deeper understanding of mosquito attraction.

3.08) Supplementary

3.08.01) UK pre-processing

Evaluating the performance of negative controls and removing contaminants

The library size (number of reads) for each of the samples (blue dots) and the negative controls (red dots) is shown in Figure 67. The red dots are in the bottom left with a library size <50,000. This supports successful collection in the samples as the negatives are blank swabs not used to collect human skin microbiome from a foot and, therefore, should contain fewer reads than the samples.

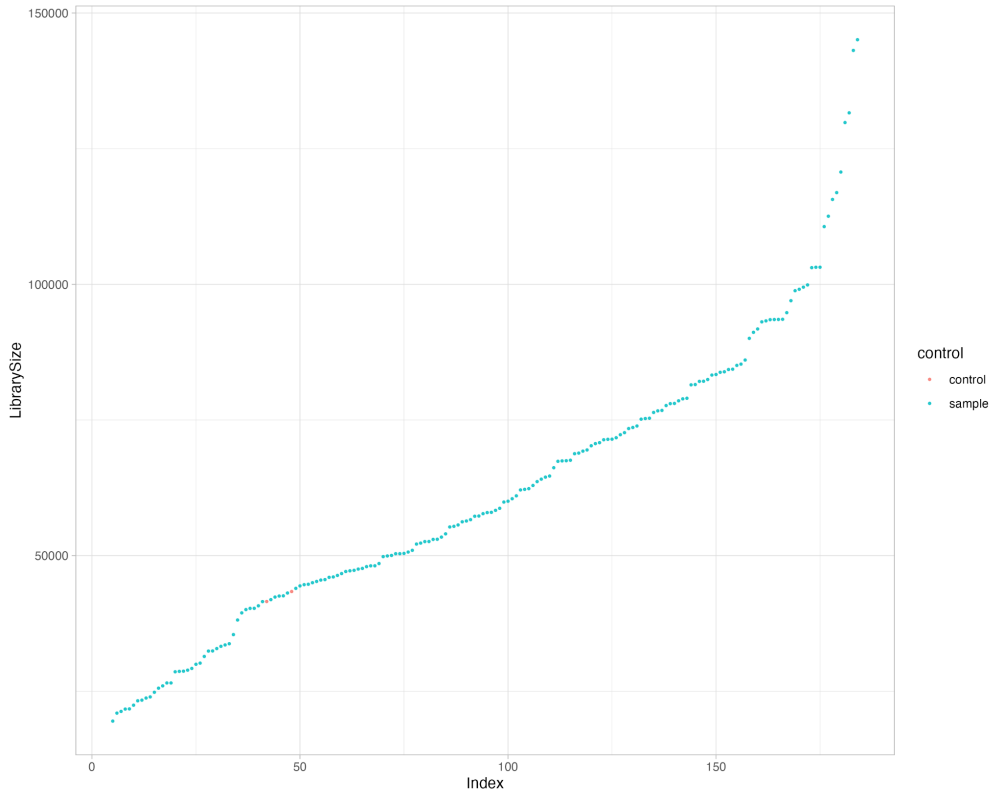

Figure 67. The library size for the samples (blue points) and the negative controls (red points) varied.

Using the decontam package [248], potential contaminant ASVs were identified and removed using the composition of the negative controls and a conservative prevalence cut-off of 0.5. These blank swabs were extracted and processed in parallel with the samples. As a result, 152 of the 3,138 ASVs were identified as contaminants and subsequently removed, and one sample was excluded. Figure 68 compares taxa bar plots at the genus level for 20 randomly selected samples before and after using decontam. The plots show the top 20 most abundant genera with all other genera combined as a single, low-abundance grey bar. Notably, the plots have high similarity pre- and post-decontam,

indicating that the decontam package has little impact on the most abundant genera. These genera are likely to be true skin microbes as they are found in higher abundance in the samples than the negative controls.

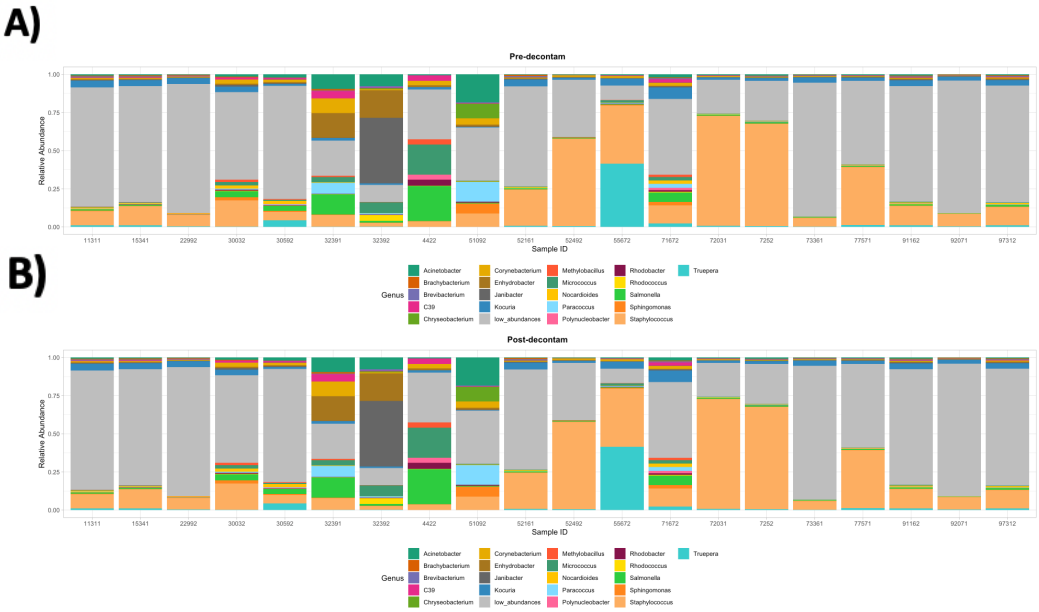

Figure 68. Comparison of 20 samples randomly selected A) Pre-decontam and B) Post-decontam. Shows the top 20 genera relative abundances. Grey is used for the other taxa not in the 20 most abundant.

The data were filtered by excluding samples containing fewer than 10 reads, leaving 184 samples. ASVs that appeared in less than 10 % of samples or a single sample were removed, resulting in 1,364 ASVs for subsequent analysis.

Evaluating the performance of positive controls

The composition of the positive controls is shown in Figure 69A as a taxa barplot. The positive controls underwent the same processing as the samples except for exclusion from the filtering step that removed the low abundant ASVs not present in 10 % of samples. This exception was made because some of the bacteria in the mock communities were not present in the samples, so applying the same filtering process would have removed the ASVs required to compare the composition of the positive controls to the expected composition.

The comparison between the actual and expected relative abundances of the taxa in D6306 (the DNA control) and D6300 (the extraction control) is shown in Table 12. These controls are expected to have equal proportions (12 % each) of *Pseudomonas, Eschericia, Salmonella, Lactobacillus, Enterococcus, Staphylococcus, Listeria* and *Bacillus*. Additionally, they were expected to include smaller quantities (2 % each) of *Saccharomyces*

and *Cryptococcus,* which are fungi and undetectable with 16S primers. In both D6306 and D6300, *Eschericha* and *Salmonella* were overrepresented, whereas *Pseudomonas* and *Staphylococcus*, *Listeria* were slightly less abundant than expected. The main difference between the DNA control (D6306) and extraction control (D6300) was that *Lactobacillus* was less abundant in the sequencing control, which was DNA extracted alongside the samples, while *Bacillus* was more abundant in the extraction control compared to the DNA control. This discrepancy suggests the DNA extraction may exhibit slight bias, over-representing *Bacillus* and under-representing *Lactobacillus*. Despite this, all expected genera were identifiable in the DNA and extraction control.

Table 12. Summary of the anticipated versus observed relative abundances, presented as percentages, for D6306 (DNA control) and D6300 (extraction control) for each of the genera in the positive controls.

Taxa	**Expected %**	**D6306 Actual %**	**D6300 Actual %**
Pseudomonas aeruginosa	12	5.27	4.67
Eschericia coli	12	16.41	20.13
Salmonella enterica	12	18.28	21.25
Lactobacillus fermentum	12	14.43	5.77
Enterococcus faecalis	12	5.67	5.79
Staphylococcus aureus	12	8.97	8.69
Listeria monocytogenes	12	8.43	5.41
Bacillus subtilis	12	22.53	28.28
Saccharomyces cerevisiae	2	0.00	0.00
Cryptococcus neoformans	2	0.00	0.00

Table 13 provides a summary of the expected and actual relative abundances of the taxa in D6310 (the log extraction control) and D6311 (the log DNA control). These controls were expected to have log abundance distributions of *Listeria, Pseudomonas, Bacillus, Saccharomyces, Eschericia, Salmonella, Enterococcus, Cryptococcus* and *Staphylococcus* over a broad range (from 10^2 to 10^8 cells). There was a slight overabundance of *Listeria*, which was the most abundant taxa in both the extraction and DNA control. Conversely, *Pseudomonas* appeared in smaller quantities than expected in both controls. Among the less abundant taxa *Bacillus*, *Eschericia, Salmonella were* detected in both controls at similar abundances to expected. As anticipated, the yeasts *Saccharomyces* and *Cryptococcus* are not detected.

Table 13. Summary of the anticipated versus observed relative abundances, presented as percentages, for D6310 (extraction control) and D6311 (DNA control) for each of the genera.

Taxa	**Expected %**	**D6310 Actual %**	**D6311 Actual %**
Listeria monocytogenes	89.1	96.3	98.5
Pseudomonas aeruginosa	8.9	2.8	1.0
Bacillus subtilis	0.89	0.90	0.50
Saccharomyces cerevisiae	0.89	0.00	0.00
Eschericia coli	0.089	0.015	0.008
Salmonella enterica	0.089	0.0240	0.0136
Lactobacillus fermentum	0.0089	0.0027	0.0000
Enterococcus faecalis	0.00089	0.00000	0.00000
Cryptococcus neoformans	0.00089	0.00000	0.00000
Staphylococcus aureus	0.000089	0.000000	0.000000

3.08.02) Gambian pre-processing

Evaluating the performance of negative controls and removing contaminants

In Figure 69, the library size (number of reads) is shown for each sample (blue dots) and negative controls (red dots). Figure 70 excluded any sample or control that exceeded 125,000 reads, leading to 10 samples and 1 control being excluded from the plot. Notably, most of the negative controls have low library sizes, below 50,000 reads. This supports successful collection in the samples as the negative controls should have fewer reads than the samples. During the normalisation step prior to sequencing, substantial quantities of the negative controls were added to the pool compared to the samples. It is plausible that this contributed to the unexpected outcome of one of the negative controls having a higher number of reads than would be expected.

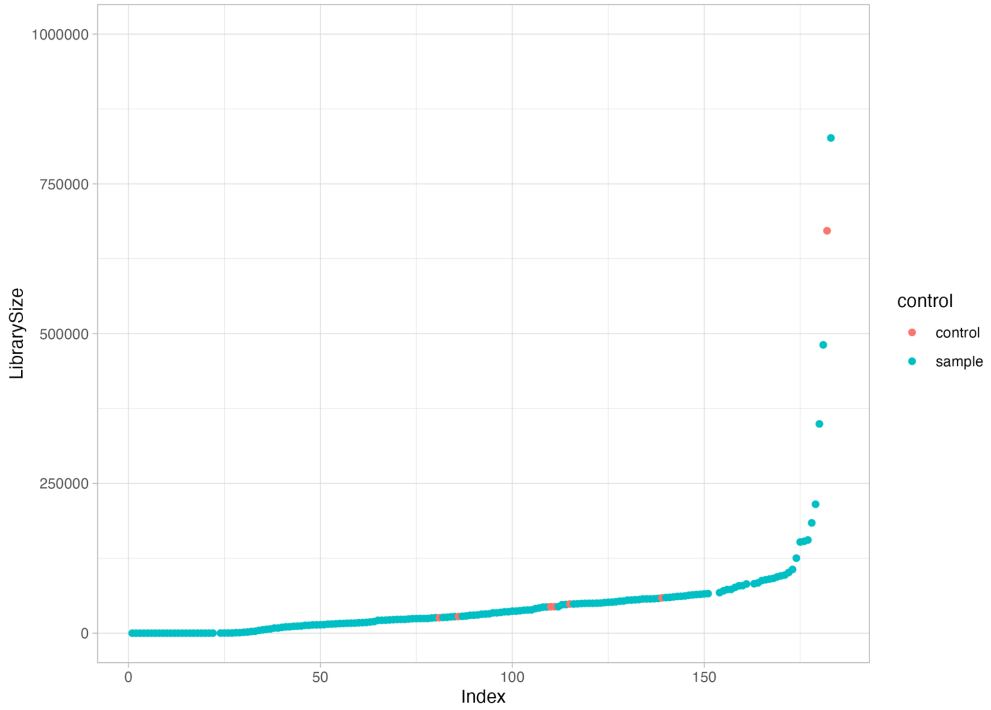

Figure 69. The library size for the samples (blue points) and the negative controls (red points) varied.

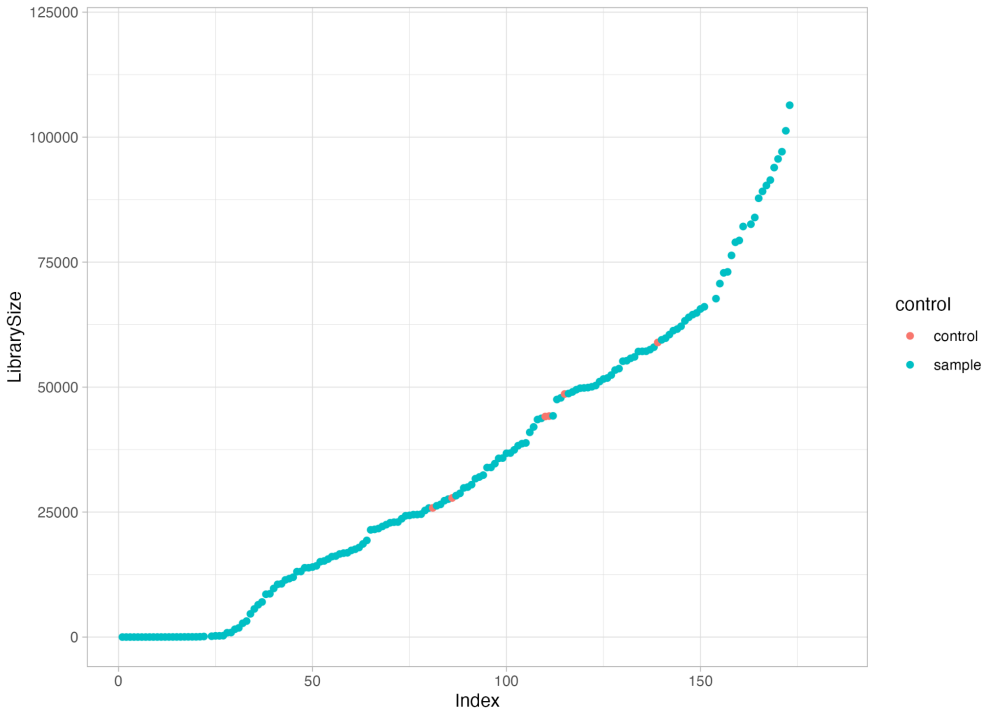

Figure 70. The library size for the samples (blue points) and the negative controls (red points) varied, with reads more than 125,000 excluded.

Using decontam, contaminant ASVs were identified and removed by using the composition of the negative controls. As there were 7 negative controls, the control with a high number of reads did not lead to decontam removing many ASVs. For this cohort, a less stringent prevalence cut-off of 0.1 was applied, compared to the 0.5 cut-off used in the UK cohort. This was because the cut-off of 0.1 resulted in significantly fewer ASVs being removed. Consequently, 139 out of 71,341 ASVs were identified as contaminants and removed. Figure 71 compares taxa bar plots at the genus level for 20 randomly selected samples before and after decontam was applied. The plots show the top 20 most abundant genera, while all remaining genera are represented as a single, low-abundance grey bar. Interestingly, there is a high degree of similarity between pre and post-decontam. This suggests that decontam has minimal impact on the most abundant genera. These prevalent genera are likely to be true components of the skin microbes, given their higher abundance in the samples compared to the negative controls.

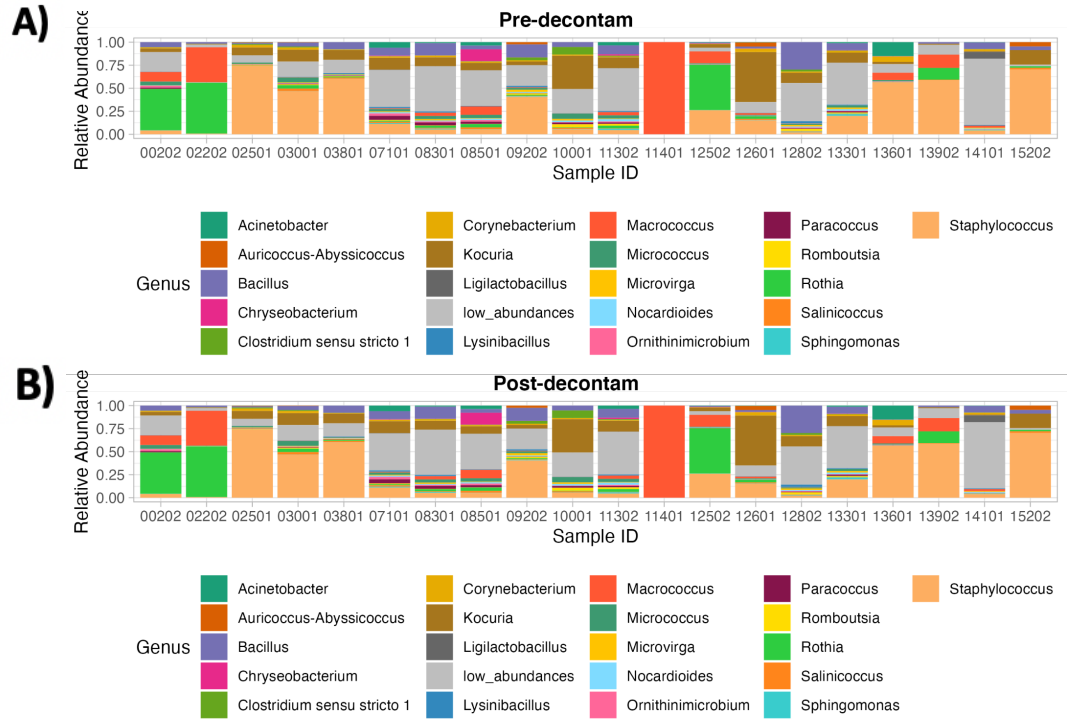

Figure 71. Comparison of 20 samples randomly selected A) Pre-decontam and B) Post-decontam. Shows the top 20 genera relative abundances grey is used for the other taxa not in the 20 most abundant.

The data were filtered by excluding samples with fewer than 10 reads, leaving 171 samples. ASVs that appeared in less than 10 % of samples or a single sample were removed, resulting in 2,560 ASVs for subsequent analysis.

Evaluating the performance of positive controls

The composition of the positive controls is shown in Figure 71A as a taxa barplot. The positive controls underwent the same processing as the samples except for exclusion from the filtering step that removed the low abundant ASVs not present in 10 % of samples.

The comparison between the actual and expected relative abundances of the taxa in D6305 (the DNA control) and D6300 (the extraction control) is shown in Table 14. As for the UK cohort, these controls are expected to have equal proportions (12 % each) of *Pseudomonas, Eschericia, Salmonella, Lactobacillus, Enterococcus, Staphylococcus, Listeria* and *Bacillus*. Additionally, they were expected to include smaller quantities (2 % each) of *Saccharomyces* and *Cryptococcus,* which are fungi, undetectable with 16S primers. In both D6305 and D6300, an overabundance of *Staphylococcus* and *Listeria* was observed. Within the DNA control (D6305), *Salmonella, Lactobacillus,* and *Enterococcus* appeared similar to expected, while *Bacillus* and *Eschericia* showed slightly higher

abundance, and *Pseudomonas* was slightly less prevalent than expected. In contrast, in the extraction control (D6300), *Pseudomonas, Eschericia, Salmonella* and *Lactobacillus* were not detected, implying some degree of bias in the extraction method. *Bacillus* were less abundant than expected, while the proportion of *Enterococcus* was around expected. However, *Staphylococcus* was found to be significantly more abundant than expected. These observed differences between the extraction, and DNA controls suggest that the DNA extraction may be somewhat biased to overrepresent *Staphylococcus* and underrepresent *Pseudomonas, Eschericia, Salmonella* and *Lactobacillus*. While all genera expected to be present could be identified in the DNA control, this was not the case for the extraction control. Additionally, some issues with D6300 were encountered due to its very low read numbers (39 reads post DADA2 compared to 78,318 for D6305), which could have been the result of poor normalisation during pooling before sequencing.

Table 14. Summary of the anticipated versus observed relative abundances, presented as percentages, for D6306 (DNA control) and D6300 (extraction control) for each of the genera in the positive controls.

Taxa	**Expected %**	**D6305 Actual %**	**D6300 Actual %**
Pseudomonas aeruginosa	12	7.16	0.00
Eschericia coli	12	13.50	0.00
Salmonella enterica	12	11.41	0.00
Lactobacillus fermentum	12	10.39	0.00
Enterococcus faecalis	12	10.04	10.26
Staphylococcus aureus	12	20.32	53.85
Listeria monocytogenes	12	14.19	30.77
Bacillus subtilis	12	13.00	5.13
Saccharomyces cerevisiae	2	0.00	0.00
Cryptococcus neoformans	2	0.00	0.00

4) Chapter 4: Volatiles and attractiveness to mosquitoes

4.01) Statement of multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper	The design of sample collection occurred before I started my PhD. My PhD uses the GC-FID and GC-MS data collected during the GenoScent project. UK volatile samples were collected in 2018 by Catherine Oke and Dr Julien Martinez (during my MSc). I was involved in running some GC-FID runs with Catherine Oke to learn the methodology. The majority of UK GC-FID samples were run by Catherine Oke and Elizabeth Pretorious. Dr John Pickett ran GC-MS at Cardiff on a subset of samples and provided chemistry support on identifying compounds. I co-led setting up fieldwork in the Gambia with Catherine Oke. The majority of the Gambian volatile samples were collected by a field team in The Gambia and shipped back to the UK due to COVID-19 disruption. Scott Tytheridge ran most of the Gambia GC-FID runs. I ran ~40 of the samples on the GC-FID for this cohort. Professor John Pickett performed GC-MS on a sub-set and provided chemistry support in identifying compounds. Me and Scott Tytheridge were able to match GC-FID and GC-MS traces manually to identify peaks that had been tentatively identified. I carried out co-injections on GC-FID to confirm the identities of compounds. I carried out all data analysis, and Dr Chrissy Roberts provided feedback on the analysis.

Student signature:

4.02) Introduction

Experimental research suggests differences in body odour play a significant role in varying levels of attractiveness [16,38,137,140–142]. Over 300 volatile organic compounds (VOCs) have been identified from human skin [145]. VOCs are small compounds (up to C20) that are characterised by having a low molecular mass, low boiling point and high vapour pressure [92]. Among the VOCs present, some compounds are linked to attracting mosquitoes [145]. Mosquitoes use body heat, visual cues, CO_2 and VOCs to find hosts [191]. There are differences between mosquito species in their odour preference, as reviewed by Coutinho-Abreu et al. (2022) [249]. Anthropophilic mosquitoes are attracted to lactic acid and CO_2, which are typical components of human sweat [249]. Smallegange et al. (2009) demonstrated that attraction can be optimised through the addition of a blend of 15 selected aliphatic carboxylic acids, revealing specific chain lengths (C3-C8 and C14) to enhance attractiveness to *An. gambiae*. The concentration and ratio of carboxylic acids in a blend are crucial determinants for attracting *An. gambiae.* Moreover, the changes in the relative amount of acids can impact the overall attractiveness of the blend [29,145]. The influence of odour goes beyond the chemical composition. The odour's evaporation rates, dosage and molecular structure, determine whether it acts as an attractant or repellent [145].

Research on human volatiles, which aims to understand why certain individuals are naturally unattractive to mosquitoes, has proposed two main mechanisms: odour masking and direct repellency [18]. The concept of odour masking is that these compounds interfere with mosquitoes' ability to detect attractive odours, essentially acting as a mask, interfering with the mosquito's capacity to detect the attractive odour and reducing the individual's overall attractiveness to mosquitos [18]. This masking effect may reduce sensitivity or responsiveness to VOCs [18]. Alternatively, specific compounds may be repellents [18]. Upon detection, these VOCs could deter mosquitoes from approaching their targets [18]. In a previous study investigating highly-attractive and poorly-attractive individuals to mosquitoes, Partial Least Squares - Discriminant Analysis (PLS-DA) was unable to significantly differentiate the highly-attractive and poorly-attractive groups based on peak areas of selected compounds [101]. Despite this, the loading plots derived from the PLS-DA suggested compounds potentially associated with each group [101]. Verhulst et al. evaluated 15 compounds: 3-methylbutanal, 2-methyl butanal, 3-hydroxy-2-butanone, 3-methylbutanoic acid, 2-methyl butanoic acid, 6-methyl-5-hepten-2-one, octanal, limonene, 2-ethyl-1-hexanol, lactic acid, nonanal, 2-phenylethanol, decanal, geranylacetone and

tetradecanoic acid [101]. The study found limonene, 2-phenylethanol, and 2-ethyl-1-hexanol were associated with poorly-attractive individuals, while lactic acid, 2-methyl butanoic acid, tetradecanoic acid and octanal were found to be associated with highly-attractive individuals. These results support previous behavioural findings with lures. Lactic acid and tetradecanoic acid were previously shown to be attractive [145]. Interestingly, Logan et al. (2008), with a different mosquito species *Aedes aegypti* reported contrasting results for octanal being associated with low attractiveness along with four other compounds not identified in Verhulst et al.'s 2013 study: 6-methyl-5-hepten-2-one, geranylacetone, nonanal, and decanal [25]. Wooding et al. (2020) used passive sampling and thermal desorption two-dimensional GC-MS to show that 2-undecanone, known to be produced by *Staphylococcus* and *Corynebacterium,* had a higher mean abundance in unattractive compared to attractive individuals but was present more often in the attractive group, although the study is limited by its attractiveness data being questionnaire based rather than behavioural assays [250]. Differences in methods across studies impact the detectability of specific VOCs. In this chapter, if VOC profiles can distinguish between attractive and unattractive groups to mosquitoes will be investigated.

VOCs are a diverse group of chemicals generated as metabolic by-products during various metabolic processes in the human body. Microbes on the skin release a wide range of VOCs through both primary and secondary metabolism [92]. These compounds include CO_2, alkenes, alcohols, ketones and sulphur-based compounds [92,251]. Culturing conditions and physiological state can influence the type and concentration of VOCs produced by microorganisms [92,202]. Volatile emissions are altered between competitive and non-competitive microbial culturing models [202]. Glucose metabolism can produce CO_2 and various intermediates that can be further biosynthesised into various VOCs [251]. Many VOCs have been explored for their short and long-range attractiveness to mosquitoes [249]. For *An. gambiae,* the main attractants include CO_2, ammonia, lactic acid and carboxylic acids [24,124,249]. In this thesis, the exploration of correlations between VOCs and microbes will allow bacterial genera to be explored. These could be targeted for future investigation to determine if they produce these volatiles and if these volatiles or attractive or repellent to mosquitoes. Attractive or repellent volatiles could be used in the future to enhance push-pull systems [252] by leveraging repellents to divert mosquitoes from homes and attractants to trap them.

Research indicates that mosquito transmitted infections can significantly alter mosquito attraction. For instance, malaria infection correlates with greater amounts of certain attractants (aldehydes: heptanal, octanal, and nonanal), whereas arbovirus infections are

characterised by reduced CO_2 output—a typical mosquito attractant—and increased levels of acetophenone [170,172,174]. This rise in acetophenone is associated with an increased abundance of *Bacillus* microbes [174]. The administration of isotretinoin, a vitamin A derivative, has been found to decrease acetophenone levels and subsequently reduce viral transmission [174]. The impact of flu compared to mosquito-borne infections has yet to be thoroughly explored. However, it is suspected that additional skin microbes and VOCs play a part in influencing mosquito attraction in these contexts.

The influence of human genetics on volatile compound profiles has not been fully investigated. Kuhn and Natsch (2009) showed monozygotic (MZ) twins exhibit more similar axilla odour profiles than unrelated individuals [177]. Using a small cohort of participants, the authors observed the clustering of odour profiles among the twin pairs. By systematically collecting samples from the same individual over several days, Kuhn and Natsch explored daily and sample-to-sample variations noting differences in volatile profile on different days but consistent clustering of twin pairs [177]. Previous research also suggests a correlation between VOC profiles and ethnicity, with differences between ethnic groups. Colón-Crespo et al. used a model of 15 VOCs derived from hand samples to classify individuals as Hispanic, Caucasian or East Asian. This method achieved 72 % accuracy overall [253]. Furthermore, in another study, ethnicity has also been shown to influence human axillary odour profile, with quantitative differences in VOCs between Caucasian, African-American and East Asian ethnic groups [254]. The study will further explore the human genetic influence on odour profiles by comparing MZ, DZ, and unrelated individuals. Additionally, differences in VOCs attributed to human attractiveness to mosquitoes will be investigated between the UK and Gambian cohorts.

Participants can vary considerably in exogenous substances present in the volatile profile, with a wide array of contaminants, including short-chain alcohols (e.g. glycerol), DEET and nicotine [255]. These exogenous substances originate from sources external to the biological system. They cause challenges in identifying and quantifying endogenous metabolites of interest [255]. Interestingly, skincare products, including deodorants and soap, have been shown to reduce attractiveness to mosquitoes [105,131]. These findings show the complexity of analysing volatile profiles and the need for consistent, rigorous methods between studies.

4.03) Aims and objectives

The aim of this chapter was to investigate the contribution of odour profile to human attractiveness to *Anopheles coluzzii* mosquitoes.

This aim was achieved through the following objectives:

1) To collect body odour samples from the feet of two cohorts of twins (in the UK and The Gambia), and analyse them by GC-FID.

2) To examine if there are differences in the volatile profiles between unattractive and attractive groups of participants in both cohorts.

3) To identify microbes that correlate with volatiles of interest.

4) To estimate if the volatile profile is more similar in MZ than DZ or unrelated individuals in both cohorts.

4.04) UK Cohort

4.04.01) Sample collection by air entrainment

Participants (N = 176, 38 MZ and 50 DZ pairs) were asked to avoid washing their feet and lower legs for 24 hours prior to sample collection. To collect a body sample from the participant, the participant's right foot was placed in a prepared bag (Toastabags Roasting Bags, 25 x 38 cm, Planitproducts, UK) and clipped shut around the calf using crocodile clips, as shown in Figure 72. Bags were fitted with Swagelok fittings at the opposite corner of the bag to the air-in and connected to polytetrafluoroethylene (PTFE) tubing.

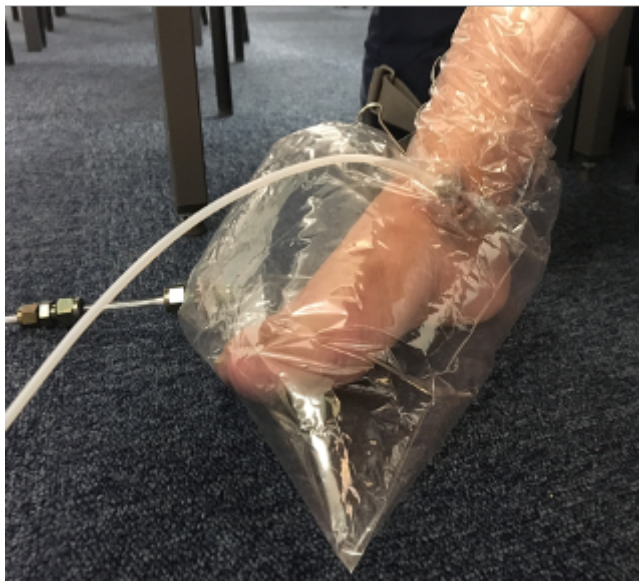

Figure 72. Sample collection of a UK participant. The participant's foot was placed inside a prepared oven bag which was clipped shut around the calf. Porapak in a sample tube for collection of odour in the bottom left of the bag.

Charcoal-filtered air was pumped into the top of the bag at 700 ml/min and vacuum pumped out of the base at 600 ml/min. The system was purged for 15 minutes before fitting the Porapak filter (50 mg of Porapak Q (mesh 50/80), Supelco Analytical, Pennsylvania, USA) as shown in Figures 72 and 73 inside a glass tube (5mm diameter) connecting the bag to the outlet. Air entrainment was carried out for 120 minutes. At the end of the sample collection, the Porapak filter was removed and sealed in a clean glass ampoule under nitrogen. Ampoules were stored in the freezer at -20 ^{o}C until sample processing.

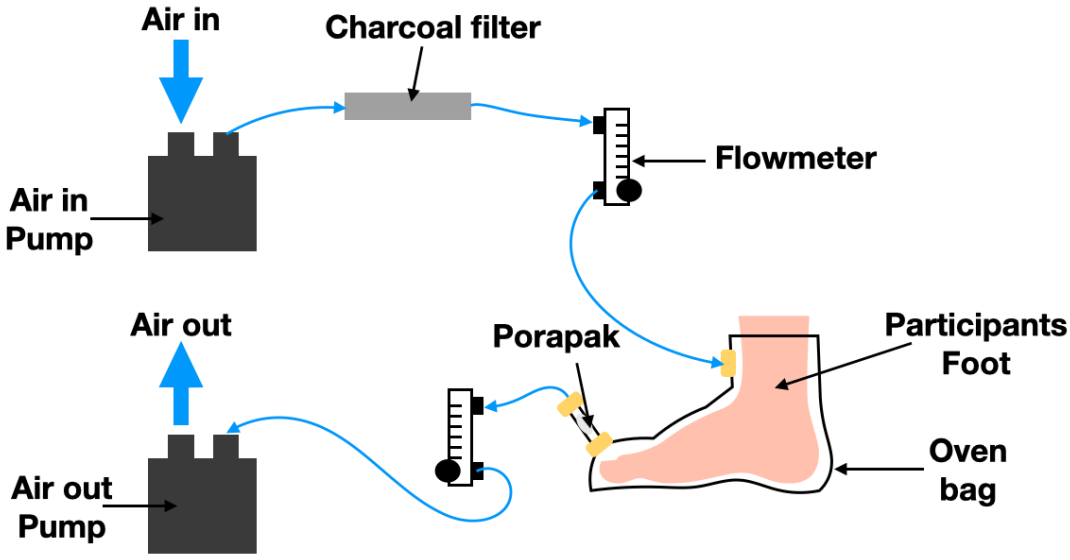

Figure 73. The direction of airflow through the system is shown by blue arrows. Thin blue arrows are PTFE tubing. The air is pumped in using an air pump through a charcoal filter and set to flow into the oven bag enclosing the participant's foot at 700 ml/min using a flow metre. A second pump pulls the air out through the Porapak enclosed in a glass tube by setting the second flow metre to 600 ml/min. Yellow circles indicate Swagelok fittings ⅛" to ¼", reducing to the diameter required at the bag end.

4.04.02) Measuring participants' body odour profile by GC-FID

On the day of sample processing, Porapak were eluted with 800 µl redistilled diethyl ether. For Gas Chromatography Flame Ionization Detector (GC-FID), the 800 µl sample was concentrated to ~50 µl under a stream of charcoal-filtered nitrogen. For storage or transport to the GC-MS, samples were re-diluted by adding 750 µl redistilled diethyl ether and sealing under nitrogen.

For GC-FID, all samples were run on an Agilent Technologies 7890A Instrument with a 50 m non-polar polydimethylsiloxane (HP1) column (50 m × 0.32 mm, solid phase thickness 0.52 µm) fitted with a cool-on-column injector and flame ionisation detector. Nitrogen was used as the carrier gas. The GC-FID temperature ramp consisted of 40 °C for 30 s, raised by 5 °C per min to 150 °C, held for 1 min, raised by 10 °C per min to 230 °C, and held for 40 min. The main components of the GC-FID are shown in Figure 74.

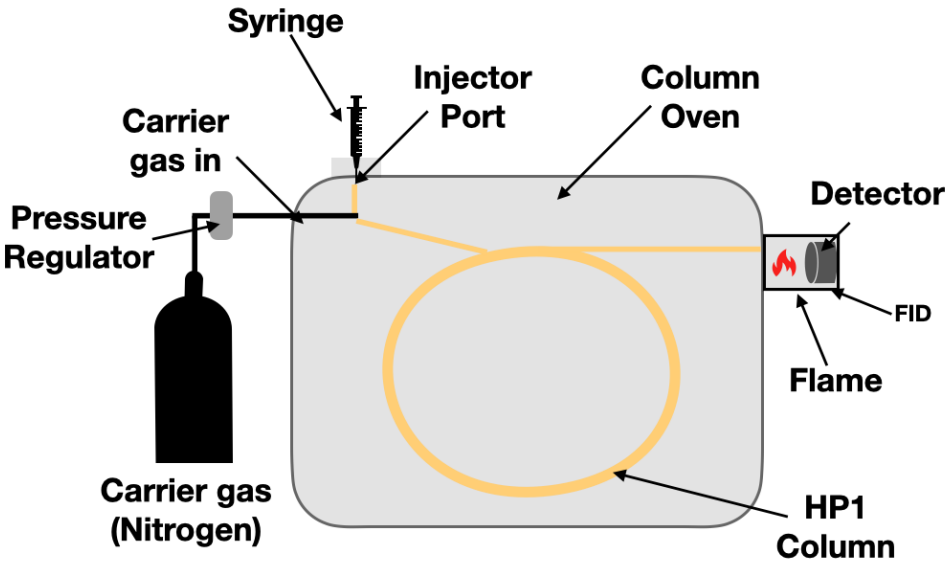

Figure 74. Diagram showing the main components of the GC-FID. The sample is injected using a syringe into the injector port onto the column. The carrier gas carries it along the column. The flame creates ions which are detected by the detector producing a signal that creates a trace.

The 4 µl sample was injected by syringe manually into the front inlet, directly into the end of the column. Half the sample went to the GC-FID and half the EAG, which we did not use in this study, meaning 2 µl sample was injected onto the column. On heating of the column, the sample was vaporised and carried through the column by the carrier gas (nitrogen). During passage through the column, compounds are separated by the oven temperature ramp and respective partition coefficients between the mobile phase (carrier gas and stationary phase (inner surface of column). Components with stronger interaction with the stationary phase will take longer to elute from the column, which results in separation between compounds. The separated components exit the column and enter the Flame Ionisation Detector (FID), which consists of a hydrogen flame. Hydrocarbons in the sample produce ions when burnt. The ions are collected by a collector electrode which generates an electric signal. The magnitude of the signal was proportional to the amount of compound that entered the flame. This signal generated was represented as a peak on the GC-FID output, a chromatogram (trace) with the retention time caused by the separation of compounds.

4.04.03) Integration

Traces were integrated using Agilent Technologies ChemStation software (C.01.04, Agilent Technologies, California, USA) and manually checked to provide outputs (raw data) that can be interrogated to examine the peaks in terms of quantity and presence/absence. The integration settings were adjusted to omit peaks below a specified area, setting the area to reject to 0.05 ng based on that week's alkane standard (~0.4). This threshold was previously established to optimally detect minor analytes without picking up too much noise [172]. Integration parameters remained at their standard values: slope sensitivity, which denotes the rate at which peak initiation occurs, was fixed at 1. Height reject was set to 0. A peak width of 0.04 was chosen to effectively differentiate between actual peaks and background noise. The integration process was initiated post the solvent peak at 3.5 minutes. Raw retention times and peak areas for each peak in the GC-FID trace were exported from Agilent ChemStation.

4.04.04) Calculating Kovats Index and compound amounts

At the start of each week, 1 µl of an alkane standard, comprising of 100 ng/µl of each of 19 alkanes from heptane C_7H_{16} to pentacosane $C_{25}H_{52}$ in hexane solvent, was analysed. The alkane in the series differed by one carbon atom, meaning they elute with an increase in retention time, as shown for an example alkane in Table 15. Each analyte should elute in the same position relative to these alkanes, but their retention times can vary due to system-specific differences. Alkanes were integrated using the same parameters for samples except for height reject, which was set to be higher than 0 to ensure only the 19 peaks for the alkanes were picked up without noise.

Table 15. Retention times (in minutes), number of carbon and areas for each in the alkane standard comprising 100 ng/µl of each compound, spanning alkanes from heptane to pentacosane.

Carbons	**Alkane**	**Retention time (mins)**	**Area**
C7	Heptane	4.41	7.17
C8	Octane	6.03	1008.31
C9	Nonane	8.70	912.55
C10	Decane	11.68	941.92
C11	Undecane	14.69	961.24
C12	Dodecane	17.61	959.74
C13	Tridecane	20.38	929.69
C14	Tetradecane	23.00	1126.16
C15	Pentadecane	25.10	971.50
C16	Hexadecane	26.76	1070.13
C17	Heptadecane	28.17	892.47
C18	Octadecane	29.40	902.80
C19	Nonadecane	30.52	832.23
C20	Icosane	31.65	885.82
C21	Heneicosane	33.01	904.67
C22	Docosane	34.69	923.28
C23	Tricosane	36.84	918.84
C24	Tetracosane	39.64	1027.52
C25	Pentacosane	43.29	916.97

The peak retention times were used to calculate Kovats Index (KI) a standardises way to compare retention times across systems and laboratories relative to the alkane standard. KIs were calculated for all peaks eluting between the first alkane in the alkane standard (heptane C_7H_{16}) to the last (pentacosane $C_{25}H_{52}$) using the equation:

$$KI = 100\,(log_{10}Rt_X - log_{10}Rt_n / log_{10}Rt_{n+1} - log_{10}Rt_n) + 100n$$

Where:

- RtX is the retention time of the compounds of interest
- Rtn is the retention time for the alkane that elutes just before the compound of interest
- Rtn+1 is the retention time for the alkane that elutes after the compound of interest
- n is the number of carbon atoms in the alkane that elutes just before the compound of interest

As for samples the raw retention times and peak areas for each peak in the GC-FID trace were exported from Agilent ChemStation. The means for alkanes C8-C25 were used to work out the mean area for 100 ng. This was used to work out the peak area for 0.05 ng, the value used to set the area reject on traces for that week during the integration of samples.

For each sample, the KI value for every peak was determined using the retention time equation. The concentration of each analyte in the injected sample was semi-quantified by comparing it to the known amount of compound in the alkane standard. The calculated area corresponding to 0.05 ng of alkane was used to estimate the amount of the analyte in the sample, expressed in ng.

4.04.05) Pre-processing of UK samples

We ran two additional external controls in addition to the alkane standard:

1) Solvent controls which contained the solvent (diethyl ether). Each new batch of solvent was run to check for contamination, i.e., no peaks except for the peak expected for the solvent. These controls are labelled as BX where X is the batch of diethyl ether. A solvent control trace is shown in Figure 75, with KI value and amount of each analyte calculated based on the alkane standard run that week. There is a single large peak where the solvent peak was expected to elute but there were several small peaks in the solvent, indicating contamination.

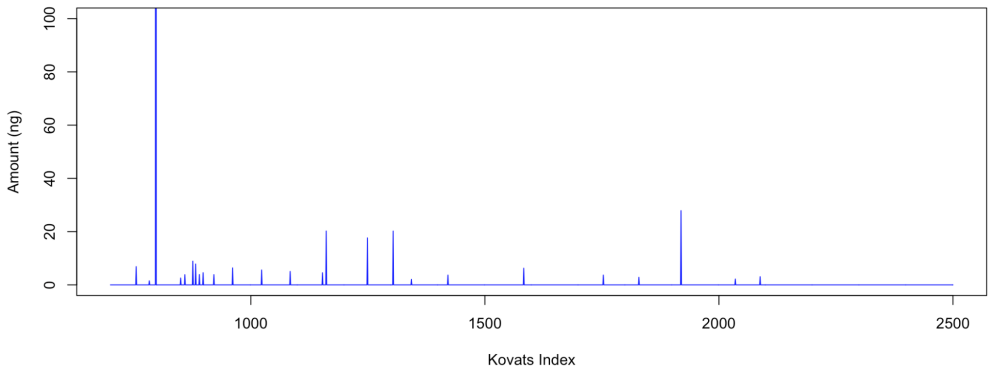

Figure 75. Solvent control trace showing Kovats index on the x-axis and semi-quantified amount relative to alkane standard in ng on y-axis. Peaks based on peak area plotted using Maldiquant in R (Gibb and Strimmer 2012).

2) Blanks, which were air entrainments without a human foot inside the bag, were conducted to establish a baseline volatile profile. This was used to differentiate between human and non-human odours. Each blank sample was labelled as GX, where 'X' denotes the sequence number of the control. An example blank control trace is shown in Figure 76, it has significantly more peaks than the solvent control.

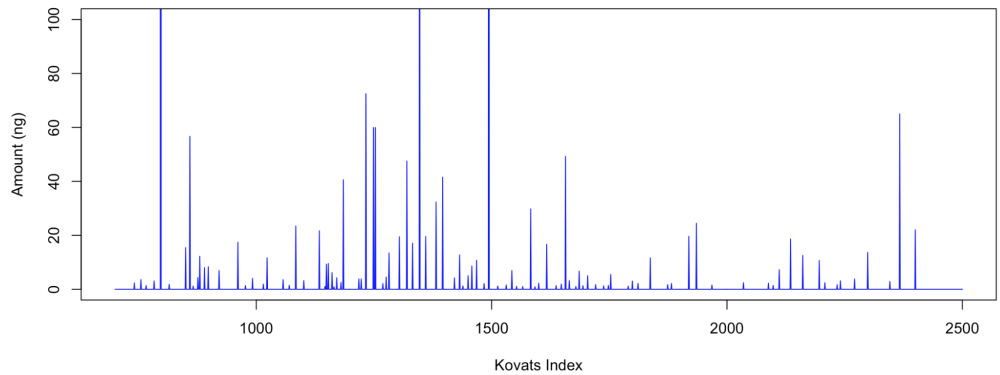

Figure 76. Blank control trace showing Kovats index on the x-axis and semi-quantified amount relative to alkane standard in ng on y-axis. Peaks based on peak area plotted using Maldiquant in R (Gibb and Strimmer 2012).

An example sample trace is shown in Figure 77. It appears to have more and larger peaks than the solvent and blank controls.

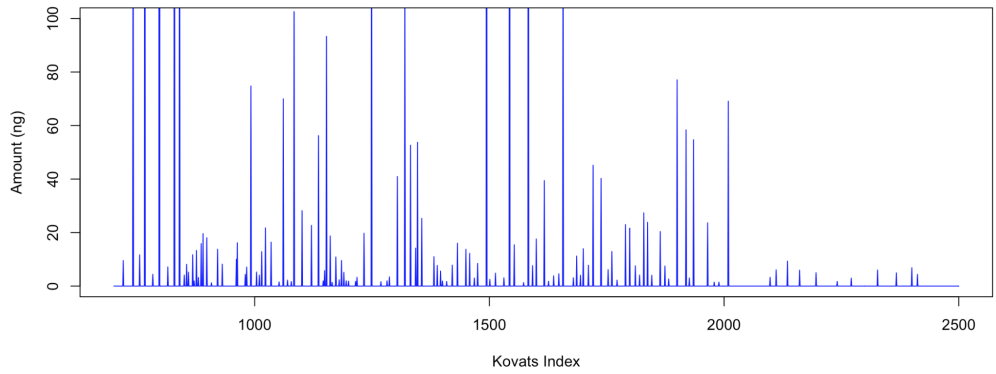

Figure 77. Sample trace showing Kovats index on the x-axis and semi-quantified amount relative to alkane standard in ng on y-axis. Peaks based on peak area plotted using Maldiquant in R (Gibb and Strimmer 2012).

GC-FID data was available for 179 samples, 15 solvent controls and 20 blank controls. We collected a single sample from each individual and ran samples once on the GC-FID. The average number of peaks was 135 for samples, 78 for solvent controls and 79 for blank controls. The number of peaks in the solvent controls was higher than expected, indicating some contamination in the solvent. These were small peaks compared to the sample and would have been present in both the samples and controls.

We had previously manually aligned the UK dataset and performed statistics that are detailed in Showering et al. (2022) [209]. In this chapter, have instead applied GCalignR, an R package that can align peak retention times and areas. This method does not compare to the alkane standard for semi-quantification. It assumes that the retention times are similar throughout for the same substances. Traces for alkane standards were compared to confirm they eluted at similar retention times throughout the sampling period.

From the GC-FID dataset, the raw retention times and areas were aligned for all peaks using the GCalignR software package [256] in R Studio 1.4.1106 for all samples, solvent and blank controls. The algorithm first addresses systematic linear shifts between samples by comparing them to a reference sample. Sample 411 was selected as the reference as it had the most peaks. The second step categorises the peaks into rows based on the closeness of retention times. Individual peaks in each sample are aligned across samples by comparing the peak retention time in that sample to the average of the preceding

samples. The final step combines homologous peaks that happen to be sorted into neighbouring rows combining rows that are putatively homologous substances.

Peaks were aligned based on retention times between 0 and 55 minutes. Substances found in the solvent or blank controls were removed from the aligned data by setting them as controls. The diagnostic plot from GCalignR is shown in Figure 78. Many GC-FID peaks detected were removed. These may be contaminants or compounds of human significance that were also found in the blank controls. After these were removed, substantially fewer peaks remained. Contamination may come from various sources, including reagents, the environment, sample preparation steps and laboratory equipment.

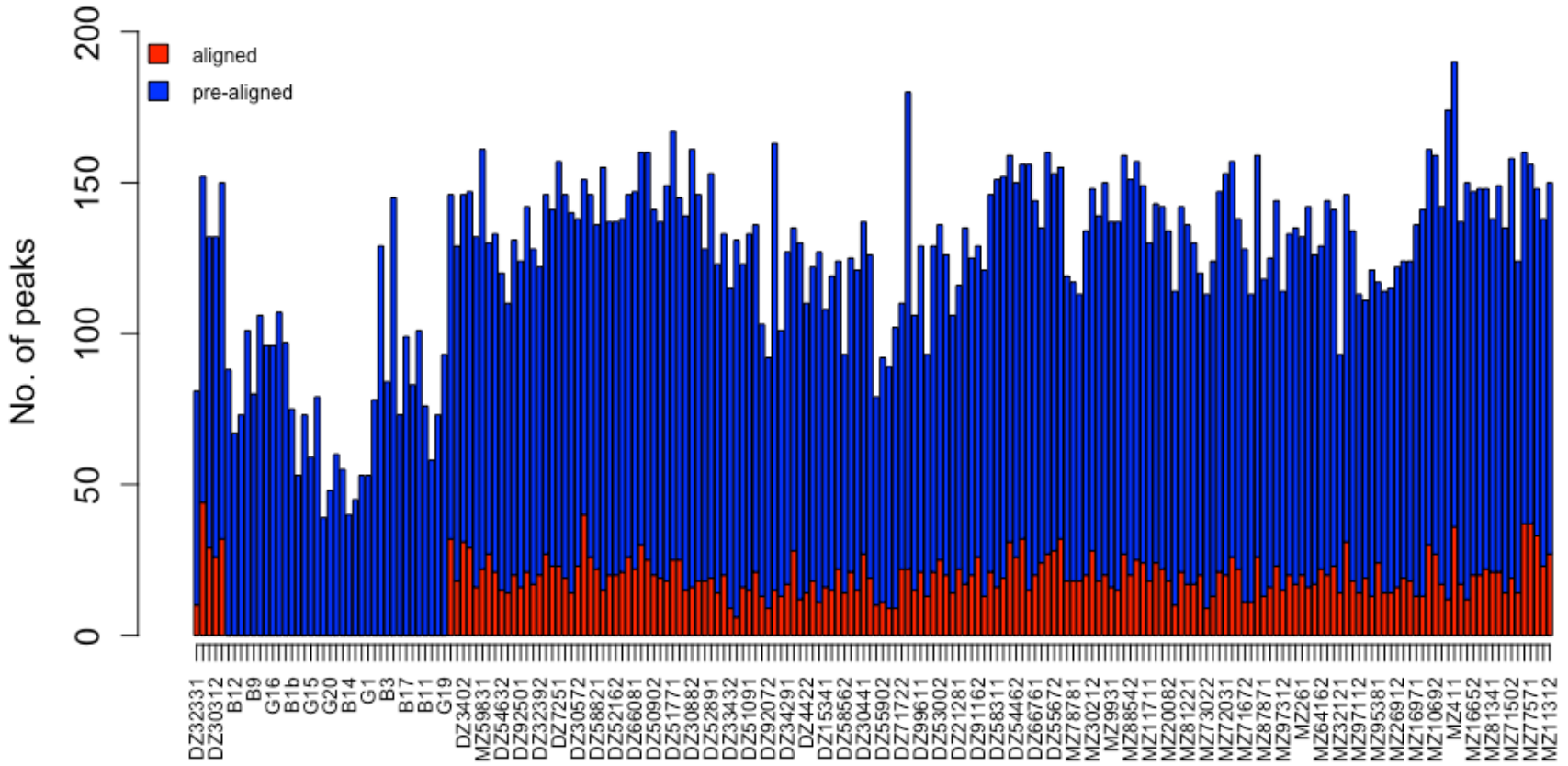

Figure 78. Diagnostic plots summarise the volatile dataset's alignment from the UK cohort, it illustrates the number of peaks present in the dataset before (blue) and after alignment (red). A subset of samples are labelled MZX or DZX, and the controls are labelled BX for solvent controls and GX for blank controls.

A total of 262 different peaks are aligned across all samples and controls. An example trace prior to the alignment and removal of peaks in the controls is shown in Figure 79.

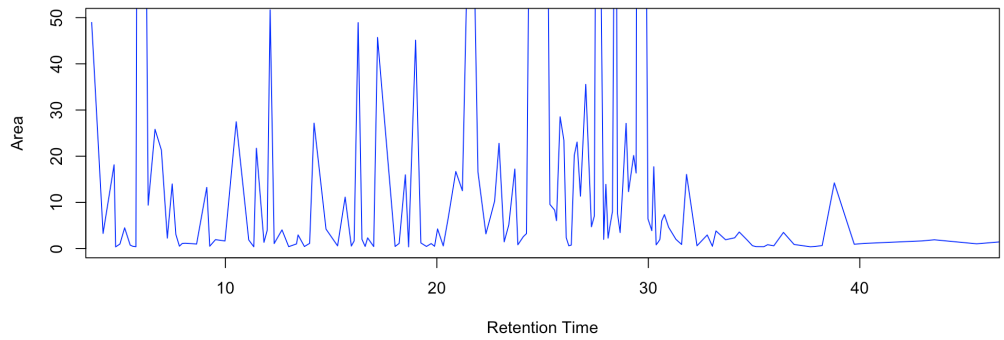

Figure 79. Sample trace prior to alignment showing retention time on the x-axis and the area on the y-axis. Plotted using Maldiquant in R (Gibb and Strimmer 2012).

The same sample after the alignment is shown in Figure 80. Many of the peaks in Figure 79 are being removed as they are present in the controls.

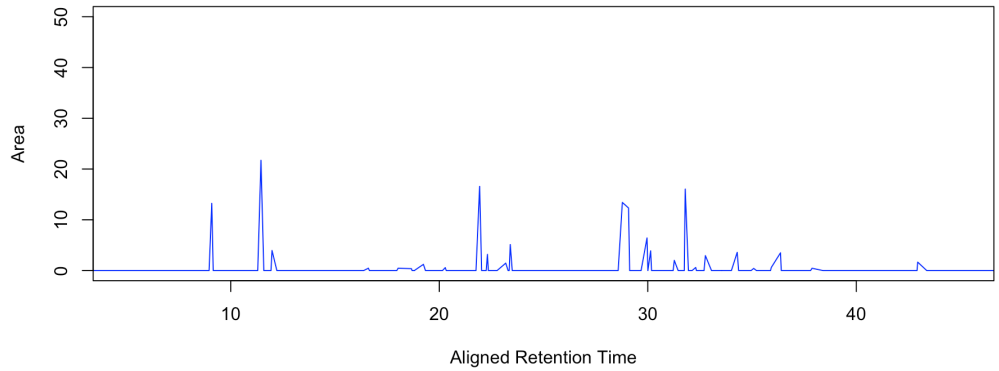

Figure 80. Sample trace post alignment showing aligned retention time on the x-axis and the area on the y-axis. Plotted using Maldiquant in R (Gibb and Strimmer 2012).

4.04.06) No evidence of a difference in the total amount of body odour between attractiveness groups

Expected results (Null):
There is no evidence of a difference in the total amount of body odour between the unattractive and attractive groups in the UK cohort.

Alternate results:
There is more total amount of body odour in people who are attractive to mosquitoes compared to those that are unattractive to mosquitoes in the UK cohort.

I first compared the total amount of VOCs (sum of area under the curve) between the unattractive (N=129) and attractive (N=49) groups, as shown in Figure 81. Attractiveness groups based on data collection described for UK participants in chapter 2, using two choice olfactometer. This was run on the aligned dataset from GCalignR with the peaks in controls removed from the samples. There was no statistical evidence of a difference in the mean amount of VOCs between the groups (t = 0.83, df = 157.7, p-value = 0.410).

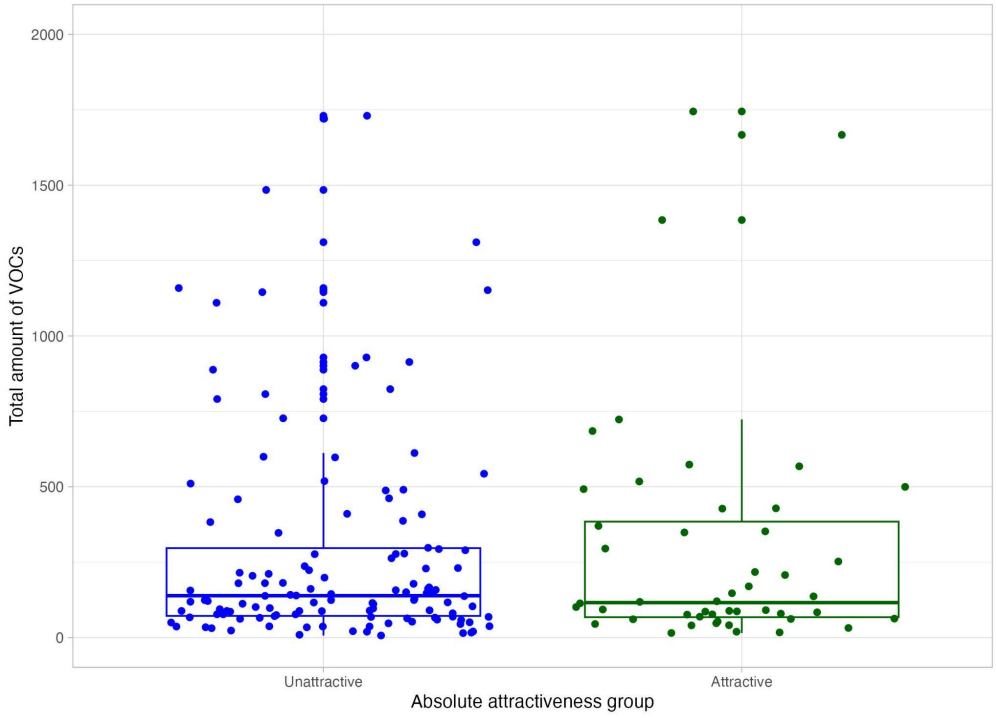

Figure 81. Boxplot comparing the total amount of VOCs between the unattractive (dark blue) and attractive (dark green) groups of human attractiveness to mosquitoes.

4.04.07) Matching GC-MS to GC-FID

GC-MS

Compounds from the literature were initially investigated (Supplementary Table 1: Known *Anopheles* active compounds) that we could identify in our samples. To identify these, a subset of samples was selected for gas chromatography-mass spectrometry (GC-MS) that showed various levels of attractiveness to mosquitoes. Professor John Pickett ran GC-MS at Cardiff University on this subset (N=14).

GC-MS was performed on a ThermoFisher Exactive GC Orbitrap gas chromatography-mass spectrometer coupled to a TRACE GC fitted with programmable temperature vaporisation injector, using a base deactivated guard column (1 m length, 0.53 mm ID, Thames Restek RE10002) and a Rxi-1ms capillary column (60 m x 0.32 mm ID, 0.50 um, Thames Restek RE13342). 1 µl of concentrated sample was injected splitless. The gas chromatogram temperature was maintained at 30 °C for 5 min and then increased to 250 °C at 5 °C/min, followed by a 10 min hold at 250 °C. Ionisation was by electron impact at 70 eV, 200 °C (source temperature). The carrier gas was helium. Professor John Pickett supplied annotated traces from the GC-MS with the known compounds annotated using the NIST database and his expertise.

Matching GC-MS to GC-FID

The GC-FID and GC-MS traces were manually aligned to estimate the retention times of these peaks of interest on the GC-FID data. Peaks in the GC-FID data were identified and their position in the GC-MS data was determined based on the shape of the peak and the trace of the hydrocarbon standard that had been run on both systems. The GC-FID KIs were compared against KI values from the literature using values from the NIST Chemistry WebBook (SRD 69, NIST, Maryland, US) on non-polar columns. The GC-FID had a HP1 column and the GC-MS a Rxi-1ms capillary column, which are both non-polar columns similar in base material and application. It would have been better to run the subset for GC-MS using a more similar program to the GC-FID to make the matching more accurate.

To confirm if identifications were correct, peak enhancement was performed on GC-FID via co-injection with commercial standards for some compounds. Successful co-injections were completed for 6-methyl-5-hepten-2-one, octanal, decanal and geranylacetone, which were of interest as being repellent. 3-methylbutanoic acid (isovaleric acid) and propanoic acid were identified from metabolic pathways in our paper (204). Some peaks investigated were

likely contaminants (2-phenoxyethanol, 4-ethyl propiophenone, biphenyl, and 2-phenylethanol). Additionally, we tentatively identified several additional compounds by comparing GC-FID and GC-MS traces for the same sample but did not co-inject to confirm retention times. These were butanoic acid, octane, 1-hexanol, heptanal, pentanoic acid (valeric acid), hexanoic acid, heptanoic acid, octanoic acid, formic acid, ethanoic acid (acetic acid), nonanoic acid, dodecane, decanoic acid, lactic acid, dodecancoic acid, tetradecanoic acid, methyl palmitate, hexadecanoic acid, indole, benzaldehyde, phenol (1-octen-3-one), 1-octen-3-ol and octadecanoic acid.

For the UK cohort, ten compounds were tentatively confirmed that had previously been reported in the literature based on retention times, of which four were successfully co-injected, as shown in Table 16.

Table 16. Suggested compound identification for the peak, associated references indicating its activity in Anopheles, the range of KIs for the compound from NIST based on non-polar columns (with variations in the inlet, column length, diameter, and thickness), and the KI value derived from the corresponding peak in the GC-FID trace. Confirmation status is determined by co-injection success and an overall assessment combining GC-MS match to GC-FID, expected KI in the range expected and co-injection results.

Compound	***Anopheles* activity Reference**	**NIST range KIs**	**KI value**	**Confirmed with co-injection**	**Confirmed identification**
Propanoic acid	[257]	683 - 748	755	Yes	Yes
Isovaleric acid	[207]	NA	850	Yes	Yes
Benzaldehyde	[172]	925 - 978	931	No	Tentatively
1-Octen-3-one	[172]	961 - 975	963	No	Tentatively
6-Methyl-5-hepten-2-one	[170,207]	964 - 994	965	No	Tentatively
Octanal	[172]	971 - 991	980	Yes	Yes
Heptanoic acid	[257]	1065 - 1083	1078	No	Tentatively
Decanoic acid	[257]	1306 - 1323	1356	No	No
Geranylacetone	[207]	1428 - 1468	1432	Yes	Yes
Dodecanoic acid	[257]	1554 - 1602	1648	No	No

4.04.08) Normalisation

The GCalignR output provided aligned retention times and areas. Peak areas were subsequently normalised to standardise the peak concentrations across samples. This normalisation step involved calculating relative abundances as a proportion of the total amount of compounds in the sample, as the total absolute area of compounds varied between samples. Relative abundances of peaks were determined by dividing the area under the curve for that peak by the cumulative areas of all the peaks in the sample. Post-normalisation, the sum of all analytes in a sample was one.

An additional filtering step was applied to remove peaks not in >10 % of samples unless the peak had a KI identified as a compound of interest based on the literature. This was to reduce noise in the data from low abundant peaks and reduce the number of peaks for multiple testing. After these steps, 46 compounds remained that were investigated in addition to 10 compounds tentatively identified, shown in Table 17. All statistics where not otherwise stated are run on aligned retention times and normalised areas with the peaks in the controls removed.

4.04.09) No evidence of a difference in the odour profile between the attractiveness group

Expected results (Null):
There is no evidence of a difference in the composition of volatile profile between the unattractive and attractive groups in the UK cohort.

Alternate results:
The attractive and unattractive groups clearly separate based on the volatile profile in the UK cohort.

The relative abundances of the highly abundant dataset (compounds present in more than 10 % of samples) were log-transformed with a +1 offset, and Principal component analysis (PCA) was performed on mean-centred data to examine the differences in volatile profiles between the unattractive (N=129) and attractive (N=49) individuals in terms of their relative attractiveness to mosquitoes (groups defined in Chapter 2). PCA is a dimensionality reduction tool which allows the visualisation of volatile profiles. Using PCA, The two groups were compared based on the volatile profiles of highly abundant VOCs, as shown in Figure 82. There was no separation between the groups on the 1st and 2nd components. Samples cluster together in the centre. PC1 explains 10.85 % of the variance, and PC2 explains 9.86 % of the variance. There was no statistical evidence of separation based on the volatile profile between the attractiveness groups (PERMANOVA, F=1.44, P =0.11). Similarly there was no separation between the 1st and 3rd components (Figure 83) or all first of the three components (Figure 84).

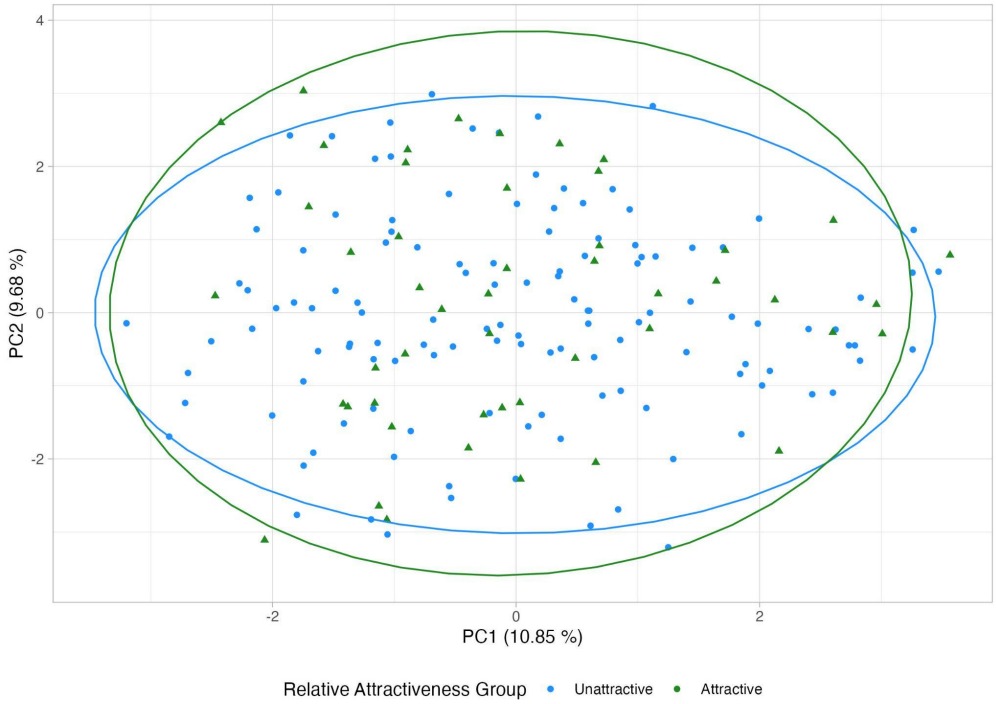

Figure 82. Principal component analysis, variation explained by the 1st and 2nd principal components for the unattractive (blue) and attractive (dark green) groups.

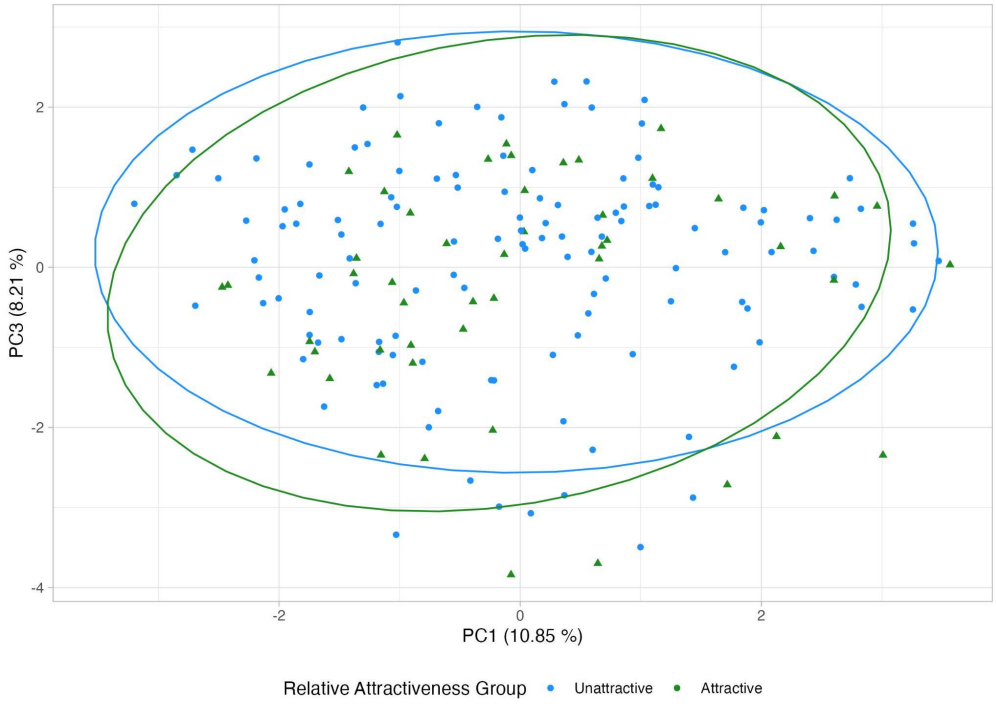

Figure 83. Principal component analysis, variation explained by the 1st and 3rd principal components for the unattractive (blue) and attractive (dark green) groups.

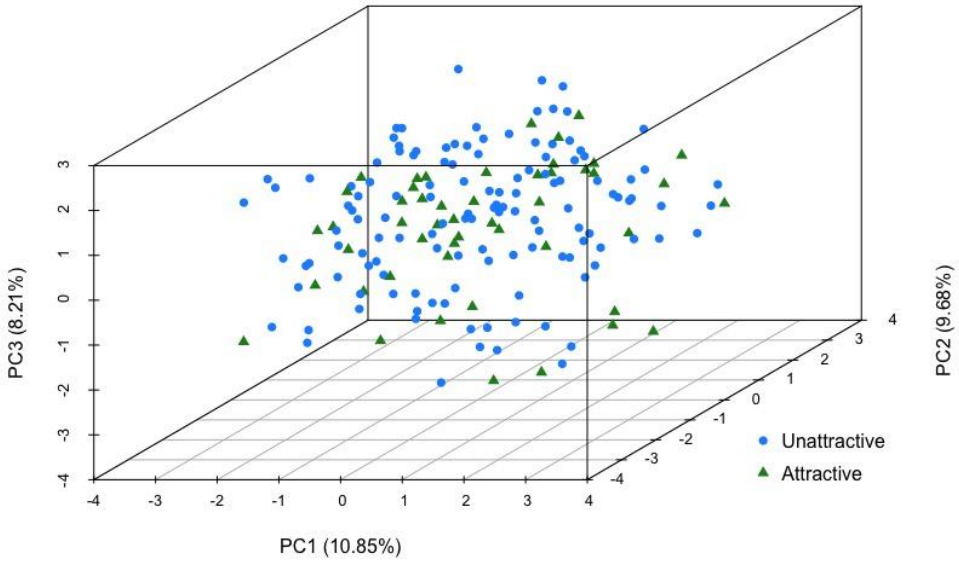

Figure 84. A 3D visualisation of the 1st, 2nd and 3rd principal components for the unattractive (blue) and attractive (dark green) groups.

Each principal component (PC) in the dataset was a linear combination of the VOCs that optimised the explained variance. A scree plot was created to illustrate the proportion of total variance accounted for by each PC, as shown in Figure 85. The plot reveals that the first two PCs account for 20.53 % of the variance, while the first five PCs collectively explain 42.16 % of the variation in the VOC profile. The more components, the more variance was explained. Therefore, less than half of the differences between the unattractive and attractive groups are explained by the first five compounds.

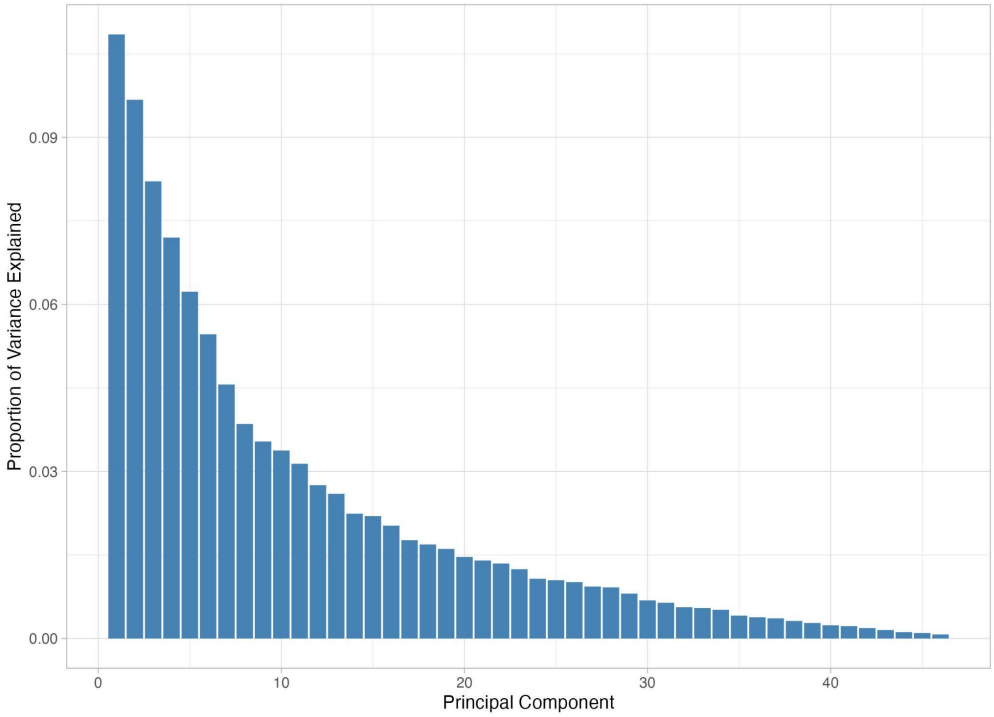

Figure 85. Scree plot from the principal component analysis shows that the variation explained increases as more principal components are added.

The loading plot of features contributing to the PCA on the first and second components are shown in Figure 86. Each bar represents a VOC. They are labelled on the y-axis by their retention time, and the x-axis shows the observed contribution (variance explained). Many VOCs explain a little of the variance on the 1st and 2nd components. On component 1, VOCs with retention times 11.14 mins and 11.448 mins have the longest bars explaining most of the difference on this component. On component 2, VOC with a retention time of 31.797 mins explains most of the variance on this component.

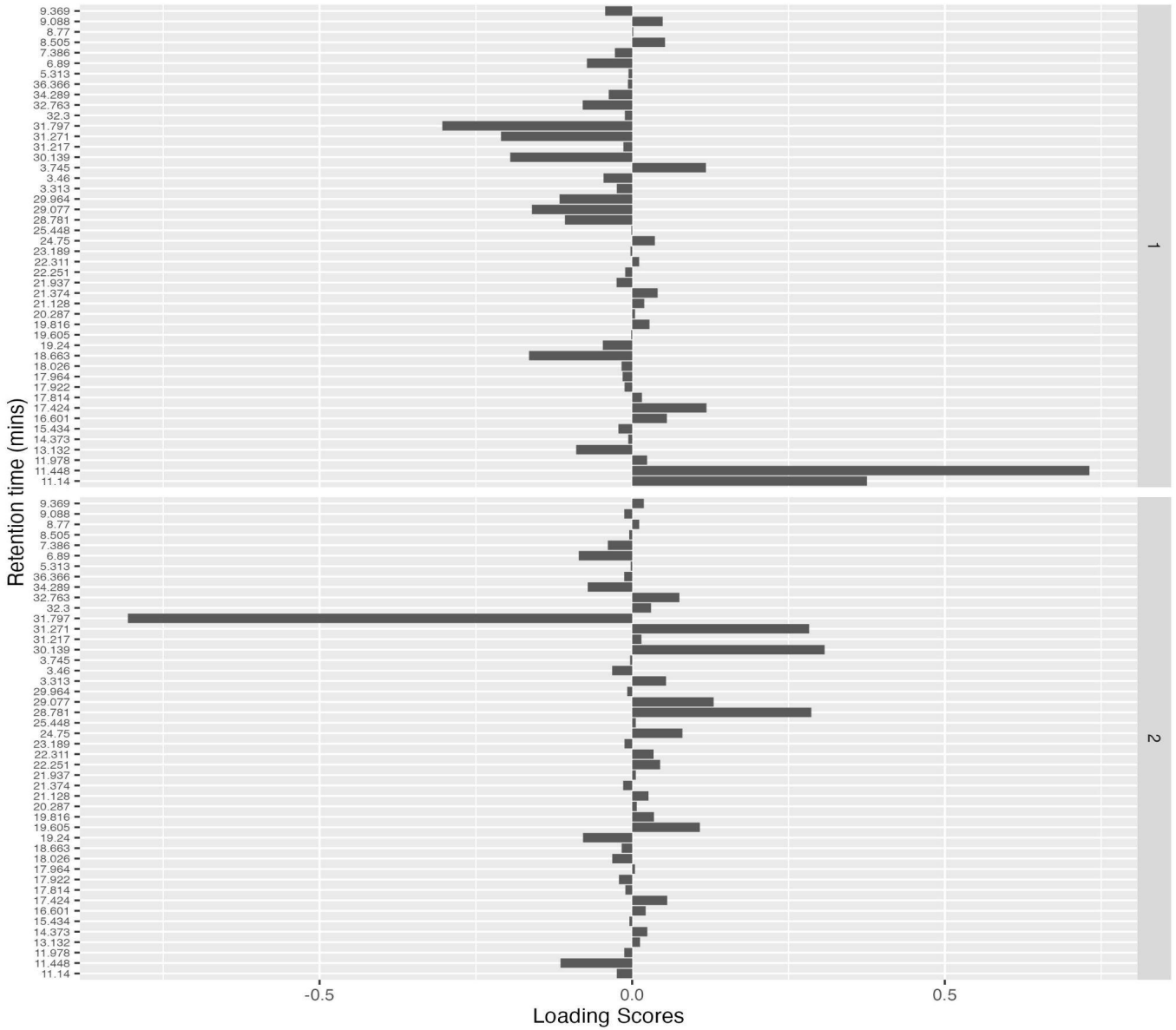

Figure 86. The y-axis shows the VOC contributors, ordered by retention time, on the 1st and 2nd principal components.

4.04.10) No evidence of a difference in known compounds between unattractive and attractive groups

Expected results (Null):
There is no evidence of a difference in the amount of any volatile between the unattractive and attractive groups in the UK cohort.

Alternate results:
There is a higher abundance of the volatile in the attractive group compared to the unattractive group if it is an attractant or higher in the unattractive group compared to the attractive group if a repellent.

The analysis presented in this section focuses on comparing volatiles between unattractive and attractive groups of humans to mosquitoes previously selected. Began exploring the ten tentatively identified compounds present after alignment: propanoic acid, isovaleric acid, benzaldehyde, 1-octen-3-one, 6-methyl-5-hepten-2-one, octanal, heptanoic acid, decanoic acid, geranylacetone and dodecanoic acid. Participants in the attractive and unattractive groups and the relative abundance percentages of each of the ten volatiles are shown in Figure 87. There are no clear, consistent trends between the two groups.

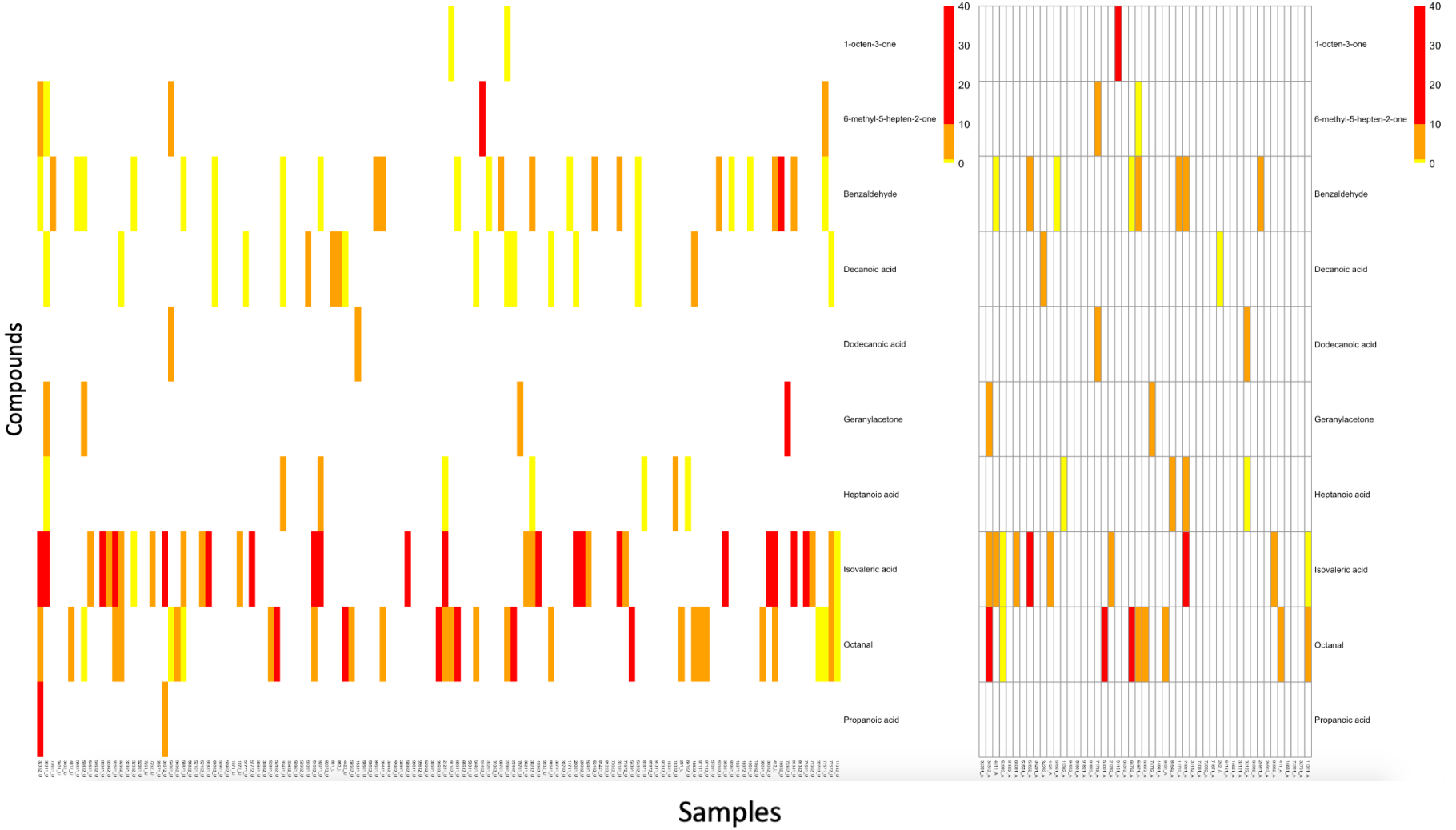

Figure 87. Heatmap showing the relative amount of tentatively identified compounds between the unattractive (no grid lines facet) and attractive (grid lines facet) individuals. Individuals are shown on the x-axis ("_U" are unattractive, "_A" are attractive to mosquitoes). White cells represent the absence of the compound, yellow cells represent very low(0.1-1 %), orange was middle (1-10 %) and red represents higher levels (10-40 %).

To interrogate these findings, the mean amount of each volatile was compared between the unattractive and attractive groups, as shown in Table 17. There was no evidence of a difference in the mean amount of any of the compounds between the attractive and unattractive groups based on t-tests with Benjamini-Hochberg adjustment.

Table 17. Comparative analysis of mean attractiveness and unattractiveness scores across tentatively identified compounds, accompanied by t-test p-values and Benjamini-Hochberg adjusted p-values.

Mean RT (mins)	**Compound**	**Mean Unattractive**	**Mean Attractive**	**P value**	**Adjusted P value**
4.870	Propanoic acid	0.112	0.000	0.446	0.638
6.890	Isovaleric acid	4.040	1.395	0.302	0.537
9.369	Benzaldehyde	0.369	0.249	0.674	0.749
10.293	1-octen-3-one	0.006	0.371	0.249	0.537
10.596	6-methyl-5-hepten-2-one	0.846	0.125	0.322	0.537
11.140	Octanal	1.491	1.159	0.166	0.537
13.573	Heptanoic acid	0.067	0.142	0.017	0.167
21.128	Decanoic acid	0.133	0.042	0.825	0.825
23.499	Geranylacetone	0.293	0.201	0.533	0.667
25.601	Dodecanoic acid	0.021	0.068	0.186	0.537

4.04.11) No evidence of a difference in unknown compounds between unattractive and attractive groups

Expected results (Null):
There is no evidence of a difference in the amount of any volatile between the unattractive and attractive groups in the UK cohort.

Alternate results:
There is a higher abundance of the volatile in the attractive group compared to the unattractive group if it is an attractant or higher in the unattractive group compared to the attractive group if a repellent.

Next, the most abundant data (peaks present in more than 10 % of samples previously filtered) were compared to repeat the analysis using an untargeted approach. First compared the 46 peaks found in at least 10 % of samples across the unattractive and attractive samples, as shown with a heatmap in Figure 88. The compound with a retention time of 11.45 mins appeared to be more abundant in unattractive people, but the patterns are complex and some attractive people also have high levels of this compound.

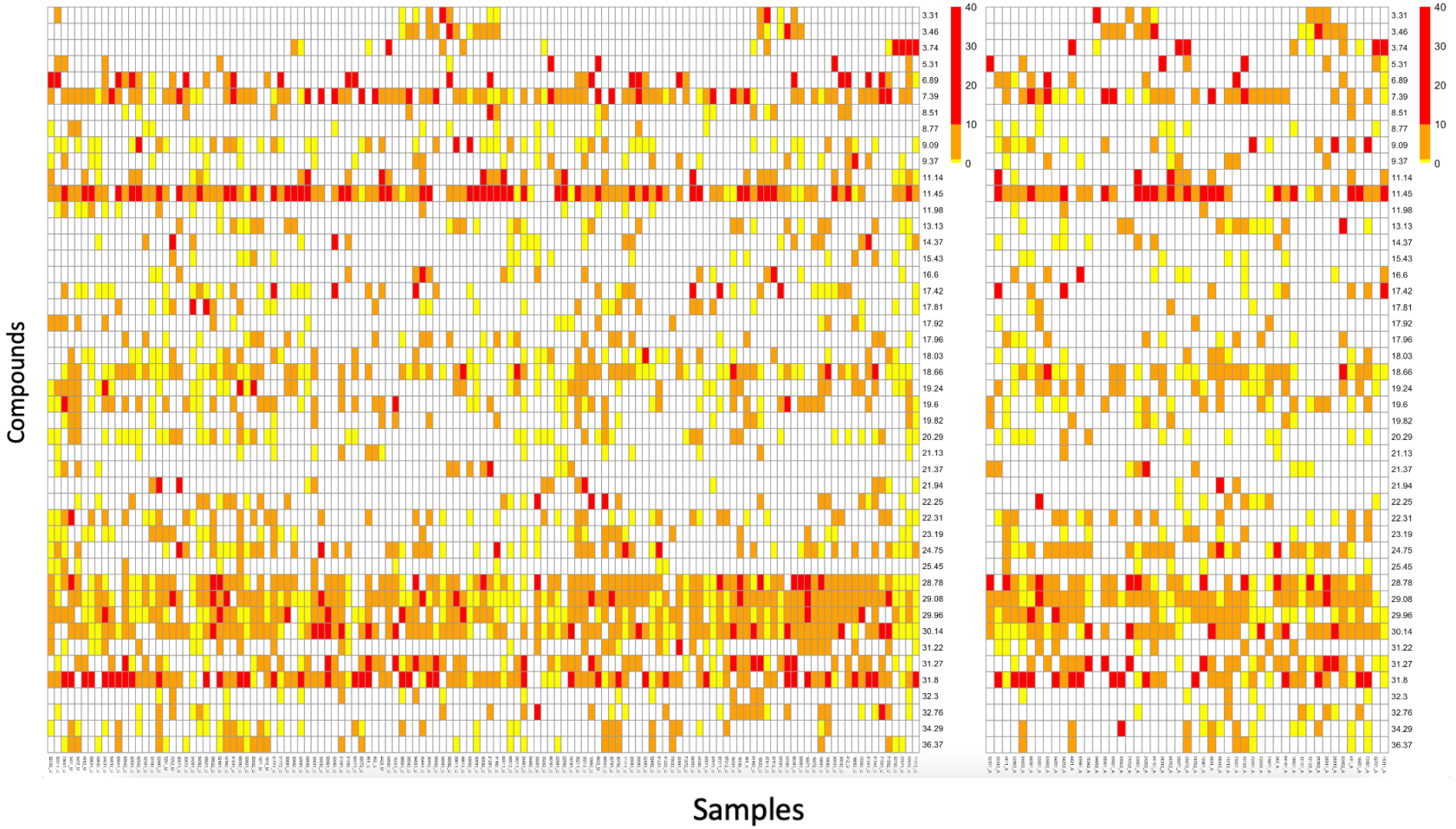

Figure 88. Heatmap showing the relative amount of the highly abundant compounds between the unattractive and attractive individuals. Individuals are shown on the x-axis ("_U" are unattractive, "_A" are attractive to mosquitoes). No compound was shown as white, very low yellow (0.1-1 %), middle orange (1-10 %) and higher levels red (10-40 %).

To interrogate these findings, the mean amount of each volatile was compared between the unattractive and attractive groups, as shown in Table 18. Three of these compounds, namely 6.890 mins (isovaleric acid), 9.369 mins (benzaldehyde), 11.140 mins (octanal), and 21.128 mins (decanoic acid), were tentatively identified and included in the previous analysis of tentatively identified compounds. There is no evidence of a difference in the mean amount of any of the compounds between the attractive and unattractive groups based on t-tests with Benjamini-Hochberg adjustment.

Table 18. Comparative analysis of mean attractiveness and unattractiveness scores across abundant compounds, accompanied by t-test p-values and Benjamini-Hochberg adjusted p-values.

Mean RT (mins)	Mean Unattractive	Mean Attractive	P value	Adjusted p value
3.313	1.228	0.965	0.416	0.988
3.460	0.521	1.186	0.936	0.988
3.745	2.034	5.142	0.934	0.988
5.313	1.158	3.235	0.129	0.988
6.890	4.040	1.395	0.172	0.988
7.386	5.785	3.367	0.707	0.988
8.505	0.374	0.123	0.784	0.988
8.770	0.088	0.069	0.675	0.988
9.088	1.875	2.513	0.689	0.988
9.369	0.369	0.249	0.602	0.988
11.140	1.491	1.159	0.921	0.988
11.448	12.867	11.228	0.357	0.988
11.978	0.138	0.110	0.829	0.988
13.132	0.421	1.105	0.091	0.988
14.373	0.581	0.229	0.888	0.988
15.434	0.085	0.086	0.634	0.988
16.601	0.746	0.592	0.924	0.988

17.424	2.034	1.826	0.555	0.988
17.814	0.760	0.125	0.964	0.988
17.922	0.212	0.116	0.836	0.988
17.964	0.299	0.575	0.988	0.988
18.026	0.614	0.189	0.599	0.988
18.663	1.963	2.205	0.821	0.988
19.240	1.038	0.929	0.807	0.988
19.605	1.273	0.675	0.338	0.988
19.816	0.462	0.442	0.810	0.988
20.287	0.365	0.205	0.591	0.988
21.128	0.133	0.042	0.287	0.988
21.374	0.255	0.692	0.579	0.988
21.937	0.848	0.462	0.391	0.988
22.251	0.771	0.373	0.618	0.988
22.311	0.824	0.535	0.975	0.988
23.189	0.341	0.232	0.745	0.988
24.750	1.841	2.420	0.508	0.988
25.448	0.089	0.068	0.018	0.841
28.781	3.856	4.800	0.534	0.988
29.077	3.973	2.967	0.801	0.988

29.964	2.910	1.868	0.979	0.988
30.139	3.812	4.638	0.049	0.988
31.217	0.590	0.428	0.793	0.988
31.271	2.974	3.676	0.896	0.988
31.797	10.518	11.433	0.845	0.988
32.300	0.140	0.066	0.974	0.988
32.763	0.611	0.304	0.603	0.988
34.289	0.430	0.770	0.806	0.988
36.366	0.405	0.376	0.962	0.988

4.04.12) Correlations of microbiome and volatiles

Expected results (Null):
There is no evidence of a correlation between any microbe and volatile combination in the UK cohort.

Alternate results:
There is evidence of a correlation between microbes and volatiles, suggesting the microbe may be producing the VOC in the UK cohort.

To investigate if there are significant correlations between microbes and volatiles, the top 10 most abundant genera with the most abundant VOCs were compared as shown in Figure 89. The strongest correlation was between *Corynebacterium* and the compound with a retention time of 6.89 mins (isovaleric acid), which has been described as having a sweaty smell. *Corynebacterium* was investigated, it is known to produce isovaleric acid based on the mVOC 3.0 database [258]. There was no report of *Corynebacterium* producing isovaleric acid in the database. There was evidence that *Staphylococcus* and *Streptococcus* produce this compound and a weak significant correlation between *Staphylococcus* and isovaleric acid. Staphylococci isolated from human feet are known to metabolise the skin-derived amino acid L-leucine to produce isovaleric acid [109,259]. This association with isovaleric acid was interesting as we identified it as more abundant in the attractive group than the unattractive group. Another strong correlation was the abundance of *Truepera* and the compound with a retention time of 36.37 mins, it is probable this was a non-volatile compound as it eluted at a high retention time. *Kocuria* and 17.92 mins, *Enhydrobacter* and 23.19 mins, and 29.08 mins have a statistically significant weak positive correlation. *Salmonella* and the compound with a retention time of 11.448 mins may be worth investigating further as we found some evidence of 11.448 mins to be more abundant in the unattractive group than the attractive group but not after adjustment for multiple testing.

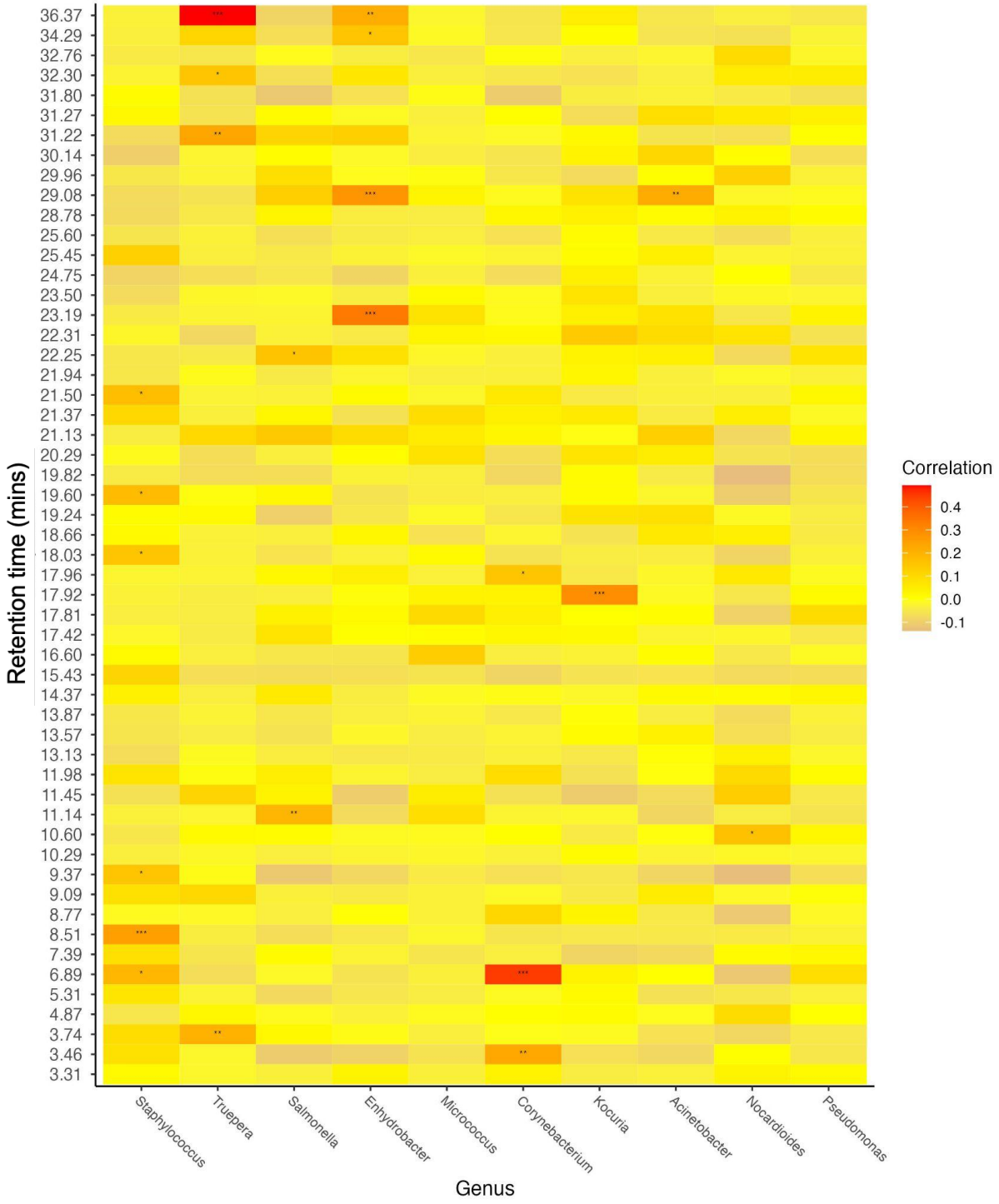

*Figure 89. Heatmap of correlations between the highly abundant genera and highly abundant compounds. The strength of correlation from the Pearson correlation coefficient colours the heatmap. A score of -1 was a perfect negative linear relationship, 0 was no correlation, and 1 was a perfect positive correlation. Yellow indicates 0, i.e. no correlation, and red 0.5, i.e. moderate positive correlation. The stars represent the significance based on the unadjusted p-value associated with the Pearson correlation coefficient *** p <0.001, ** p <0.01 or * p <0.05.*

I subsequently explored the correlations between the same VOCs and the top 20 most abundant OTUs. Notably, the sixth most abundant OTU, belonging to the *Truepera* genus, demonstrated a strong correlation with a VOC that had a retention time of 36.37 minutes. This VOC was identified at the genus level in Figure 89. Additionally, OTU13, identified as *Staphylococcus*, showed a significant correlation with a VOC having a retention time of 9.09 minutes. Interestingly, no correlation was found between *Staphylococcus* and this specific VOC in previous analyses, highlighting differences in correlations among the five Staphylococcus OTUs in Figure 90.

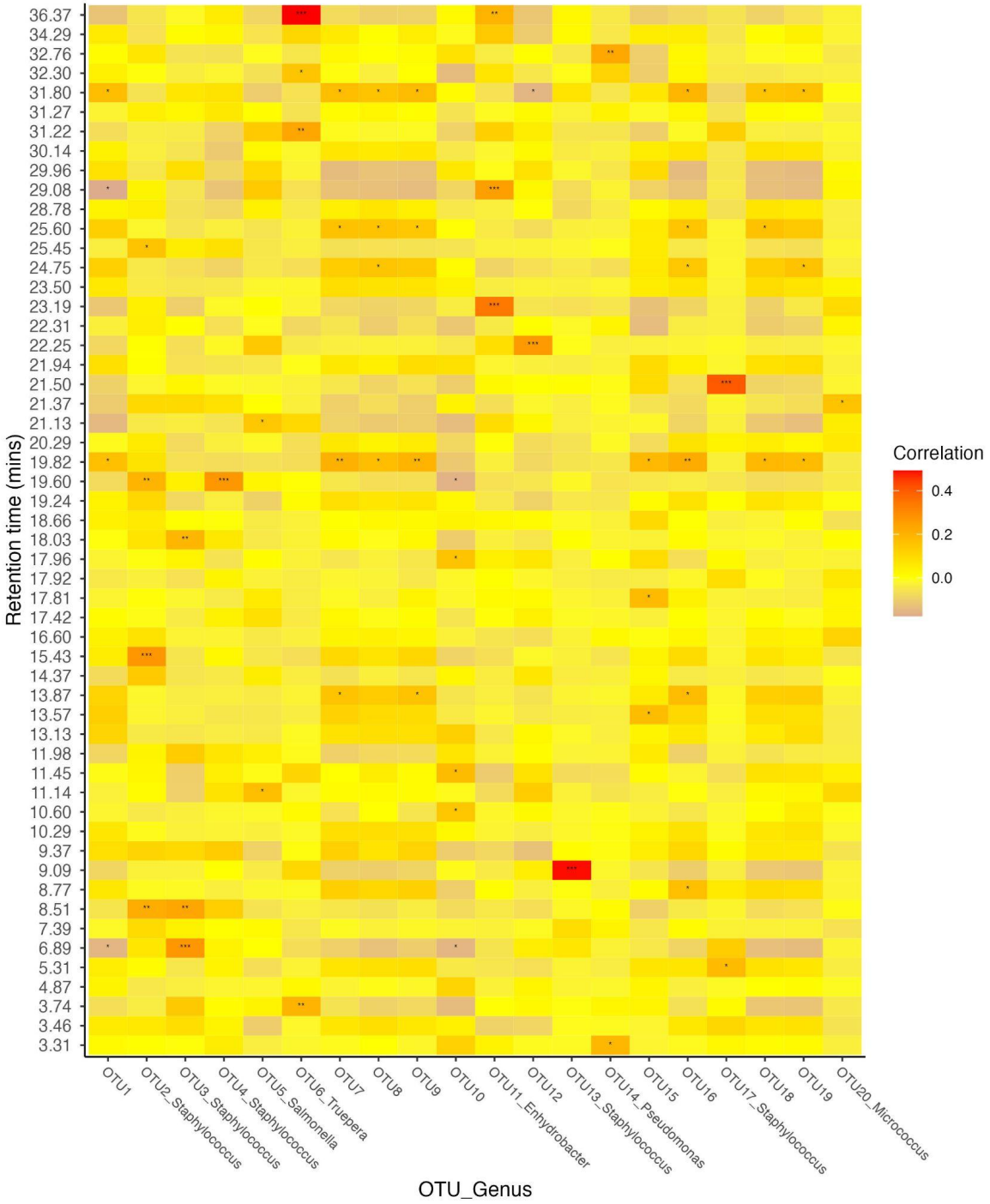

*Figure 90. Heatmap of correlations between the highly abundant VOCs and highly abundant compounds. The strength of correlation from the Pearson correlation coefficient colours the heatmap. A score of -1 was a perfect negative linear relationship, 0 was no correlation, and 1 was a perfect positive correlation. Yellow indicates 0, i.e. no correlation, and red 0.5, i.e. moderate positive correlation. The stars represent the significance based on the unadjusted p-value associated with the Pearson correlation coefficient *** p <0.001, ** p <0.01 or * p <0.05.*

4.04.13) Genetics

Expected results (Null):
No evidence of a difference in the distribution of volatile profile differences between MZ, DZ and unrelated pairs in the UK cohort.

Alternate results:
MZ pairs have more similar distribution of volatile profile differences than DZ pairs than unrelated pairs in the UK cohort.

To investigate if odour profiles were more similar for MZ than DZ than unrelated pairs, standard Euclidian distances were used to compare the distances between samples with the equation:

$$Distance = \sqrt{((1/n) * \Sigma((X\_i - Y\_i)\text{^}2))}.$$

Where X_i and Y_i are the values of the ith dimension of vectors X and Y, respectively

I compared the normalised Euclidian distance between volatile profiles between the MZ twin pairs (N=40), DZ twin pairs (N=48) and unrelated pairs (N=31774). The distribution of volatile profile differences was compared between MZ, DZ and unrelated pairs as shown in Figure 91. ANOVA to compare the means of the three groups gave no evidence of a difference (F value = 1.55, P value = 0.21). There was no difference in the distribution of the difference in odour profile per pair between MZ twins, DZ twins and unrelated pairs. The unrelated pairs have some extreme outliers, as shown in the tail. This result suggests there is no genetic component to volatile profiles.

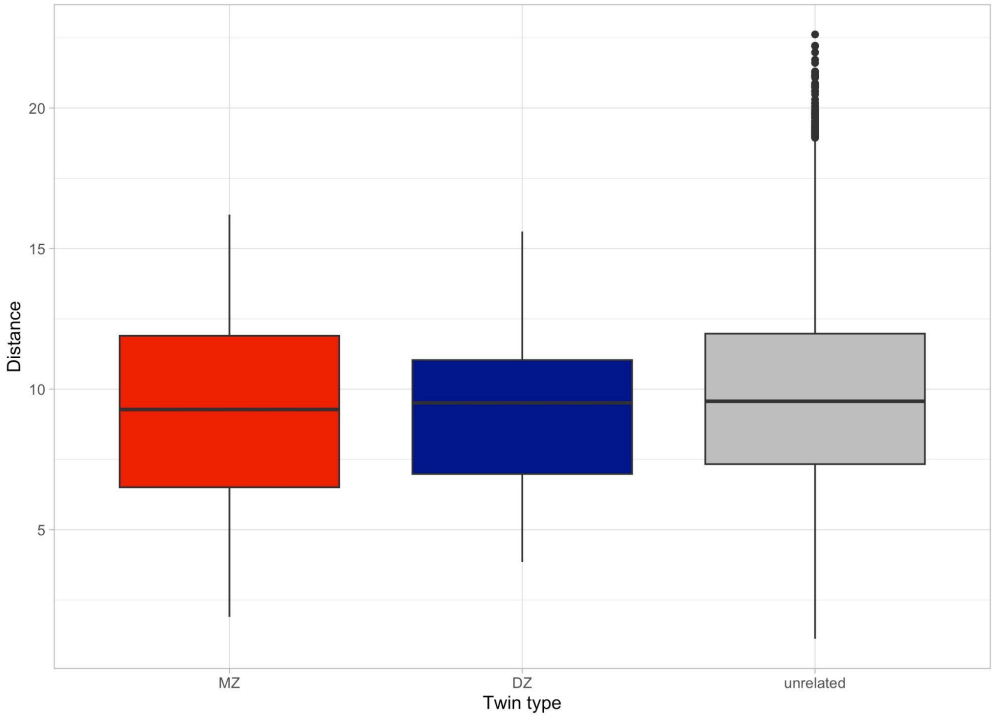

Figure 91. Boxplot comparing the normalised Euclidean distance per pair between MZ (red), DZ (blue) and unrelated (grey) pairs.

4.05) Gambian cohort

For the Gambian cohort, VOC samples were obtained through air entrainment, mirroring the procedure used for the UK cohort, as shown in Figure 92. The Gambian environment was notably dustier. Due to travel restrictions following the onset of the first COVID-19 lockdown, a field team was responsible for the collection in The Gambia. Field conditions necessitated modifications to the collection process. The absence of nitrogen meant samples could not be sealed under it, and instead, they were sealed in the air within ampoules for transportation to the UK. These samples were stored at -20°C and subsequently transported to LSHTM, London, by air on ice. Once in London, they were kept at -20°C until they were analysed using GC-FID, as detailed earlier. Some of the ampoules had minor cracks, any that were completely shattered were discarded. While we had sourced diethyl ether from Prof. John Pickett at the University of Cardiff for processing UK samples, the pandemic disrupted this supply. Consequently, we opted for an alternative from Sigma (≥99.9 %, inhibitor-free Diethyl ether (309966), Sigma-Aldrich, Missouri, US), which is evident from the solvent controls labelled SigmaX have significantly fewer peaks. Blanks are labelled ContX.

Figure 92. Sample collection of a Gambian participant. The participant's foot was placed inside a prepared oven bag clipped shut around the calf. Porapak for collection of odour at the bottom left of the bag.

4.05.01) Pre-processing of Gambian samples:

GC-FID data was available for 210 samples, seven solvent controls and 14 blank controls. The average number of peaks was 116 for samples, 10 for solvent controls and 95 for blank controls. As for the UK cohort, two control types were regularly run: solvent controls and blanks.

Peaks were aligned using the GCalignR as previously described for the UK cohort. The reference was the sample with the most peaks, sample 115_01. Substances found in the control samples were removed from the aligned data. The diagnostic plot from GCalignR is shown in Figure 93. Many GC-FID peaks detected were also present in the controls (samples on the right-hand side) and are therefore removed during alignment. After these are removed, substantially fewer peaks remained in the samples.

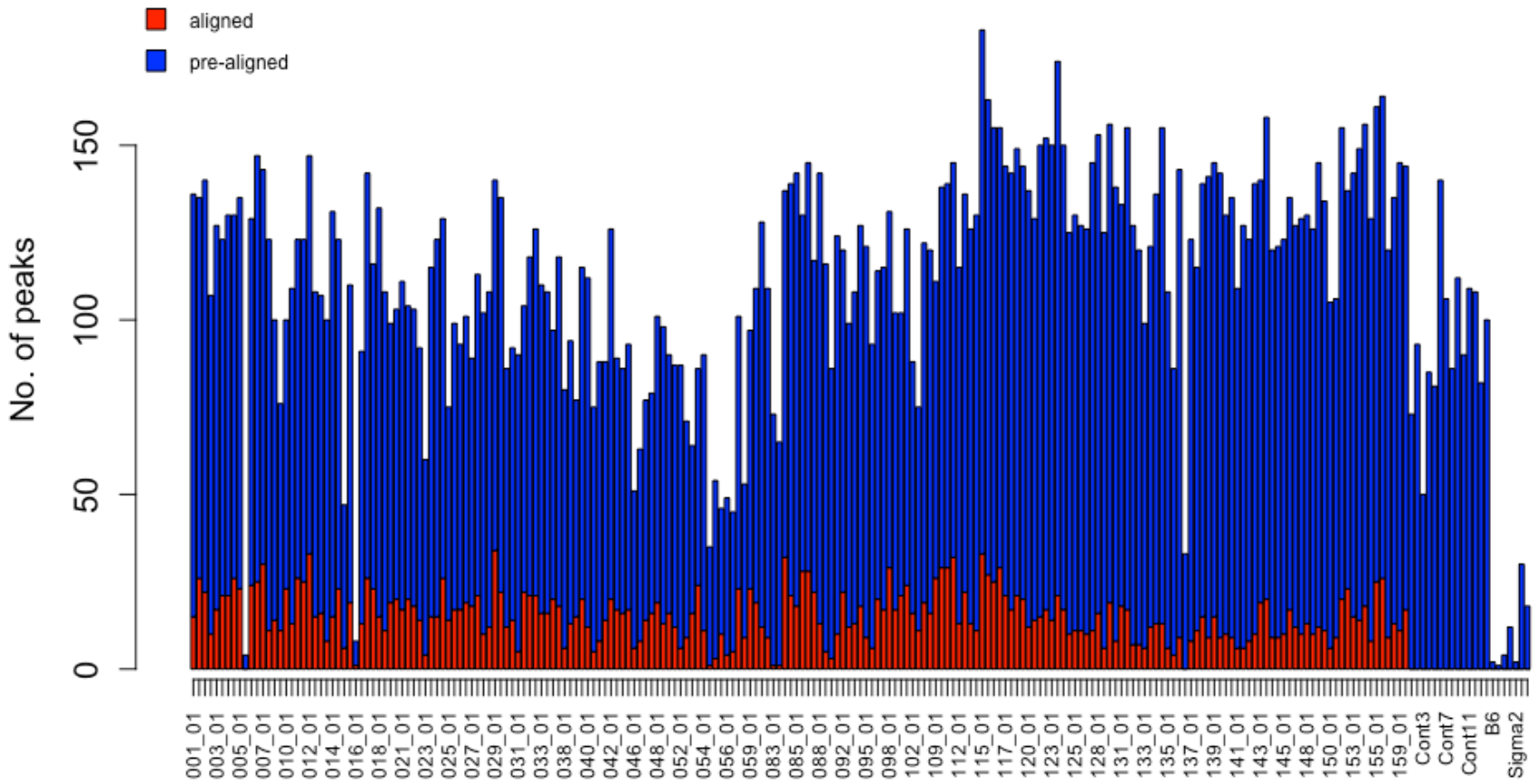

Figure 93. Diagnostic plots summarise the volatile dataset's alignment from the Gambian cohort, and illustrate the number of peaks present in the dataset before (blue) and after alignment (red). The samples are labelled XXX_01 or XXX_02 and the controls are labelled BX or SigmaX for solvent controls and ControlX for blank controls.

Across all the samples and controls, 248 compounds are aligned. An example trace prior to the alignment and removal of peaks in the controls is shown in Figure 94.

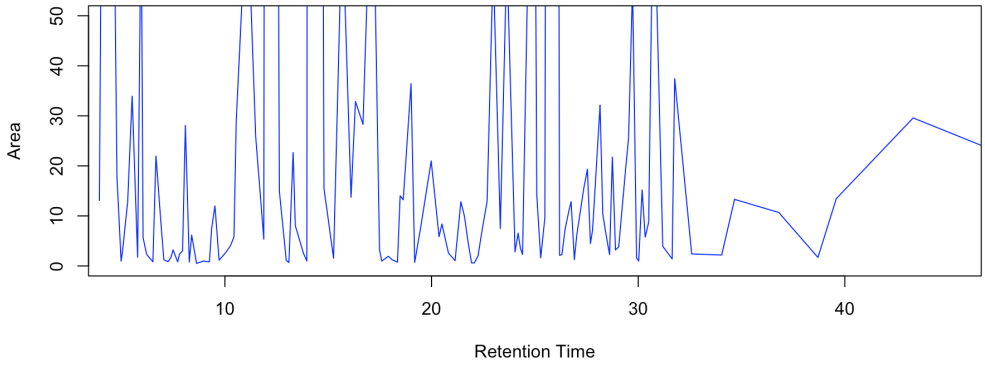

Figure 94. Sample trace prior to alignment showing retention time on the x-axis and the area on the y-axis. Plotted using Maldiquant in R (Gibb and Strimmer 2012).

The same sample after the alignment is shown in Figure 95. Many of the peaks in Figure 94 are being removed as they are present in the controls.

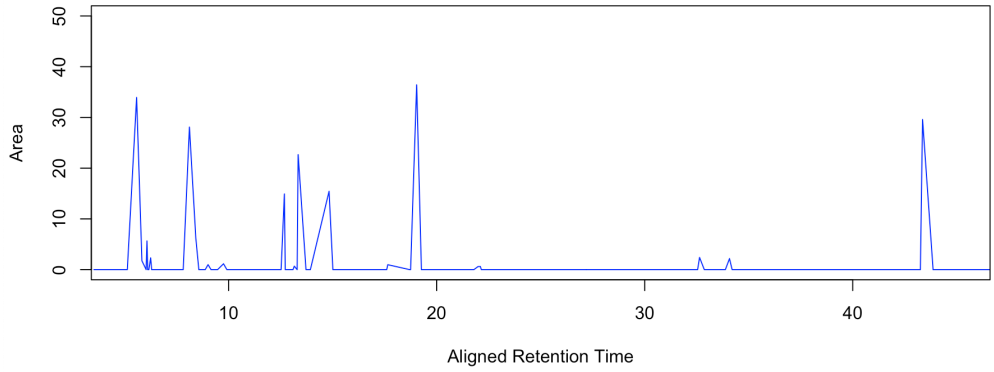

Figure 95. Sample trace post to alignment showing adjusted retention time on the x-axis and the area on the y-axis. Plotted using Maldiquant in R (Gibb and Strimmer 2012).

4.05.02) No evidence of a difference in the total amount of body odour between attractiveness groups

Expected results (Null):
There is no evidence of a difference in the total amount of body odour between the unattractive and attractive groups in the Gambian cohort.

Alternate results:
There is more total amount of body odour in people who are attractive to mosquitoes compared to those that are unattractive to mosquitoes in the Gambian cohort.

I first compared the total amount of VOCs (sum of area under the curve) between the unattractive (N=124) and attractive (N=46) groups, as shown in Figure 96. Attractiveness groups based on data collection described for Gambian participants in chapter 2, using cage assay. There is no statistical evidence of a difference in the mean amount of VOC between the groups (t = -0.78, df = 75.08, p-value = 0.440), indicating no difference in the total VOC amount between attractive and unattractive groups.

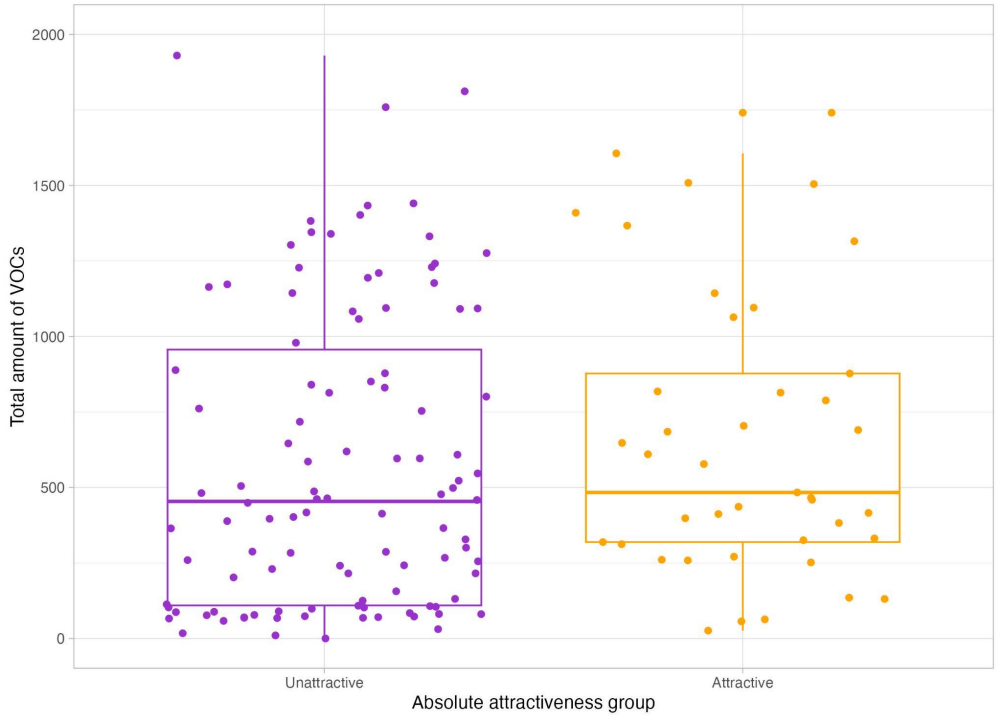

Figure 96. Boxplot comparing the total amount of VOCs between the unattractive (dark purple) and attractive (dark orange) groups of human attractiveness to mosquitoes.

The aligned dataset was then normalised as previously described to standardise the concentration of peaks across samples by calculating relative abundances as percentages. Aligned data was used to identify the retention times of known compounds. Six compounds were identified by John Pickett on GC-MS and identified on GC-FID based on retention times and comparing them to known KI values, as shown in Table 19. Compounds not present in >10 % of samples were removed for exploratory analysis. Following this step, 55 compounds remained for investigation in addition to the six compounds tentatively identified.

Table 19. Suggested compound identification for the peak, associated references indicating its activity in Anopheles, the range of KIs for the compound from NIST based on non-polar columns (with variations in the inlet, column length, diameter, and thickness), and the KI value derived from the corresponding peak in the GC-FID trace. Confirmation status is determined by co-injection success and an overall assessment combining GC-MS match to GC-FID, expected KI in the range expected and co-injection results.

Compound	**Anopheles activity Reference**	**NIST range KIs**	**KI value**	**Confirmed with co-injection**	**Confirmed identification**
Butanoic acid	[257]	789 - 856	779	No	No
Octane	[172]	861 - 866	797	No	No
1-Octen-3-one	[172]	961 - 975	963	No	Tentatively
Octanoic acid	[257]	1165 - 1194	1168	No	Tentatively
Dodecane	[172]	1556	1300	No	No
Dodecanoic acid	[257]	1554 - 1602	1648	No	No

4.05.03) No evidence of a difference in the odour profile between the attractiveness group

Expected results (Null):
There is no evidence of a difference in the composition of volatile profile between the unattractive and attractive groups in the Gambian cohort.

Alternate results:
The attractive and unattractive groups clearly separate based on the volatile profile in the Gambian cohort.

Principal component analysis (PCA) was used to examine the differences in volatile profiles between the unattractive (N=124) and attractive (N=46) individuals in terms of their relative attractiveness to mosquitoes (groups defined in Chapter 2). The relative abundances of the highly abundant dataset (compounds present in more than 10 % of samples) were log-transformed with a +1 offset, and the PCA was performed on mean-centred data. Using PCA, compared these two groups based on the volatile profiles of highly abundant VOCs, as shown in Figure 97. There was no separation between the groups on the 1st and 2nd components. Samples cluster together in the centre. PC1 explains 13.92 % of the variance, and PC2 explains 7.99 % of the variance. There was no statistical evidence of separation based on the volatile profile between the attractiveness groups (PERMANOVA, $F=0.85$, $P=0.637$).

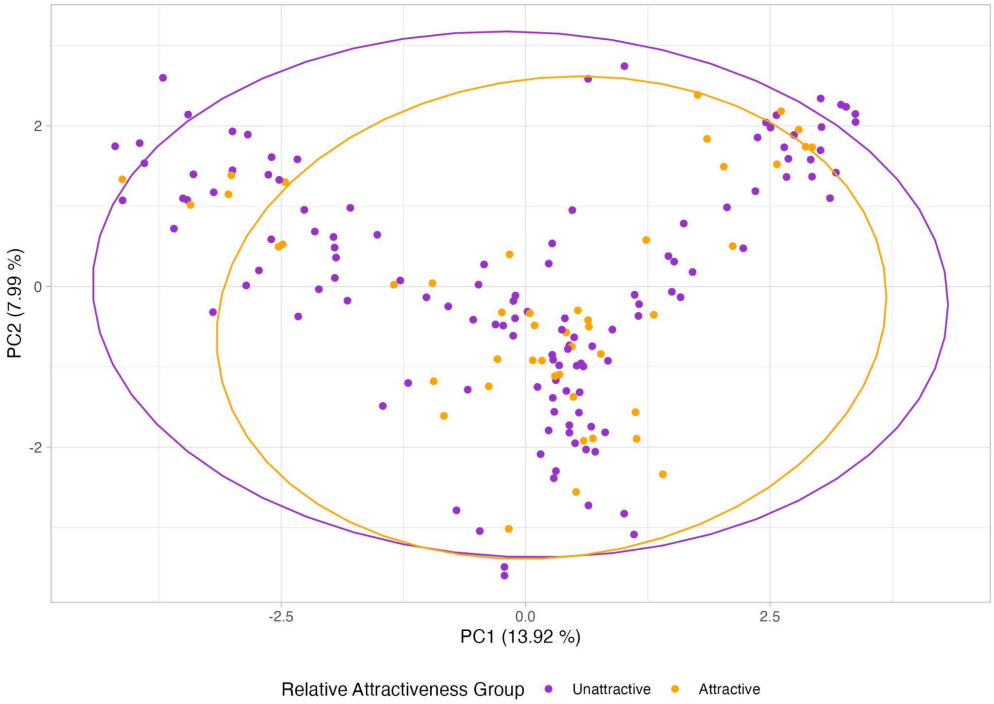

Figure 97. Principal component analysis, variation explained by the 1st and 2nd principal components for the unattractive (dark purple) and attractive (dark orange) groups.

I created a scree plot to illustrate the proportion of total variance accounted for by each principal component, as shown in Figure 98. The plot reveals that the first two PCs account for 21.90 % of the variance, while the first five collectively explain 38.01 % of the variation in the VOC profile.

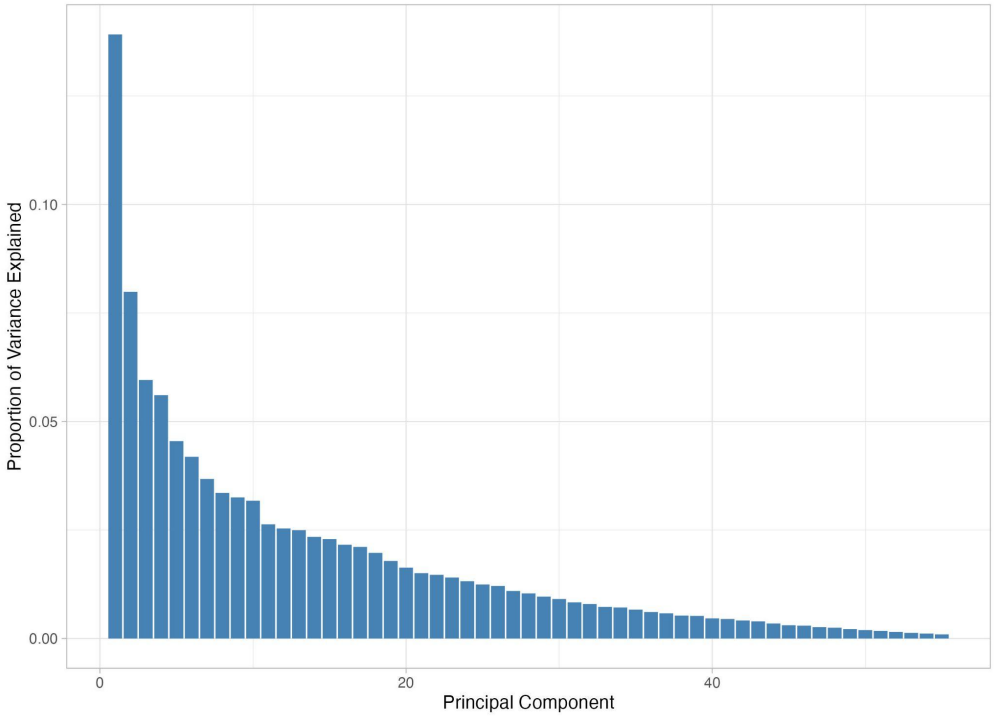

Figure 98. Scree plot from the principal component analysis shows that the variation explained increases as more principal components are added.

A loading plot of features contributing to the PCA on the first and second components was created as shown in Figure 99. The VOCs are labelled by their retention time on the y-axis, and the x-axis shows the variance each VOC explains. Many VOCs explain a little of the variance on the 1st and 2nd components, meaning no single VOC discriminates between the groups. The VOC with a retention time of 9.469 mins contributes the most to components 1 and 2. On component 1, VOCs with retention times 6.034 mins, 24.41 mins and 28.757 mins are also contributing.

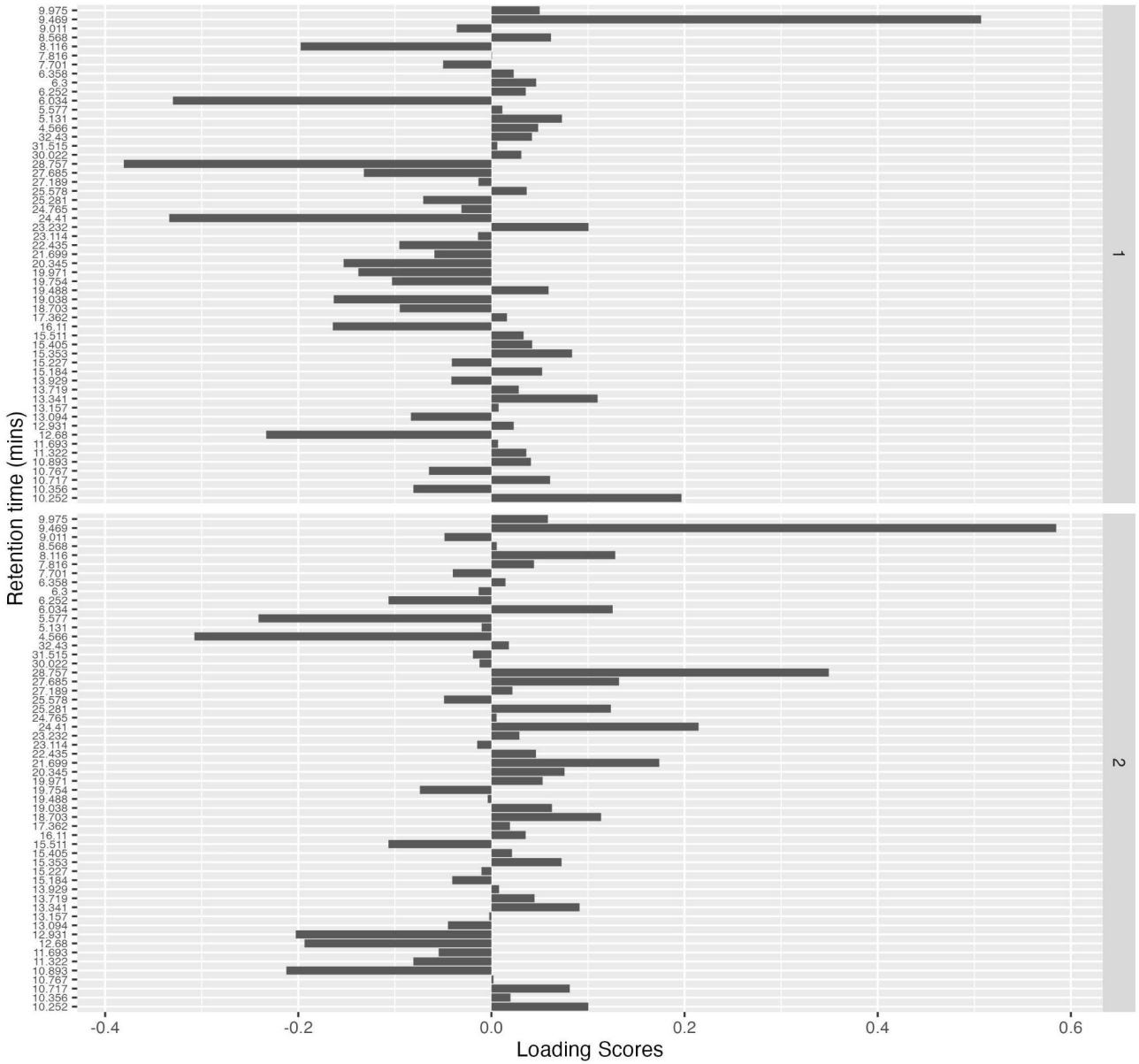

Figure 99. The y-axis shows the VOC contributors, ordered by retention time, on the 1st and 2nd principal components.

4.05.04) No evidence of a difference in known compounds between unattractive and attractive groups

Expected results (Null):
There is no evidence of a difference in the amount of any volatile between the unattractive and attractive groups in the Gambian cohort.

Alternate results:
There is a higher abundance of the volatile in the attractive group compared to the unattractive group if it is an attractant or higher in the unattractive group compared to the attractive group if a repellent.

Compounds from the literature we could identify in our samples were initially investigated. Some were previously identified in the UK cohort by comparing the GC-FID and GC-MS traces and performing peak enhancement on GC-FID via co-injection. Additionally, we tentatively identified additional compounds from the literature by comparing GC-FID and GC-MS traces for the same sample but did not co-inject to confirm their retention times. Six compounds were tentatively identified that were present after alignment: butanoic acid, octane, 1-octen-3-one, octanoic acid, dodecane and dodecanoic acid, which are all known to be involved in the attractiveness of *Anopheles* to humans. Figure 100 compares unattractive and attractive participants and the amounts of each of the six volatiles. There are no clear, consistent trends between the attractive and unattractive groups.

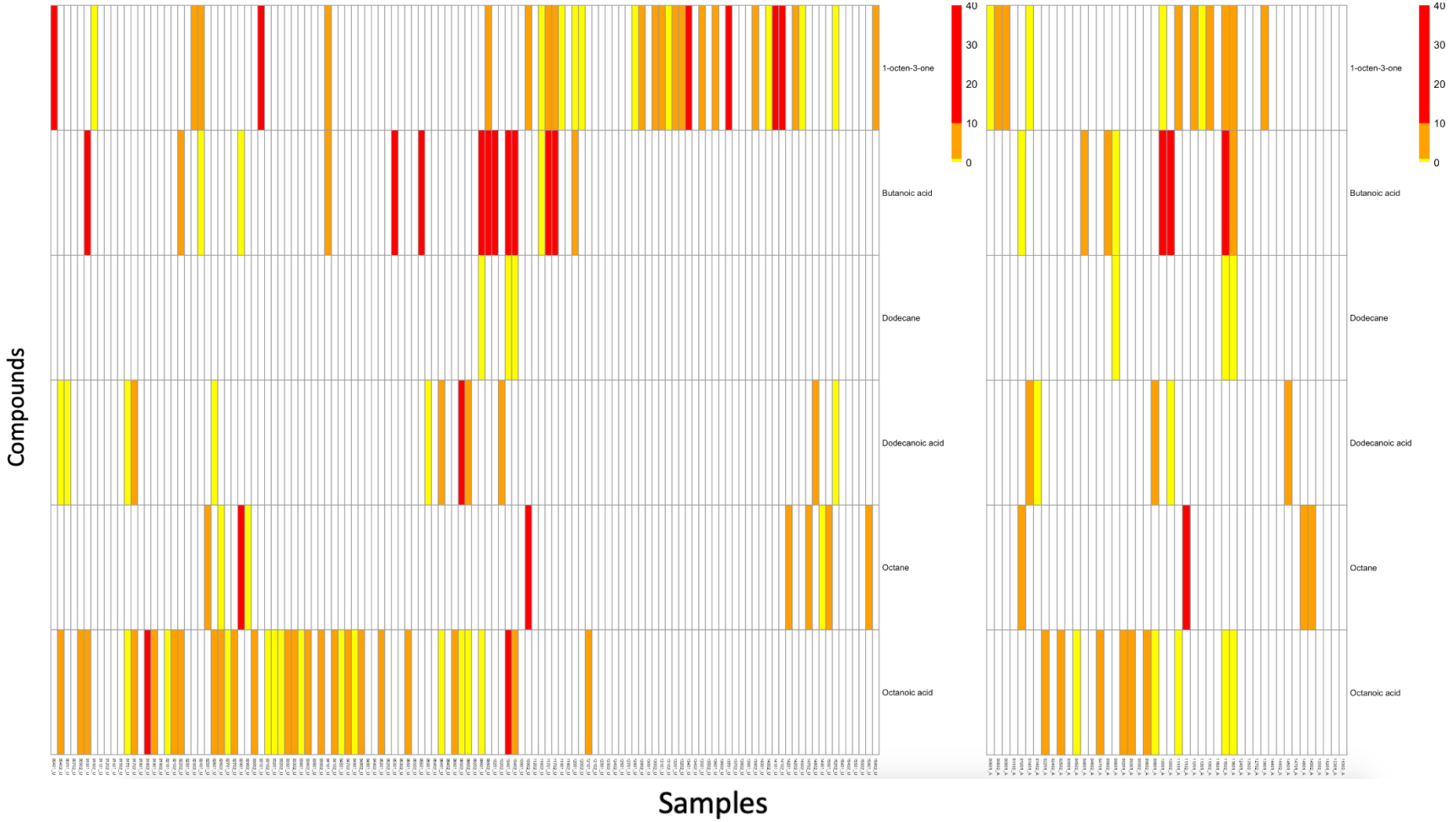

Figure 100. Heatmap showing the relative amount of tentatively identified compounds between the unattractive and attractive individuals. Individuals are shown on the x-axis ("_U" are unattractive, "_A" are attractive to mosquitoes) separated with unattractive participants in the left facet, attractive right. No compound was shown as white, very low yellow (0.1-1 %), middle orange (1-10 %) and higher levels red (10-40 %).

I examined the findings by comparing the average quantities of each volatile between the attractive and unattractive groups, as illustrated in Table 20. Based on t-tests with Benjamini-Hochberg adjustment, there's no evidence of a difference in the mean amount of any of the compounds between the attractive and unattractive groups.

Table 20. Comparative analysis of mean attractiveness and unattractiveness scores across tentatively identified compounds, accompanied by t-test p-values and Benjamini-Hochberg adjusted p-values.

Mean RT (mins)	Compound	Mean Unattractive	Mean Attractive	P value	Adjusted P value
5.577	Butanoic acid	3.923	4.321	0.727	0.825
5.831	Octane	0.651	1.005	0.782	0.825
10.252	1-octen-3-one	1.655	0.543	0.106	0.318
16.110	Octanoic acid	1.184	0.499	0.094	0.318
17.301	Dodecane	0.010	0.039	0.536	0.825
25.622	Dodecanoic acid	0.342	0.171	0.825	0.825

4.05.04) No evidence of a difference in unknown compounds between unattractive and attractive groups

Expected results (Null):
There is no evidence of a difference in the amount of any volatile between the unattractive and attractive groups in the Gambian cohort.

Alternate results:
There is a higher abundance of the volatile in the attractive group compared to the unattractive group if it is an attractant or higher in the unattractive group compared to the attractive group if a repellent.

Next, the most abundant data (peaks present in more than 10 % of samples previously filtered) was used to repeat the analysis using an untargeted approach. First, the 55 peaks were compared across the unattractive and attractive samples, as shown in Figure 101.

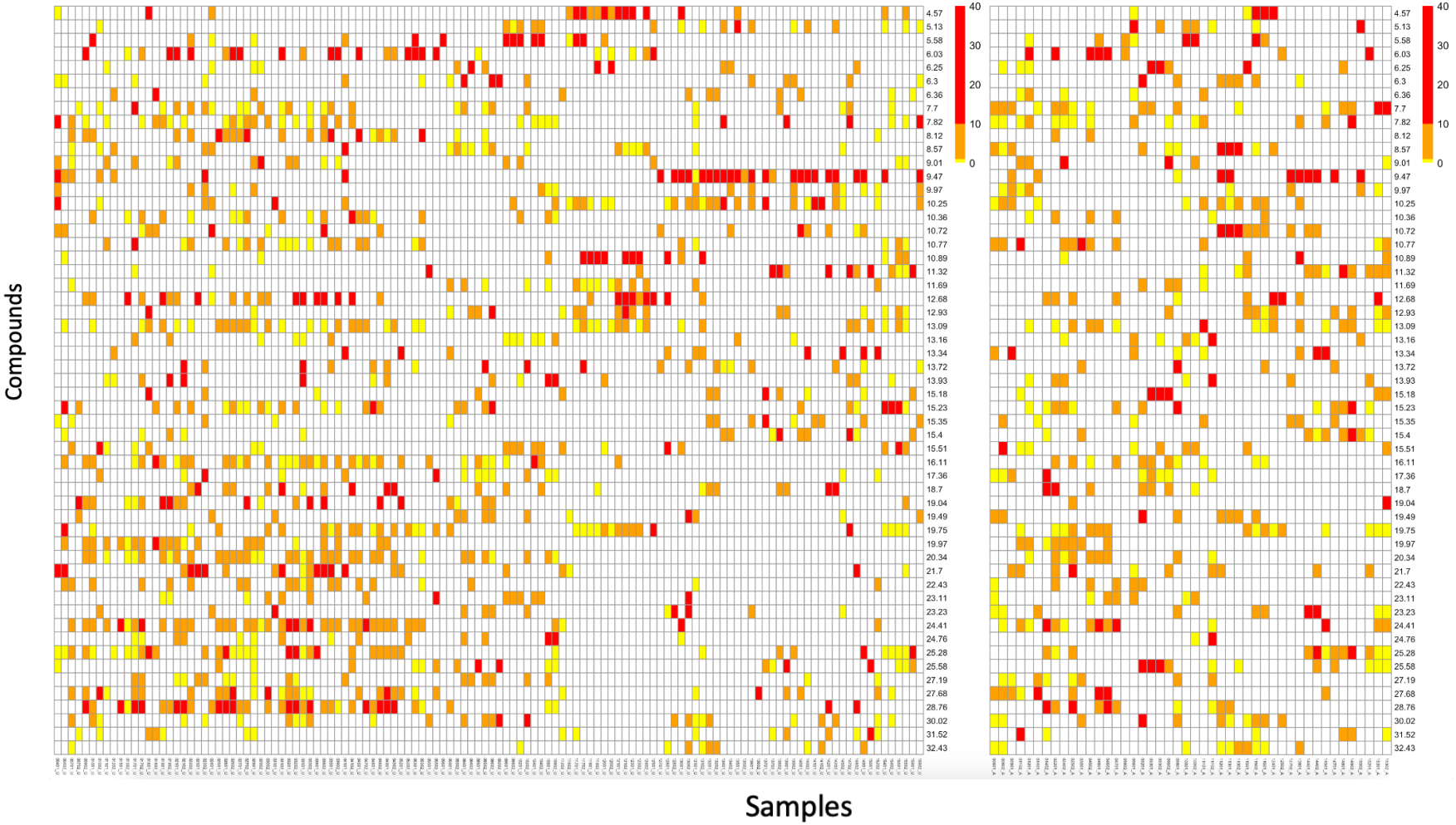

Figure 101. Heatmap showing the relative amount of the highly abundant compounds between the unattractive and attractive individuals. Individuals are shown on the x-axis ("_U" are unattractive, "_A" are attractive to mosquitoes). No compound was shown as white, very low yellow (0.1-1 %), middle orange (1-10 %) and higher levels red (10-40 %).

I analysed the findings by comparing the average quantities of each volatile between the attractive and unattractive groups, as presented in Table 21. Among these compounds, the one with a retention time of 10.252 mins (1-octen-3-one) had been tentatively identified and was part of the prior analysis of tentatively identified compounds. There was no evidence of a difference in the mean amount of any of the compounds between the attractive and unattractive groups.

Table 21. Comparative analysis of mean attractiveness and unattractiveness scores across abundant compounds, accompanied by t-test p-values and Benjamini-Hochberg adjusted p-values.

Mean RT (mins)	Mean Unattractive	Mean Attractive	P value	Adjusted p value
4.566	2.182	1.571	0.601	0.780
5.131	0.399	0.961	0.276	0.660
5.577	3.923	4.321	0.889	0.928
6.034	3.509	5.491	0.415	0.780
6.252	1.445	1.925	0.730	0.837
6.300	0.583	0.840	0.680	0.830
6.358	0.314	0.160	0.394	0.780
7.701	0.749	2.944	0.166	0.660
7.816	1.356	0.596	0.225	0.660
8.116	1.267	0.362	0.035	0.607
8.568	0.361	1.650	0.131	0.660
9.011	0.715	1.544	0.462	0.780
9.469	8.187	6.173	0.485	0.780
9.975	0.238	0.360	0.549	0.780
10.252	1.655	0.543	0.066	0.607
10.356	0.322	0.171	0.273	0.660

10.717	0.649	1.959	0.194	0.660
10.767	0.472	2.389	0.208	0.660
10.893	2.022	0.443	0.033	0.607
11.322	2.031	1.025	0.268	0.660
11.693	0.125	0.172	0.709	0.830
12.680	4.397	2.313	0.167	0.660
12.931	0.726	0.669	0.876	0.928
13.094	0.521	1.588	0.230	0.660
13.157	0.161	0.895	0.223	0.660
13.341	2.803	3.302	0.818	0.900
13.719	1.362	1.246	0.919	0.936
13.929	0.904	0.442	0.276	0.660
15.184	0.579	2.000	0.190	0.660
15.227	2.883	1.025	0.144	0.660
15.353	0.349	0.353	0.984	0.984
15.405	0.467	0.772	0.468	0.780
15.511	1.084	0.895	0.806	0.900
16.110	1.184	0.499	0.065	0.607

17.362	0.476	0.426	0.895	0.928
18.703	1.116	0.798	0.523	0.780
19.038	2.442	0.364	0.030	0.607
19.488	0.588	0.857	0.529	0.780
19.754	0.989	0.713	0.477	0.780
19.971	0.922	0.559	0.329	0.754
20.345	0.487	0.394	0.594	0.780
21.699	1.933	1.402	0.541	0.780
22.435	0.412	0.513	0.706	0.830
23.114	0.384	0.171	0.234	0.660
23.232	1.157	2.274	0.455	0.780
24.410	1.982	3.158	0.429	0.780
24.765	0.908	0.310	0.343	0.754
25.281	1.771	1.317	0.559	0.780
25.578	0.713	1.462	0.264	0.660
27.189	0.239	0.188	0.610	0.780
27.685	1.825	1.419	0.702	0.830
28.757	3.752	1.753	0.053	0.607

30.022	0.612	0.869	0.559	0.780
31.515	0.281	0.462	0.569	0.780
32.430	0.099	0.208	0.197	0.660

4.05.05) Correlations microbiome and volatiles

Expected results (Null):
There is no evidence of a correlation between any microbe and volatile combination in the Gambian cohort.

Alternate results:
There is evidence of a correlation between microbes and volatiles, suggesting the microbe may be producing the VOC in the Gambian cohort.

To investigate if there are significant correlations between microbes and volatiles, the top 10 most abundant genera were correlated with the most abundant VOCs as shown in Figure 102. The strongest correlations were seen for *Auricoccus* and VOCs with retention time of 11.32 mins and 31.52 mins, *Macrococcus* and VOC with retention time of 13.16 mins, *Bacillus* and VOC with retention time of 13.72 mins, *Parococcus* and VOC with retention time of 25.62 mins, and *Acinetobacter* and VOCs with retention times of 5.13 mins, 11.69 mins and 15.18 mins. These VOCs could not be further explored as we were unable to confirm the identity of these compounds.

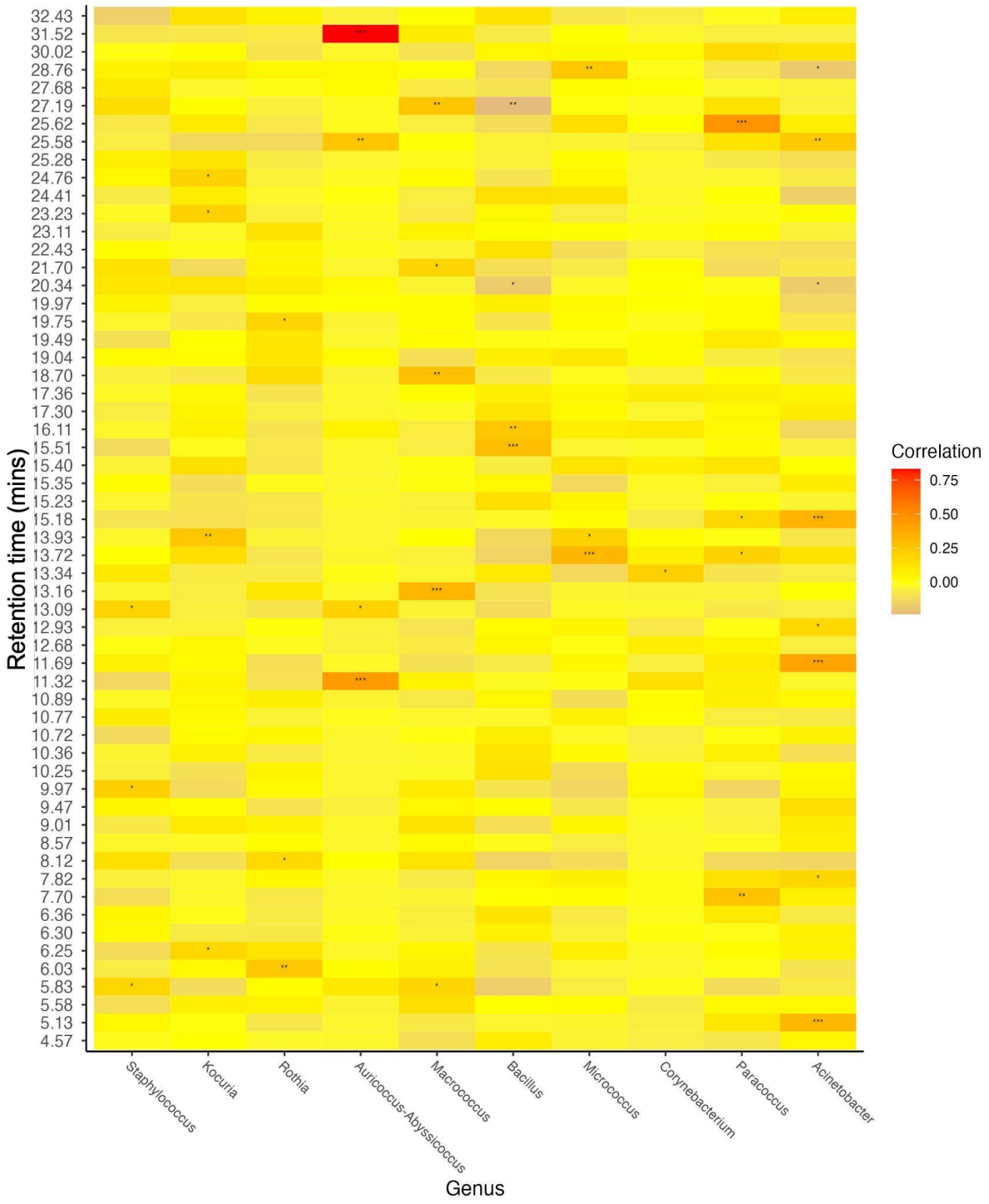

*Figure 102. Heatmap of correlations between the highly abundant genera and highly abundant compounds. The strength of correlation from the Pearson correlation coefficient colours the heatmap. It was between -1 and 1, where -1 was a perfect negative linear relationship, 0 was no correlation, and 1 was a perfect positive correlation. Yellow indicates 0, i.e. no correlation, and red 0.5, i.e. moderate positive correlation. The stars represent the significance based on the unadjusted p-value associated with the Pearson correlation coefficient *** p <0.001, ** p <0.01 or * p <0.05.*

I subsequently explored the correlations between the same VOCs and the top 20 most abundant OTUs. Notably, the *Auricoccus* genus, which includes the fourteenth most abundant OTU, exhibited a pronounced correlation with a VOC recorded at a retention time of 36.37 minutes, as detailed in Figure 102 at the genus level. Additionally, OTU7 and OTU15, both classified as *Macrococcus*, demonstrated a marked correlation with a VOC characterised by a 13.16-minute retention time, a finding noted in the genus-level analysis. No *Bacillus* OTUs were among the top 20 in abundance; therefore, the association between *Bacillus* and the VOC with a 13.72-minute retention time is not depicted in Figure 103. Similarly, the relationships between *Parococcus* and a VOC with a 25.62-minute retention time, as well as *Acinetobacter* and VOCs with retention times of 5.13, 11.69, and 15.18 minutes, were also not observed.

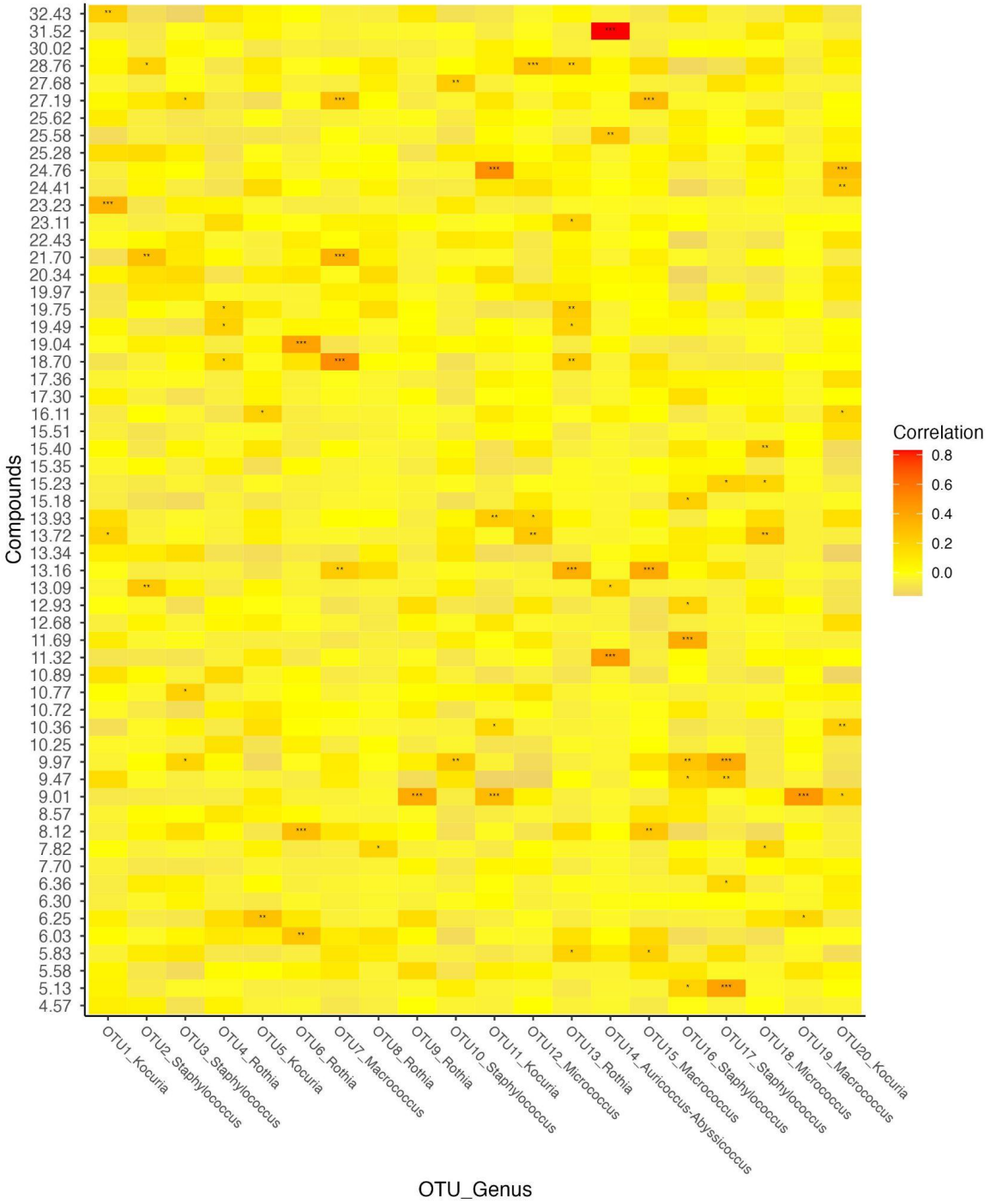

*Figure 103. Heatmap of correlations between the highly abundant OTUs and highly abundant compounds. The strength of correlation from the Pearson correlation coefficient colours the heatmap. It was between -1 and 1, where -1 was a perfect negative linear relationship, 0 was no correlation, and 1 was a perfect positive correlation. Yellow indicates 0, i.e. no correlation, and red 0.8, i.e. strong positive correlation. The stars represent the significance based on the unadjusted p-value associated with the Pearson correlation coefficient *** p <0.001, ** p <0.01 or * p <0.05.*

4.05.06) Genetics

Expected results (Null):
No evidence of a difference in the distribution of volatile profile differences between MZ, DZ and unrelated pairs in the Gambian cohort.

Alternate results:
MZ pairs have more similar distribution of volatile profile differences than DZ pairs and unrelated pairs in the Gambian cohort.

To investigate if odour profiles were more similar for MZ than DZ and more similar for MZ than unrelated, standard Euclidian distances were used to compare the distances between samples. The normalised Euclidian distance between volatile profiles between the MZ twin pairs (N=43), DZ twin pairs (N=42) and unrelated pairs (N=28645) were compared. Figure 104 shows a boxplot comparing the distribution of volatile profile differences between MZ, DZ and unrelated pairs. ANOVA to compare the means of the three groups gave evidence of a difference (F value = 11.8, P value = 7.56e-06). Tukey's Honestly Significant Difference (Tukey HSD) test was performed on pairs. For MZ-DZ (Difference = -0.54, 95 % CI = -2.16-1.09, p-value = 0.72), DZ-unrelated (Difference = 1.40, 95 % CI = 0.26-2.54, p-value = 0.011), and MZ-unrelated (Difference = 1.93, 95 % CI = 0.78-3.09, p-value = <0.001). This gives very strong evidence for a difference between MZ and unrelated and evidence of a difference between DZ and unrelated, but no evidence of a difference between MZ and DZ. The boxplots show smaller distances per pair for attractiveness to mosquitoes between MZ and DZ than unrelated. The density plot shows little difference in the distribution of the difference in attractiveness between the MZ and DZ twins. In conclusion there is weak evidence for a genetic component.

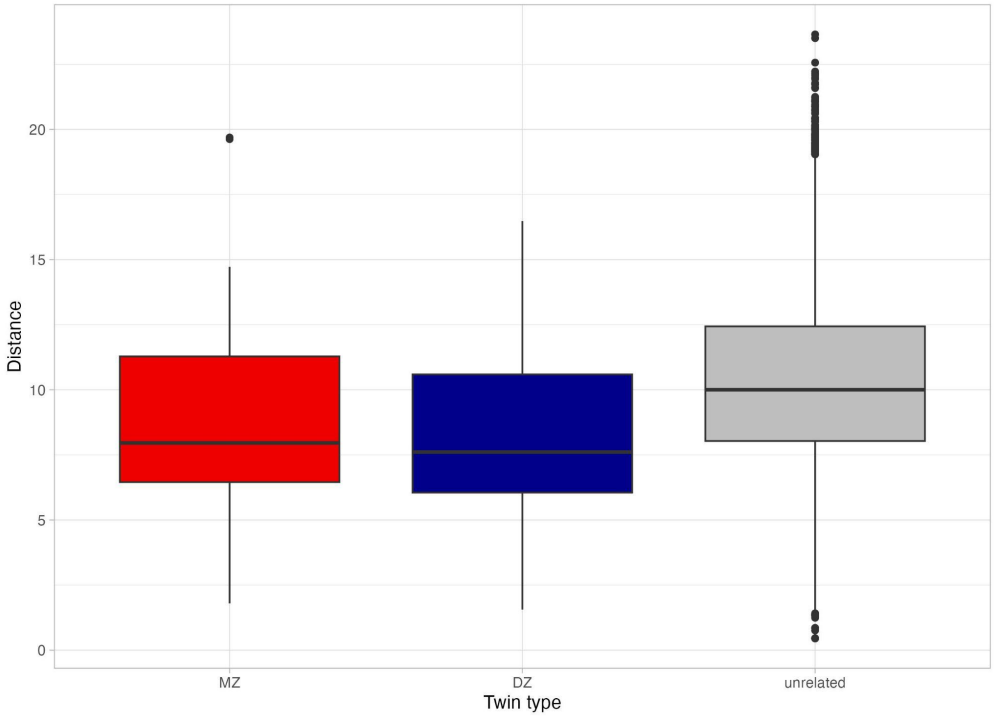

Figure 104. Boxplot comparing the normalised Euclidean distance per pair between MZ (red), DZ (blue) and unrelated (grey) twins.

4.06) Discussion

4.06.01) Methods Comparison

To reduce potential contamination, most studies include a 24-hour wash-out period. During this time, participants are asked to avoid using deodorant and cosmetics and to only wash with odour-free soap to reduce contaminants [95]. In this chapter, we also included a 24-hour washout period, but many endogenous compounds were still identified in our samples.

There are several methods available for collecting VOCs. Solvent extraction is a common method in which the skin is sampled with a cotton pad, or sweat is directly extracted using a solvent, such as hexane or ether [95]. However, this technique can isolate compounds that are not volatile at body temperature [95]. Another option is dynamic headspace collection onto a porous polymer such as Tenax or Porapak Q. Traditionally, samples were first adsorbed onto an intermediate material, e.g. cotton pads, which had drawbacks relating to the intermediate medium not absorbing certain compounds and difficulty making intermediates analytically sterile [95]. More recently, the use of absorbent traps without intermediate absorption has been achieved [25,260], as used in this chapter (Porapak). Our method relied on solvents that can become contaminated and vary between batches. This method usually requires a concentration step, as performed in our study with nitrogen, which risks losing low molecular weight VOCs. To overcome these limitations, solvent-free methods like Solid Phase Microextraction (SPME) and dynamic headspace collection can be used, where the trapping tubes are directly inserted into the GC for thermal desorption. These methods can detect lower molecular weight VOCs without a solvent peak. GC-MS studies can be performed untargeted, able to capture and identify all compounds. Although, the choice of column and method can result in significant variation between studies.

Analysing skin volatiles requires a highly sensitive technique capable of detecting low concentrations of VOCs emanating from the skin. Gas chromatography-mass spectrometry (GC-MS) is often considered the gold standard. It can analyse hundreds of compounds and is highly sensitive, allowing for untargeted investigation of samples with low concentrations of compounds [102]. As we only have GC-FID in-house at LSHTM, we ran all samples by GC-FID and calculated retention times using a method previously optimised for semi-volatiles. A subset of samples were analysed by GC-MS by our collaborator Prof John Pickett at Cardiff University. It would have been interesting to also analyse more volatile compounds (<C8), we were unable to analyse with our method, for example using thermal desorption GC-MS.

PCA was used for volatile analysis rather than sPLS-DA as used for the microbiome. SPLS-DA is a supervised method that knows the group membership (attractive vs unattractive) to model and discriminate between. It is advantageous when the number of variables (ASVs) is greater than the number of observations (individuals). sPLS-DA performs the variable selection and classification simultaneously. It can deal with sparsity and high-dimensionality data like microbiome and allows identification of the ASVs contributing most to differences between the groups. Whereas PCA is an unsupervised method that does not consider group membership. Instead, it seeks to explain variance in the dataset by reducing dimensionality by projecting the data into a smaller subspace while retaining as much of the original data as possible. PCA is useful for visualising high-dimensional data. It is widely used in metabolomics [261]. PCA is a powerful tool that uncovers patterns and detects outliers by effectively reducing the dimensionality of data while retaining variability [262]. This makes it particularly useful in identifying the key metabolites that contribute to the observed variance [261]. Alternatively, could have applied sPLS-DA to the metabolomics data but it would not have been appropriate as the main objective was to explore the data to identify patterns. As only the most abundant and known compounds were investigated the metabolomics data is not high-dimensions (there are more individuals than volatiles investigated), so sPLS-DA would not have had many advantages and may have led to overfitting.

4.06.02) Main discussion

This chapter explores the role of volatile organic compounds on human attractiveness to mosquitoes. The findings indicate that the volatile profile could not separate the attractive and unattractive groups of participants to *An. coluzzii*. Specifically, my analysis did not reveal differences in the amount of body odour or composition of the volatile profile between the unattractive and attractive groups.

There was no separation in the volatile profiles for the attractive and unattractive groups on the basis of the proportion of the total profile made up of specific VOCs. No statistical evidence exists for a difference in volatile profiles for the UK or Gambian cohort. Previously Verhulst et al. (2013) used PLS-DA and similarly showed that the profiles could not be fully discriminated [101], although they were able to use the loadings from the PLS-DA to associate compounds with the highly-attractive and poorly-attractive groups. Many individual volatile compounds have previously been shown to be electro-physiologically active with *An. gambiae* mosquitoes [18,257], and we identified several of these same compounds in our foot odour samples, including butanoic acid, octanoic acid, dodecanoic acid, propanoic acid, decanoic acid. Prior research has identified VOCs with differential abundance between attractive and unattractive groups. For instance, Logan et al. identified eight compounds that had higher levels in unattractive individuals relative to attractive individuals. However, this study focused on attraction to the mosquito species *Aedes aegypti* [25]. Similarly, De Obaldia et al. (2022) identified certain carboxylic acids, including pentadecanoic, heptadecanoic, and nonadecanoic, which were found in higher concentrations in individuals attractive to *Aedes aegypti* compared to those less attractive [16]. In my comparison of the abundance of known compounds and highly abundant compounds between the attractive and unattractive groups to *Anopheles coluzzii,* there was no significant difference in their mean quantities. Many peaks detected were discarded during analysis, as they were also present in the blank controls (air entrainment without a foot). This suggests that only a few human-specific compounds were detected. While this could be a sampling issue, our laboratory group have successfully used this method previously [172]. An alternative analysis method would have been to remove the mean amount of compounds in the blank instead of removing the entire compound if present in the blank. The way attractiveness was categorised (in Chapter 2) could explain the lack of a difference in volatile profile between the groups in our study. It might be easier to discern differences in extremely attractive and unattractive groups than in the broad categories used

in this chapter. Overall, no statistically significant differences in the volatile profiles was observed between the attractiveness groups in either cohort studied.

The comparison of samples and controls showed significantly more peaks in the samples than in the solvent or blank controls. When the total amount of VOCs between the attractive and unattractive groups was compared, there was no statistical evidence of a difference in the mean amount of compounds between the groups. These results give no statistical evidence of a difference in the amount or the composition of VOCs in our study in either the UK or Gambian cohort. The lack of a statistical difference in volatile profiles between the attractive and unattractive groups in our study may indicate an absence of any biological difference. However, this result could also be attributed to limitations in our capacity to detect subtle odour differences or very volatile compounds using our methodology. Given the extreme sensitivity of mosquito olfaction, as highlighted by the impact of minute changes in CO_2 [263], there might be subtle variances that our method failed to capture. Notably, numerous peaks, especially small peaks, were discarded since they were also observed in the controls. Some ether controls yielded more peaks than expected, likely contamination as the batches from Sigma had far fewer non-solvent peaks. Our blank controls showed more peaks than we had anticipated. Some of these might be due to contamination or human compounds from the ambient air that were inadvertently eliminated through the method used. Variations in the amount and the combination of VOCs are important [28,124,191]. Previous research has shown that the ratios between compounds can have an important effect if a repellent effect is seen [93]. As there was no evidence of differences with PLS, models to predict the attractiveness groups were not built, as the differences between the groups were inconsistent.

There was some evidence for correlations between microbes and volatiles. Some of these were known, like Staphylococci producing isovaleric acid. Other associations were stronger such as *Corynebacterium* and isovaleric acid. This high-level analysis would be more informative for looking at individual species. Previous research has demonstrated variations in the volatile profiles of different *Staphylococcus* species when analysed using HS-SPME GC-MS [241] Specifically, *Staphylococcus hominis* and *Staphylococcus saprophyticus* exhibit distinct volatile profiles, with a limited overlap where only 8 out of 20 compounds were identified in both species, highlighting their unique chemical signatures between species [241] Fungi could be producing some of the VOCs characterised, which limits the correlation with microbiome work [264]. To confirm would need to culture and air entrain individual microbes to show that they produced these VOCs, and for those that appeared to be more highly abundant in the attractive or unattractive group, conduct behavioural

assays with mosquitoes. The assays could be run with these differing VOCs at concentrations that would be expected naturally on human skin.

I found no evidence that MZ twins were more similar in VOC profile than DZ or unrelated pairs in the UK cohort and weak evidence in the Gambian cohort. Kuhns and Natsch previously found a genetic effect when comparing odour profiles between MZ twins and unrelated individuals [177]. In their study, they took multiple samples from the same individual and used a more sensitive LECO Pegasus 4D GC x GC–ToF-MS system [177] than our Agilent Technologies 7890A GC. Kuhns and Natsch also treated the extract with recombinant enzyme N-acetyl glutaminase for hydrolysis of glutamine conjugates [177]. This may have helped to improve resolution and sensitivity by breaking down complex compounds into constituents.

There were several limitations to this study. Firstly, our study design involved collecting a single sample from each participant and running it once on the GC-FID. A more comprehensive approach would be to collect samples over multiple days and run them on the GC-FID in triplicate. Kuhn and Natsch (2009) previously collected samples over multiple days and were able to uncouple day-to-day variation from interpersonal variation [177]. However, given that our sample collection took 2 hours of the participant's time and additional samples would have required additional visits, we chose to collect a single sample. We chose to inject samples onto the GC-FID manually as we wanted to ensure that the remaining sample in the vial did not evaporate in case, we wanted to run a sample or run a sample by GC-MS later. However, it can lead to inconsistencies in the amount of samples loaded. Auto-injection minimises discrepancies in peak retention time and variation in the sample volume injected and allows samples to be ran over night. Using ether to elute our samples resulted in an ether peak appearing in the GC-FID trace. This could pose challenges in distinguishing more volatile compounds with a small molecular weight from the solvent peak at the retention time of ether elution. Thermal desorption could have been a potential alternative to overcome this limitation, given that it does not produce a solvent peak. Other parts of the method could have been altered, such as the trapping agent (Porapak), the flow rate in collection or altering the column for a more polar column. We only injected 4 ul of our 50 ul sample, and we had a 50:50 split between the GC-FID and EAG, meaning approximately 5 % of the sample was analysed. We opted not to use internal standards in our study, although they would have helped with alignment because they would have to be introduced directly into the samples. Once added, these standards cannot be removed and can mask peaks of interest if they elute at the same time. We retained samples for potential GC-MS analysis and also considered future GC-EAG tests to evaluate

electrophysiological responses. Although internal standards have been used in some studies, Wooding et al. (2020) used eucalyptol as an internal standard [250]. Therefore we did not get absolute quantification in this study. We could have applied external multiple-point quantification [265]. However, the FID (detector) may have variable responses to distinct compounds, meaning quantification might not be precise unless done with the actual compound of interest. This was further complicated as many peaks of interest were small. We used different samples for behavioural and odour analysis, i.e. socks for behavioural assays and air entrainment from the foot for gas chromatography. We cannot confirm the same odour profile was on both materials. An alternative would have been to collect socks, air entrain half and use half in behavioural assays. There are alternative gas chromatogram options that are more sensitive and may have allowed us to identify small peaks. We analysed all samples using GC-FID, but GC-MS would be preferred to give suggested identifications for all samples by comparing the profiles against a database such as NIST. VOCs present in a few people might be important - these were not explored in this exploratory approach as the low abundant peaks were removed to reduce the number of VOCs when comparing compounds between the attractiveness groups.

The use of GC-FID is a limitation of all analysis discussed in this chapter. While FID detects volatile and combustible compounds ionisable in a flame, it falls short in effectively identifying non-volatile or non-combustible substances, which are commonly found in biological samples. A significant shortcoming of FID compared to mass spectrometry is its inability to provide structural information. This impedes the identification of unknown metabolites, as it cannot be cross-referenced with database spectra. Moreover, GC-FID is less sensitive than GC-MS. FID may fail to recognise trace metabolites that are pivotal in studies such as mosquito attraction. Furthermore, FID is less appropriate for untargeted metabolomics due to its limited data complexity, rendering data interpretation challenging. Due to these limitations, other techniques are often preferred for untargeted metabolomics studies. High-Resolution Mass Spectrometry techniques, like GC×GC-TOF-MS, offer several advantages over GC-FID. They have higher sensitivity and accuracy even at low concentrations, crucial for low levels of unknown compounds. They allow for structural elucidation based on mass and fragmentation patterns, have a wider dynamic range and higher throughput in analysis. Additionally, the software available on GC-MS systems can handle more complex data sets providing more comprehensive analysis. In conclusion, while GC-FID remains a useful tool and was the only option available at LSHTM, it is less appropriate for untargeted metabolomics. High-resolution GC-MS techniques stand as a powerful alternative, providing superior resolution, sensitivity, and accuracy for in-depth metabolomic studies.

Overall, in both cohorts, we found no evidence of a difference in amounts of odour between the attractive and unattractive groups. The PCA did not give evidence of the separation between the attractiveness groups. There was no evidence that volatile composition or the total amount of VOCs was important for discriminating the groups.

5) Chapter 5: Skin microbiome transfers proof of concept study

5.01) Introduction

Skin-associated diseases such as acne, atopic dermatitis and dandruff are associated with alterations in the skin microbiome termed dysbiosis [266–268]. The idea of manipulating the skin microbiome to treat skin diseases has been proposed [269]. Previous investigations have shown successful outcomes of microbiome manipulation utilising faecal microbiome transfers to treat *Clostridium difficile* infection and irritable bowel syndrome [270,271]. There are differences in the skin microbiome composition between attractive and unattractive groups of people to mosquitoes [137,209]. However, at the time of writing (2023), there were no published reports of using SMT to change human attractiveness to mosquitoes.

The potential to manipulate an individual's skin microbiome across different body sites has shown the feasibility of microbiome transfers and subsequent engraftment within an individual. Costello et al. (2009) transferred the microbiome from the tongue to the forehead or forearm. Their findings revealed that at 2, 4 and 8 hours post-transfer, the microbial profile of the forearm was more similar to the tongue, whereas the forehead maintained its native profile [113]. It was observed that the environmental characteristics may have a more substantial role in shaping the microbial communities at sebaceous sites like the forehead than at dry sites like the forearm [113]. A simple method of skin microbiome community transfer was proposed by Perin et al. (2019). The skin microbiome was transferred between the forearm and back of an individual without any pre-treatment [272]. A single transfer resulted in some unique arm species absent on the back before the transfer, appearing immediately post-transfer (median 34 species), with a median of 4 arm species persisting 24 hours post-transfer. The presence of three arm-only species was confirmed by culture, but most species cultured were common to both the back and arm. The researchers could not show if the microbiome transfer were active or passive [272]. Overall, there is some evidence of viable transfers between body sites within a person. The success of transfers may be influenced by the distinct microenvironment of body sites, which can create adverse conditions preventing the growth of transferred microbes or, conversely, facilitate the growth of transferred microbes creating a competitive environment.

Skin microbiome transplantation can also be applied between people. In an innovative approach to treating malodour, Callewaert et al. (2017) used axillary skin microbiome transfers between siblings, where one sibling had a strong body odour, to combat malodour [273]. They hypothesised that transfers between related individuals were more likely to succeed [273]. In contrast to Perin et al.'s method, which avoided pre-treatment, Callewaert et al. first disrupted the microbiome community of the recipient using antibacterials and

antibiotics [272,273]. The transfer reduced strong body odour and resulted in a microbial shift, with increased *Staphylococci* and fewer *Corynebacteria* post-transfer [189,273]. In addition to direct transfer, researchers have also explored skin bacteriotherapy. This strategy typically involves the application of defined combinations of microbes, often consisting of one or multiple pure cultures. Paetzold et al. (2019) developed a method for modulations targeting specific *Cutibacterium acnes* strains [269]. They applied direct transfers, which in contrast to the previous studies described, included a culturing step that could potentially of biased the microbial community compared to the donor. Additionally, they formulated specific strains of *Cutibacterium acnes* of interest for application to the skin. They found specific microbial strain formulations were more effective than whole community transfers, and a mixture of *C. acnes* strains had the best results. They achieved long-term engraftment (up to 52 days) following three days of repeated application [269]. Similarly, Callewaert et al. reported that applying *Staphylococcus epidermidis* cultures could reduce odour scores, a similar outcome to their previous whole microbiome transfer study [189,273]. Many microbes are co-dependent, relying on each other for mutualistic metabolism or cross-feeding to survive [274]. Skin microbiome transfers may transfer co-dependent microbes allowing them to persist in the recipient. In this chapter, direct skin microbiome transfers were performed between participants from the foot, without culturing or pre-treatment and the impact on human attractiveness to mosquitoes was assessed.

Skin microbiome modulation to reduce human attractiveness to mosquitoes could be achieved through several approaches. Firstly, direct manipulation of the skin microbiome composition, either by introducing microbes that generate unattractive metabolites or by eliminating microbes that generate attractants through phage therapy, as proposed by Lucas-Barbosa et al. (2022) [90]. The potential of this strategy is supported by culture studies that showed changing the ratios of *Staphylococcus epidermidis, Pseudomonas aeruginosa, Corynebacterium minutissimum,* and *Brevibacterium epidermidis* could reduce attractiveness to *Aedes* mosquitoes [202]. Furthermore, Lucas-Barbosa et al. (2023) demonstrated the significance of microbial competition and showed distinct outcomes on mosquito attractiveness when culturing microbes in shared media instead of separating them. It was shown that competition is essential for differences in mosquito attractiveness to be seen when microbial ratios are manipulated, a finding relevant to skin microbiome transfers. A similar idea suggested was to add microbial diversity to reduce attractiveness to mosquitoes [202]. This was based on the results of Verhulst et al., which showed alpha diversity was higher in people that are less attractive to mosquitoes [137]. There was no evidence to support this finding in Chapter 3. Likewise, in Chapter 2, there was no evidence of a group of participants naturally repellent to *Anopheles coluzzii*. Thus, I did not

hypothesise that SMT from unattractive to attractive individuals would produce a repellent outcome; but that it might decrease attractiveness. Blocking quorum sensing has been proposed as an approach to reduce attractiveness [204]. Skin microbiome transfers could alter quorum sensing by changing the profile of quorum sensing or transferring quorum sensing inhibitors. Another strategy that has received attention relies on deriving repellent metabolites from people's body odour. This led to the discovery of compounds, including 6-methyl-5-hepten-2-one and geranylacetone, which in a 1:1 mixture, outperformed DEET [93]. The appeal of microbes is due to the potential for a prolonged duration of repellency. Typically VOCs evaporate off the skin within a few hours, protecting humans from bites for up to 8 hours. However, if we could apply bacteria that generate repellent VOCs to the skin, the protection period from bites could be significantly longer [90]. This research aimed to assess the effectiveness of skin microbiome transfers in reducing human attractiveness to mosquitoes and to determine the longevity of any reduction in attractiveness.

While microbial composition significantly influences mosquitoes' attractiveness, human attractiveness may vary based on the mosquito species [90,205]. The odour profiles that attract both *Anopheles* and *Aedes* have been shown to overlap, suggesting the presence of shared olfactory cues despite evolutionary convergence between species [90]. The daytime biting behaviour of *Aedes* mosquitoes can make behavioural assays easier with human participants. Overlapping VOCs contributing to human attractiveness to *Aedes* and *Anopheles* have been identified. Lucas-Barbosa et al. found 13 VOCs to be exploited by *Aedes aegypti, Aedes albopictus* and *Anopheles gambiae ss.* propanoic acid, lactic acid, butanoic acid, acetone, sulcatone, heptanoic acid, octanoic acid, tetradecanoic acid, dimethyl sulphide, ammonia, 3-methyl-1-butanol, 1-octen-3-ol, heptanal [9,90,275]. Additional comparisons of odour profiles and attractiveness across mosquito species were conducted by Coutinho-Abreu et al. (2022), who reviewed the compounds known to be involved in the attractiveness of *Anopheles gambiae, Aedes aegypti* and *Culex quinquefasciatus* [249]. Furthermore, models developed by Lucas-Barbosa et al. (2023), which are based on behavioural information with *Anopheles*, revealed differences in attractiveness to *Aedes* when microbial ratios were altered in culture [202]. This suggests microbial VOCs overlap in the odour profiles between the species. This chapter focuses on *Aedes* mosquitoes for selecting participants for skin microbiome transfers and the resulting impacts. The diurnal activity of *Aedes* allowed assays with participants to be conducted in daylight. Moreover, repellents are currently the primary method to prevent the transmission of *Aedes*-borne diseases such as Dengue and Zika viruses. Therefore, the potential of new approaches to prevent *Aedes* bites is of particular interest.

5.02) Aim and objectives

In the summer of 2021, I worked with a master's student to do a small study and establish a method for foot-on-cage testing to test participants' attractiveness to *Aedes aegypti* mosquitoes and Skin Microbiome Transfers (SMTs) from low-attractive to high-attractive participants. In this chapter, SMTs are repeated with a larger sample using a 1:many design.

Aim

The aim of this chapter was to establish skin microbiome transfers from poorly to highly attractive participants and test if this reduces human attractiveness to *Aedes* mosquitoes.

Objectives

1. To investigate if there are differences in the donor and recipient group's skin microbiome composition before skin microbiome transfer.
2. To establish if the SMT method can successfully transfer skin microbes between people.
3. To examine how long transferred bacteria persist in the recipient skin microbiome post-transfer.
4. To examine if SMT reduces human attractiveness to *Aedes* mosquitoes.

5.03) Methods

5.03.01) Overview of the study design

The experimental design focused on transferring skin microbiomes from participants that were poorly attractive to mosquitoes to those that are highly attractive to mosquitoes (Figure 105). The study aimed to test if transfers would reduce the attractiveness of the highly attractive participants to mosquitoes.

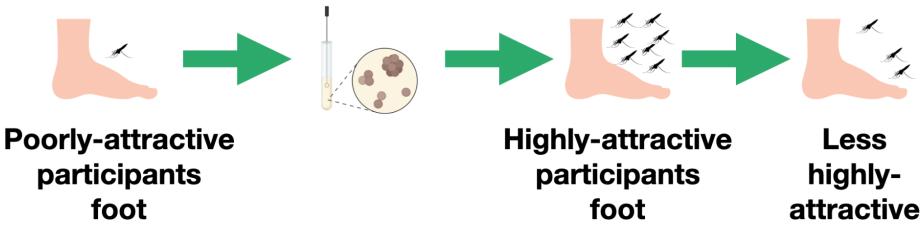

Figure 105. Overview of the skin microbiome transfer (SMT) study design where an SMT sample is transferred from a poorly-attractive "donor" to a highly-attractive "recipient" to reduce how attractive they are to mosquitoes.

A previous study [Halina Potangwa MSc project, 2021] found variability in the transfer success and attributed this to donor differences [276]. Given the logistical constraints on the sample size, a 1-to-many design was selected to reduce donor variability, as shown in Figure 106.

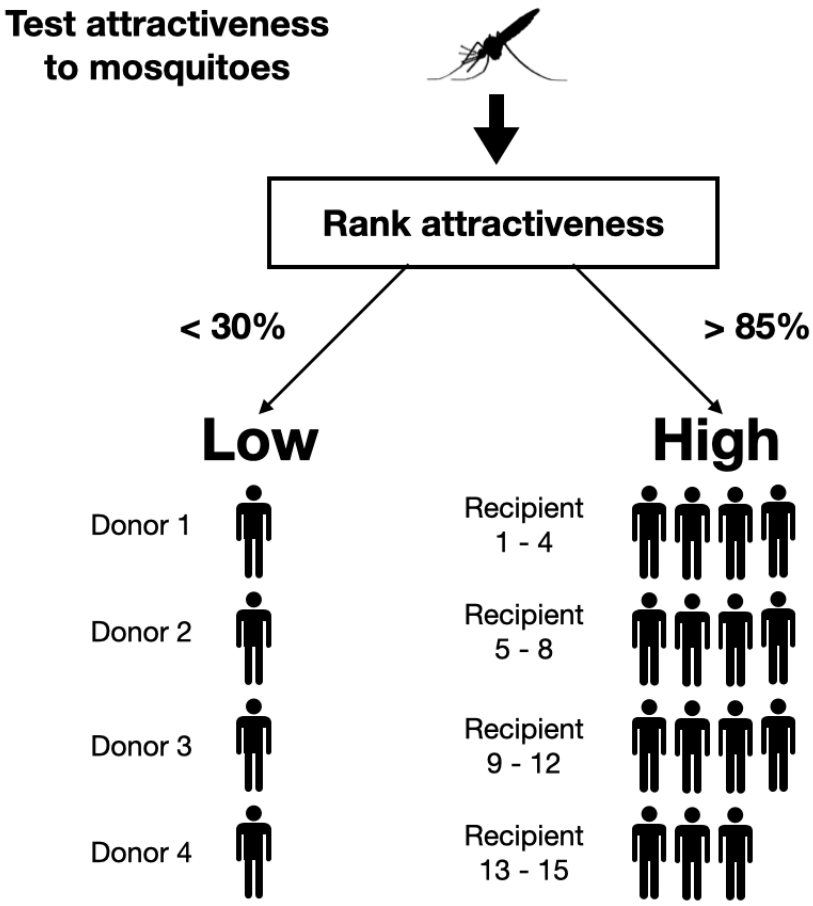

Figure 106. Selection of low and high-attractive groups and individual participants as donors 1-4 and recipients 1-15

5.03.01) Recruitment and Screening

Participant Recruitment

This study was approved by the London School of Hygiene & Tropical Medicine (LSHTM) MSc Ethics Committee (Ethics ref: 26659) and was conducted at LSHTM. Potential participants were provided with information sheets and screened for eligibility against inclusion and exclusion criteria. The eligibility criteria included participants aged 18-50 years with no history of severe health complications. Those with visible or past skin conditions, recent antibiotic use (within the last six months), and vulnerable populations were excluded. Participants who met the criteria and opted to join provided written consent. Before each session, participants were instructed to abstain from alcohol, spicy foods, and the use of fragranced products such as soaps, lotions, and perfumes for 24 hours. Out of 102 participants screened, 14 were deemed ineligible due to recent antibiotic use. Using a foot-

on-cage assay, those who met the eligibility criteria were then assessed for their attractiveness to *Aedes aegypti* mosquitoes.

Rearing

All SMT behavioural studies used non-blood-fed *Aedes aegypti* mosquitoes (AEAE strain, originated PRE 1926 in West Africa) maintained in a colony at LSHTM. Before the experiment, mosquitoes were maintained at 26 ± 1 °C and 70 % humidity under a 12/12 light/dark cycle. Larvae were reared using tap water and hamster pellets (Pets at Home Guinea Pig Nuggets, Pets at Home, Cheshire, UK). Non-blood-fed experimental adults, 5-8 days old, were fed on 10 % glucose solution and housed in 15 x 15 x 15 cm fabric cages (BugDorm-4M1515 Insect Rearing Cage, BugDorm, Taichung, Taiwan). The evening before testing *Ae. aegypti* were starved overnight (for approximately 16 hours) by replacing the sugar stock solution with water. On the day of testing, cups of 20 unfed 5-8 day-old females were prepared by aspirating mosquitoes into cups.

Foot-on-cage assay

I screened participants' attractiveness to mosquitoes to get an attractiveness score that was not confounded by personal perceptions of attractiveness or bite reactions. A 30 x 30 x 30 hard-edge cage (BugDorm-1 Insect Rearing Cage, BugDorm, Taichung, Taiwan) was set up with a batch of 20 mosquitoes previously released from the cup inside the cage. The mosquitoes were given at least 30 minutes to adjust to the testing room, which maintained a temperature range of 25 - 29 °C and a humidity of 65 % - 75 %. The participant was instructed to remove the sock from their right foot for the experiment. Breath was blown on top of the cage to activate the mosquitoes as a source of CO_2. Subsequently, a frame was placed on the cage, and the participant's bare foot was positioned on top of the frame, creating a safe gap between the foot and the cage. To ensure probing without bites, the frame's optimal width was determined to be 15 mm. Three identical frames were 3D printed by Dr Chrissy Roberts at LSHTM. Figure 107 displays an actual setup of the cage with 20 *Ae. aegypti* mosquitoes. Cages and frames were cleaned with 20 % ethanol between assays. The thickness of the frame and length of time for the assay had previously been optimised in an MSc project I supervised [276]. The cage contained 20 female *Ae. aegypti* mosquitoes. After 2 minutes, the number of mosquitoes probing the foot within the cage area was recorded. The attraction scores were then calculated using the following formula:

$$Attraction\ score\ =\ Number\ probing\ foot\ /\ total\ number\ mosquitoes$$

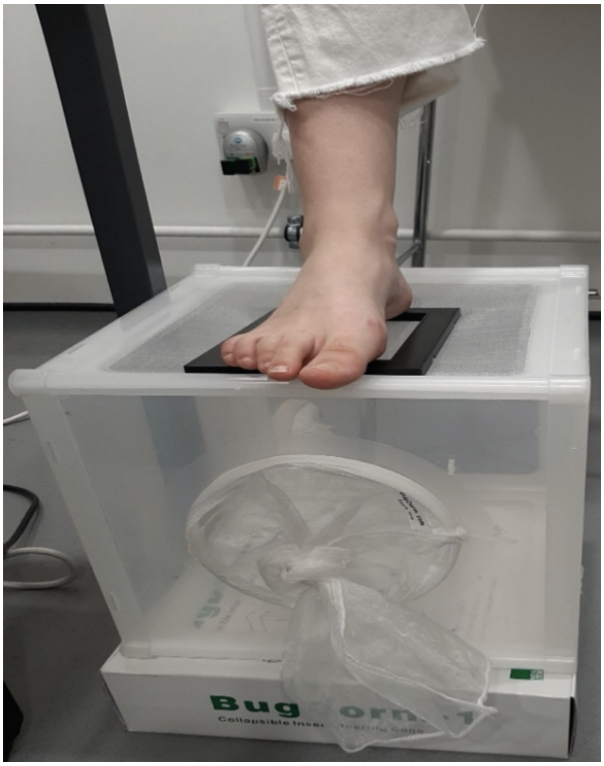

Figure 107. The foot-on-cage assay set-up consisted of a hard-edge 30 x 30 x 30 BugDorm cage containing 20 Aedes aegypti mosquitoes, a plastic frame on top of the cage to prevent bites on which the participant's foot is placed.

The design of the cage assay was optimised during an MSc project I supervised in the summer of 2021. We tested over a 5 minute period and found that between 75 and 120 seconds after placing the foot had the most probing from mosquitoes, as shown in Table 22 (data from [276]). I, therefore, chose to use 2 minutes in this assay.

Table 22. Testing the optimum length of time before measuring attractiveness to mosquitoes using the same participant's foot with four separate cages of Aedes aegypti mosquitoes over a 5-minute period. Scores are the proportions of responses out of the 20 mosquitoes in each test. The average is across the four tests. Data from MSc project (Potangwa 2021).

Time (Seconds)	Test 1	Test 2	Test 3	Test 4	Average
0	0.00	0.00	0.00	0.00	0.00
30	0.15	0.10	0.05	0.05	0.09
45	0.20	0.15	0.05	0.00	0.10
60	0.15	0.15	0.05	0.05	0.10
75	0.20	0.15	0.05	0.05	0.11
120	0.20	0.15	0.05	0.05	0.11
240	0.10	0.05	0.05	0.00	0.05
300	0.05	0.00	0.00	0.00	0.01

Study participants

The eligible participants (N=88) were only screened with *Ae. aegypti* used the foot-on-cage assay once to get an initial attractiveness score to select people as attractive (>=85 %) as “recipients” and unattractive (<=30 %) as “donors”. Both groups were re-screened before the transfer. This gives two behavioural replicates per participant before the transfer.

5.03.03) SMT donation

SMTs were run over four weeks with a different donor each week. At the beginning of each transferring day, the donor was re-screened for attractiveness to mosquitoes to check if they were still low attractive. 20 skin swabs were collected from the sole of the foot of the “donor” participant using Isohelix DNA buccal swabs, as shown in Figure 108. The buffer was the same as for the previous microbiome experiments. In brief, Tris buffer with pH 7.9, 0.5 % tween-20 and 1 ml of Ethylenediaminetetraacetic acid. Each swab was dipped into the buffer and rubbed vigorously for 30 seconds, 15 seconds on each side of the swab from

the toes towards the heel and stored into 2 ml Eppendorf tubes containing 1 ml of the same buffer described above at room temperature.

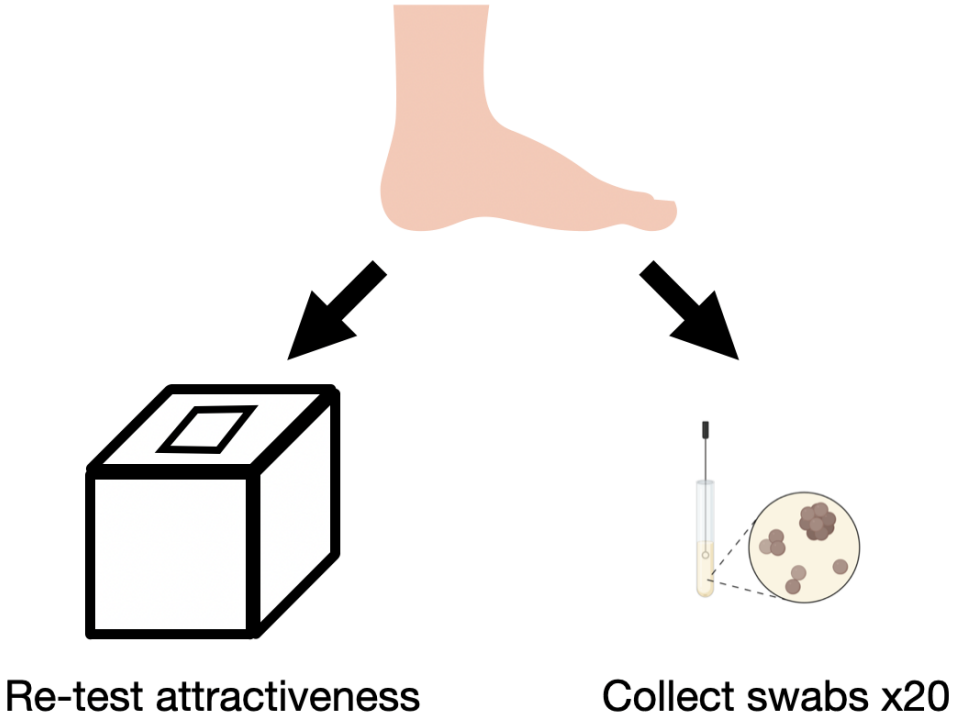

Figure 108. The procedure for donors was to re-test attractiveness using the foot-on-cage test and to collect 20 microbiome swabs for the SMT.

After collection, the buccal swabs from the donors were removed from the Eppendorf tubes using tweezers. Eppendorf tubes were centrifuged at 2000 rcf for 5 minutes with the front of the lid facing inwards and again at 2000 rcf for 5 minutes with the front part of the lid facing outwards to ensure that the pellet was evenly distributed. 800 µl of the supernatant was removed, and the remaining 200 µl, including the pellet, was left at the bottom of the tubes. The contents of all 20 Eppendorf tubes, each with 200 µl of pellet solution, were pooled to yield approximately 4 ml of microbiome-containing solution, which was then gently vortexed. This was then split into:

1. 4 tubes of 500 µl for applying to participants (500 µl x 4 = 2 ml)
2. 500 µl for DNA extraction (any extra put in here)

All prepared microbial samples were stored at 37 °C in a thermomixer until they could be transferred to the recipients. Aimed to do all transfers within 3 hrs of collecting the bacteria. The microbial samples for sequencing were stored at -20 °C until DNA extraction.

5.03.04) SMT and recipient

The four-time points (0, 6, 24 and 72 hours) for each recipient and the experiments carried out at each are shown in Table 23.

Table 23. Overview of experimental procedures performed at each time point. At the initial 0-hour mark, attractiveness was assessed using the foot-on-cage assay and skin microbiome samples collected. After applying an SMT to the foot, the foot-on-cage assay and skin microbiome sampling are repeated. At all other time points (6, 24 and 7h hours post-transfer), attractiveness is assessed, and skin microbiome samples are collected.

Time post-transfer (hours)	**Task for recipient**
0	Re-screen participant with foot-on-cage test and collect skin microbiome swab, apply the SMT from the donor. Then foot-on-cage test and collect skin microbiome swab
6	Foot-on-cage test and collect skin microbiome swab
24	Foot-on-cage test and collect skin microbiome swab
72	Foot-on-cage test and collect skin microbiome swab

0 hours post transfer

At the initial visit, skin swabs were collected from the test foot of the recipient as a baseline control. Then the attractiveness towards the *Ae. aegypti* was assessed through the foot-on-cage assay to check attractiveness pre-transfer.

The test foot was divided into approximately four sections using a permanent marker pen, as shown in Figure 109. Subsequently, the SMT from the donor was pipetted onto the area inside the large rectangle. A plate spreader ensured even distribution of the SMT across the entire base of the foot. This precaution ensured that during sample collection for sequencing at intervals of 0, 6, 24, and 72 hours post-transfer, the applied SMT remained undisturbed for subsequent collection points.

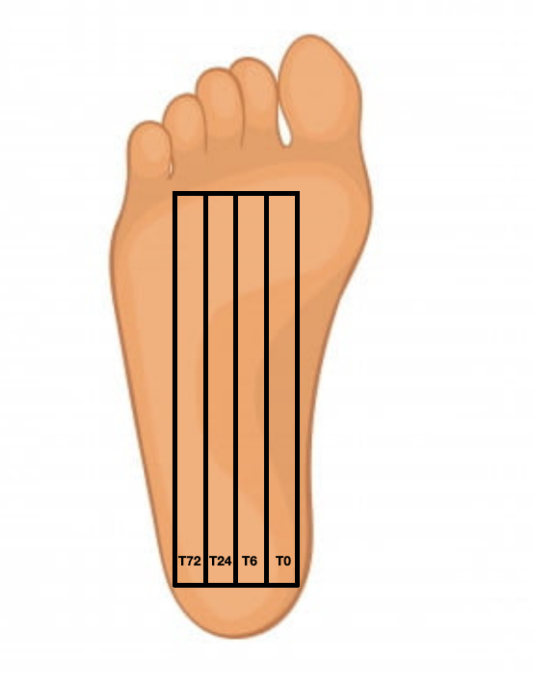

Figure 109. Cross section of the base of the foot and the four areas where swabs were collected from post-transfer.

The foot was given at least two minutes to dry. Two swabs were then collected from the section of the foot marked T0 hours and immediately placed into the Eppendorf tube containing the buffer. Swabs were stored at -20 °C until DNA extraction. The test foot receiving the swab was then re-screened for attractiveness towards *Ae. aegypti* through the foot-on-cage assay test. Between visits post-transfer participants were allowed to wear their own shoes and socks.

6 hours post-transfer

After six hours, the participant was invited again for the foot-on-cage assay and the attractiveness was assessed only on the test foot which received the transfer. Then two skin swabs were collected from the section of the test foot marked 6 hours.

24 hours post-transfer

After 24 hours, the participant was invited again for the foot-on-cage assay and the attractiveness was assessed only on the test foot which received the transfer. Then two skin swabs were collected from the section of the test foot marked 24 hours. At this point, it was recorded if the recipient had washed their feet and whether they had showered.

72 hours post-transfer (optional visit)

After 72 hours, the participant was invited again for the foot-on-cage assay and the attractiveness was assessed only on the test foot which received the transfer. Then two skin swabs were collected from the section of the test foot marked 72 hours. Recorded if the recipient had washed their feet and whether they had showered.

5.03.05) Controls

Before conducting the transfer, re-assessed the attractiveness of both donors and recipients to mosquitoes, which took place between 4 and 14 weeks following the initial screening. However, longitudinal microbiome samples were not collected to confirm that the skin microbiome was consistent over time prior to the transfer. Previous research has demonstrated that attractiveness to *Aedes aegypti* remains consistent over several months [16]. The microbiome controls used were negative controls, swabs that were opened in the testing room and processed alongside the samples. The positive controls were ZymoBIOMICS D6300 community standard and ZymoBIOMICS D6305 DNA standard. It was assumed that the skin microbiome composition would be the same across the foot. To validate this assumption should have collected additional controls from all four sites on the foot to confirm the homogeneity of composition across the foot. It would have been beneficial to incorporate attractiveness controls, which could have been achieved by evaluating the attractiveness of the participant's other foot, which did not receive a transfer with the foot that did receive an SMT. However, I had concerns that such a setup would lead to cross-transfer between the participant's feet. To enhance reliability, multiple behavioural tests at each time point could have been conducted. To do this, it might have been more effective to collect samples like socks at each time point, permitting multiple subsequent tests.

5.03.06) DNA extractions

Bacterial DNA was extracted from 71 samples, 6 negative controls and the extraction positive control. As for the Gambian samples (chapter 3 Gambian cohort methods), the QIAamp DNA microbiome kit was used (Qiagen). Bacterial cells were lysed using pathogen lysis tube L, vortexing on vortex genie at maximum speed for 10 minutes. The addition of benzonase was avoided.

5.03.07) 16S rRNA sequencing

The 16S rRNA amplicons were amplified through PCR by targeting the 16S V3/ V4 region as for the previous microbiome work following the Illumina 16S Metagenomic Sequencing Library Preparation protocol [225]. The amplicon PCR included 12.5 µL of Kapa Hifi HotStart ReadyMix (2X), 5 µL of DNA template, 2.5 µL each of forward and reverse primers (1 µM), and 2.5 µL of H_2O. The PCR program consisted of an initial denaturation step at 95 °C for 3 mins, followed by 30 cycles of denaturation at 98 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute. The final extension step was carried out at 72 °C for 5 minutes. AMPure XP beads were used to purify the PCR product.

The Nextera XT Index Kit v2 Illumina sequencing indexes (Illumina, Inc) and Kapa Hifi HotStart ReadyMix (2X) were used for the Index PCR to add the Nextera XT barcodes. The amplification program consisted of initial denaturation at 95 °C for 3 mins, followed by eight cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 5 mins. AMPure XP beads were used to purify the indexed PCR product. The library was prepared and quantified as previously described (Chapter 3) and pooled with a final concentration of 4 pM and 20 % PhiX [225]. The samples were sequenced using the 2 x 300 base pair MiSeq V3 kit on the Illumina MiSeq in-house at LSHTM. The demultiplexed Fastq files were downloaded for analysis.

5.04) Results

5.04.01) Screening results

The initial screening results were used to classify participant attractiveness to mosquitoes and select the donors and recipients for the transfers. Of the 88 eligible participants, 5 had attractiveness <=30 % and 24 had attractiveness scores >=85 %. Of these, 4 poorly attractive participants were chosen as “donors” and 15 highly attractive as “recipients”. Figure 110 summarises the number of participants recruited for the study and where they were excluded.

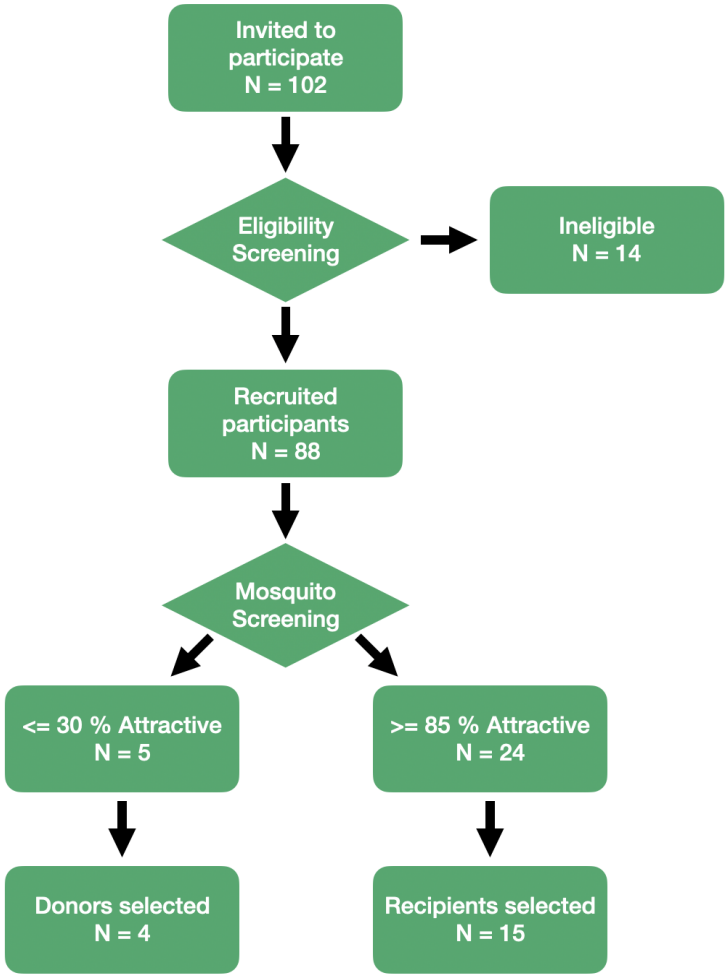

Figure 110. Flow diagram of participant recruitment, which passed the screening and the participants selected as low (<=30 %) or high (>85 %) attractive.

5.04.02) Attractiveness: Does SMT reduce human attractiveness to *Aedes* mosquitoes

Expected hypothesis (H0): There is no difference in recipients' attractiveness to mosquitoes after SMT compared to before the transfer.

Alternative hypothesis (H1): There is some reduction in recipients' attractiveness to mosquitoes after SMT compared to before the transfer.

Distribution of attractiveness scores

A histogram of attractiveness scores at the initial screening is shown in Figure 111. Some people have very low scores (<30 %), most people are in the middle, and some are highly attractive (>85 %).

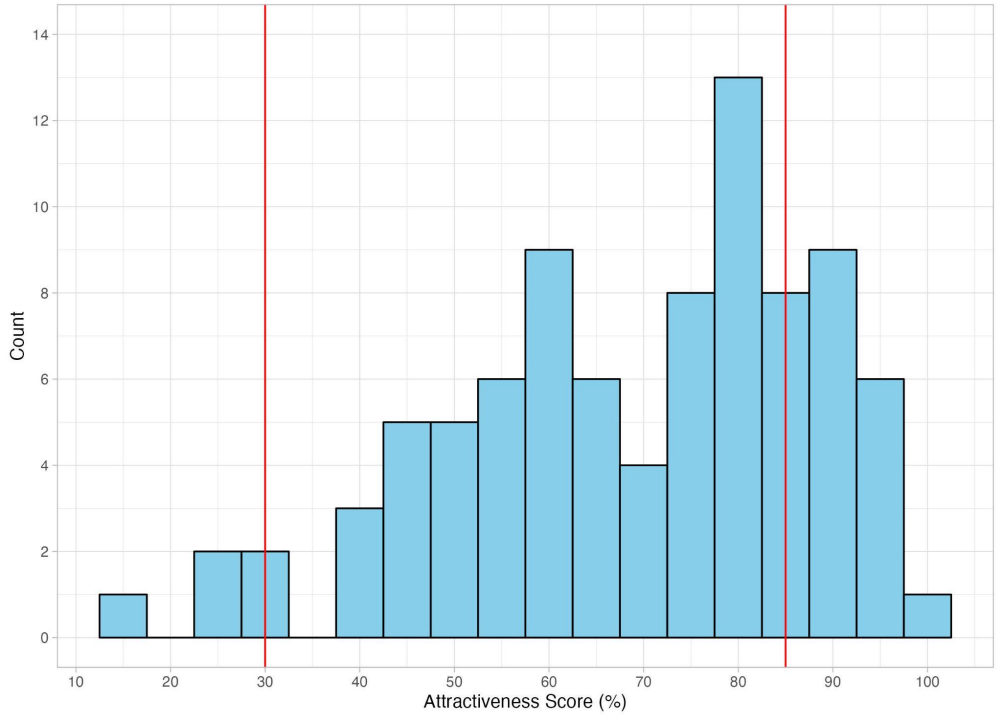

Figure 111. Histogram of attractiveness scores, the bins are 5 wide. Red lines represent the cut-off to be considered low and high attractive at 30 % and 85 %.

Differences in attractiveness between donors and recipients

Before the transfer, the attractiveness of both donors and recipients was evaluated a second time. Figure 112 illustrates a distinct difference in attractiveness scores between the chosen donors and recipients. Donors consistently received lower scores, while recipients consistently ranked higher. The interval between the preliminary and the follow-up screening, just before the SMT, varied between 4 to 14 weeks. On the donation day, donors D3 (110) and D4 (117) increased their attractiveness scores to 60%. In contrast, donors D1 (192) and D2 (182) retained their low scores, 35% and 20%, respectively.

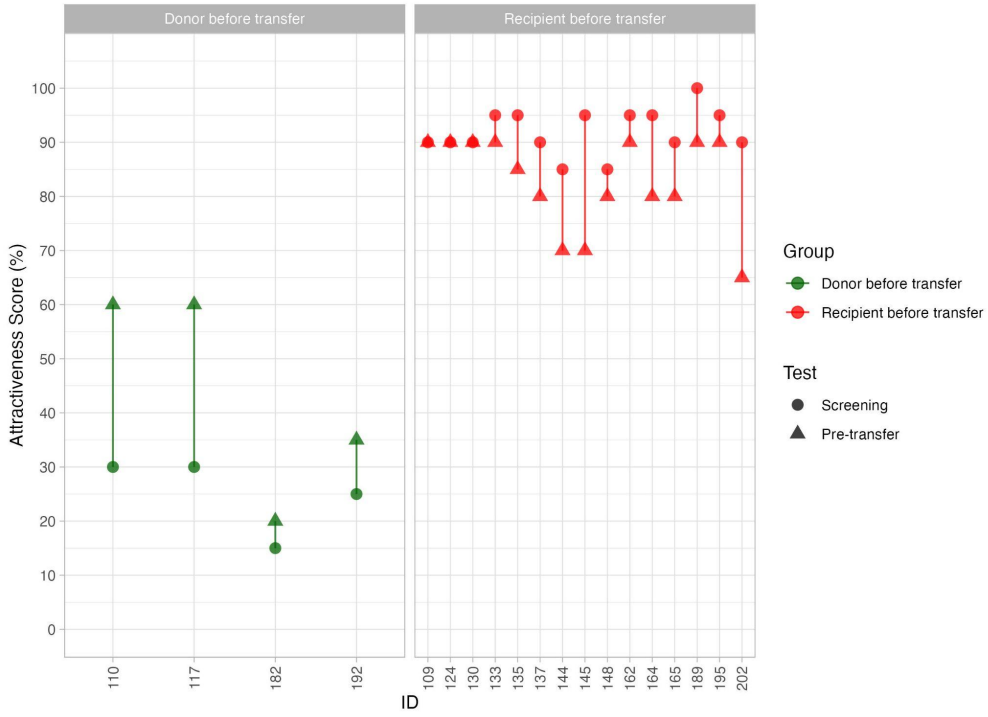

Figure 112. Attractiveness to mosquitoes of the donors (green) and recipients (red) at the initial screening (circles) and pre-transfer (triangles).

Absolute attraction scores reduced post-transfer

Figure 113 shows the recipient attractiveness before and after transfer for each recipient, faceted by the donor they received the transplant from. For donor D1, post-transfer reductions are noted in three out of the four recipients. Two recipients exhibit reductions between 0-6 hours post-transfer. One recipient experienced a reduction in the time period between 6-24 hours, while no reductions were observed in any recipients between 24-72 hours. For donor D2, post-transfer reductions in attractiveness for two out of the four recipients. Three recipients exhibit reductions between 0-6 hours post-transfer. One recipient experienced a reduction between 6-24 hours, while one recipient had a reduction at 72 hours post-transfer. For donor D3, there is a slight reduction in one of the three

recipients post-transfer. Two recipients exhibit reductions between 0-6 hours post-transfer. One recipient experienced a reduction in the between 6-24 hours post-transfer, while no recipient had a reduction at 72 hours post-transfer. For donor D4, post-transfer reductions are noted in two out of the four recipients. No reductions between 0-6 hours post-transfer. Two recipients experienced a reduction in the time period between 6-24 hours. One recipient was unable to return at the time point. Their attractiveness score 24 hours post-transfer was 80 %. There is variability in mosquito response and only 20 mosquitoes in each cage. The recipients who received transfers from D1 and D2 appear to have better reductions in attractiveness post-transfer on average, indicating a clearer effect from these donors (192 and 182 respectively in Figure 112) which may be linked to more consistent low attractiveness in these donors compared to the other donors.

Figure 113. Changes in attractiveness over time (before, immediately after, 6, 24 and 72 hours post transfer) faceted and coloured by the donor. Panel is the donor: D1 = 192, D2 = 182, D3 = 110 and D4 = 117. Each transfer is represented by a different line (i.e. 3 or 4 donors per recipient). The black line shows the average attractiveness of recipients at each point.

The absolute attractiveness scores (number of mosquitoes trying to probe the foot within the frame out of 20 mosquitoes released) before and at 0, 6, 24 and 72 hours after transfer is shown in Figure 114. There appears to be a trend in the median attractiveness reducing post-transfer compared to before. There is a slight reduction in median attractiveness, this reduction would not move them from the highly-attractive (>= 85 %) attractive to the poorly-attractive (<= 35 %) group.

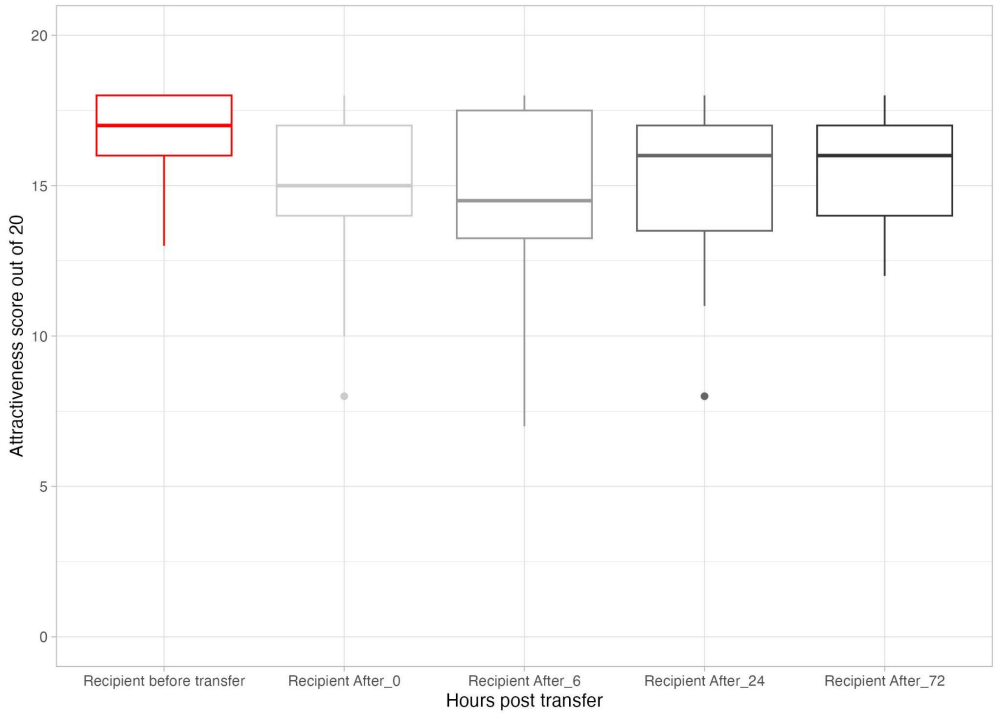

Figure 114. Boxplot of attractiveness scores for recipients before and 0/6/24/72 hours post-transfer.

As the data were not normally distributed, a Wilcoxon signed rank test with continuity correction was used to compare the medians before and after transfer with Benjamini-Hochberg correction for multiple testing. The null hypothesis was the median difference between before and after transfer was zero. There was some statistical evidence of a reduction in attractiveness immediately post-transfer, indicating a trend towards reduced mosquito attraction post-transfer (median difference= -2, V=50, Adjusted p-value=0.054). The biggest reduction in attractiveness to mosquitoes was seen 6 hours post-transfer (median difference= -2.5, V=48.5, Adjusted p-value=0.054). There was suggestive evidence of a statistical reduction in attractiveness 24 hours post-transfer (median difference= -1, V=48, Adjusted p-value=0.054) but no evidence of any reduction after 72 hours (median difference= -1, V=23, Adjusted p-value=0.143).

5.04.03) Investigating if there are differences between the donor and recipient groups skin microbiome composition before skin microbiome transfers

79 microbiome samples were successfully sequenced (Samples N=71, (N=4 donors, N=14 recipients pre-transfer, N=15 recipients 0 hours post-transfer, N=14 recipients 6 hours post-transfer, N=15 recipients 24 hours post-transfer and N=9 recipients 72 hours), Positive controls =2 and Negative controls =6 microbiome controls described in Chapter 3).

Expected hypothesis (H0): There is no difference in skin microbiome composition between donors and recipients prior to SMT.

Alternative hypothesis (H1): There is some difference in skin microbiome composition between donors and recipients prior to SMT.

Sparse partial least squares discriminant analysis (sPLS-DA) was used to compare microbiome composition between donors (green) and recipients prior to transfer (red) groups based on beta diversity filtered by adding an offset of 1, removing low counts (<0.01%) and CLR transforming as shown in Figure 115. N=18 samples remained (donors N=4, recipients pre-transfer N=14). Components 1 and 2 combined to explain 14 % of the differences between attractiveness groups. The centroids for the groups are separated on the 1st and 2nd components. The 95 % confidence intervals do not overlap. The separation between the groups gives some evidence of a difference in dispersion, but there is no evidence of a difference in dispersion between the centroids (PERMANOVA, $P = 0.61$). Additionally, density pots on the outside show clear separation in the distribution, especially on component 1.

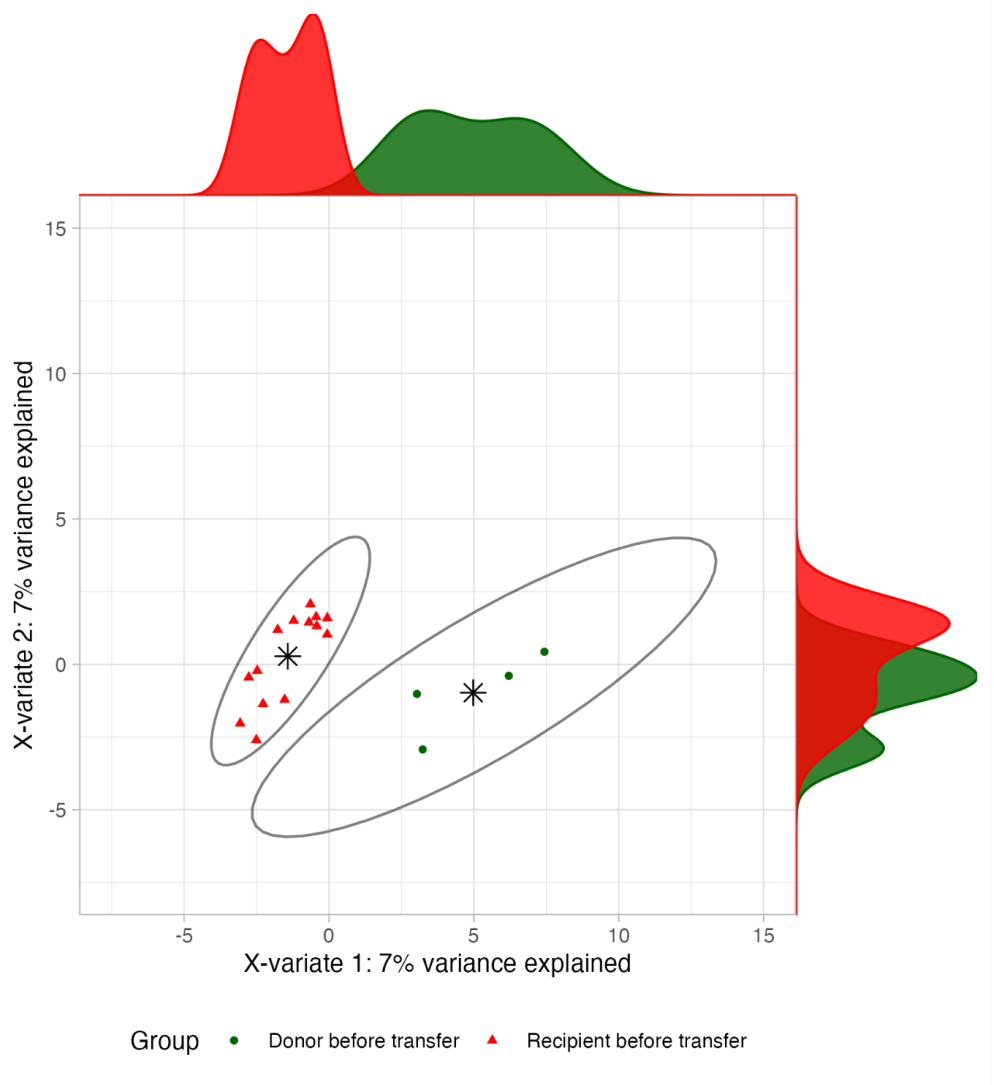

Figure 115. Sample plot visualises differences between the groups from a sparse partial least squares discriminant analysis (sPLS-DA) implemented in the MixOmics package (Rohart et al. 2017). It compares skin microbial composition (beta diversity) between the donors (green) and recipients (red) prior to the transfer. Individuals are presented as small circles (donors) or small triangles (recipients). Microbiome data were pre-processed by filtering, CLR transformed and scaled (centred and standardised). The centroids, depicted as black stars, represent the average microbiome composition in the two-dimensional space on the first and second components of the analysis for each group. The enclosing ellipses (large circles) represent the 95 % confidence intervals for these groups, providing a visual estimate of the dispersion around the group means. The first component explains 7 % variance, and the second component is 7 % variance, summing to a cumulative variance of 14 %. Additionally, density plots are placed above and to the right of the loading plot presenting the distribution of the scores for the first and second components for each group. These provide a view of the spread of the data for each component within the donor and recipient groups.

5.04.04) SMT method is not successful at transferring major taxa between donors and recipients

Expected hypothesis (H0): There is no difference in recipients' skin microbiome after SMT compared to before the transfer.

Alternative hypothesis (H1): There is some difference in major taxa in the recipients' skin microbiome after SMT compared to before the transfer. Taxa present in the donor but absent in the recipient prior to the transfer become detectable after the transfer.

5.04.05) Exploratory analysis of compositional changes post-transfer

Figure 116 shows the changes in microbial composition in each recipient post-transfer, based on the top 20 most abundant genera. Each recipient is presented in a separate grouped bar plot. The plots are arranged in rows based on the donor they received a transfer from. Each plot has up to six bars showing the microbial composition of the donor and then the recipient at distinctive time points: before the transfer, immediately after, and at 6, 24 and 72 hours post-transfer. The stacked bar chart does not sum to one, as only the top 20 most abundant genera are displayed, not all genera. This focus enables visualisation of the changes in the recipient after transfer and allows for comparison to the donor they received the transfer from. Though there are observable shifts in the relative abundance of certain genera post-transfer, there is no clear trend of donor-specific genera appearing in the recipients over time. Overall, the recipient's microbiome post-transfer based on the top 20 most abundant genera appears to stay more similar to the native state than to the donors.

In the first panel, which focuses on donor D1 and recipient R1, there is a difference in the microbial composition between the donor and recipient. The donor has a higher relative abundance of *Staphylococcus* (orange bar). However, R1's microbiome remains relatively stable before the transfer, immediately after, and 6 hours post-transfer. However, 24 hours post-transfer, there is an increase in *Cutibacterium* observed in R1, but the donor does not exhibit a high abundance of this genus. Therefore, this increase in *Cutibacterium* may not be directly attributable to the transfer. It may instead be due to the extraction process or sequencing. Multiple samples taken at each time point could have confirmed this.

In the panels for D1R2, D1R3, D2R2 and D2R4, the microbiome composition is consistent in the recipients before and after transfer. Whereas D3R2 has an unexpected trend with the presence of *Deinococcus* in the recipient before the transfer but not afterwards, along with an increase in *Haematobacter* post-transfer. Given the low/absence of *Haematobacter* in the donor, this change may be due to disruptions caused by the transfer. These observations, made at the genus level, indicate limited overall compositional changes attributable to the donor suggesting the SMT was unsuccessful.

Figure 116. Visualisation of the top 20 most abundant genera over time (colours) for each recipient. The figure combines 15 grouped bar plots, one per recipient. The plots are organised in rows, grouped by the donor the recipient received the transfer from. Each plot represents the relative abundance of the genera displayed on the y-axis at six-time points on the x-axis: the Donor before the transfer, Recipient before the transfer, immediately post-transfer (Recipient After_0), 6 hours post-transfer, 24 hours post-transfer and 72 hours post-transfer.

5.04.06) Exploratory Differential Abundance Analysis Immediately after transfer

I then used sPLS-DA to compare microbiome composition between donors before transfer (green) and recipients before transfer (red) groups and recipients immediately after transfer (grey) based on beta diversity filtered by adding an offset of 1, removing low counts (<0.01%) and CLR transformed. N=33 samples remained (donors N=4, recipients pre-transfer N=15 and post-transfer N=14). Components 1 (5 %) and 2 (4 %) combined explain 9 % of the differences between the groups, as shown in Figure 117. The centroids for the groups are separated on the 1st and 2nd components, although the 95 % confidence intervals overlap. While the 95 % confidence intervals show some overlap, there seems to be a greater difference in the positioning of the points on the first and second components when comparing donors and recipients. However, both points and confidence intervals overlap when examining the recipient groups before and immediately after the transfer. Despite some visual separation between the groups, there's no statistically significant evidence of a difference in dispersion between the centroids (as confirmed by PERMANOVA, P=0.933).

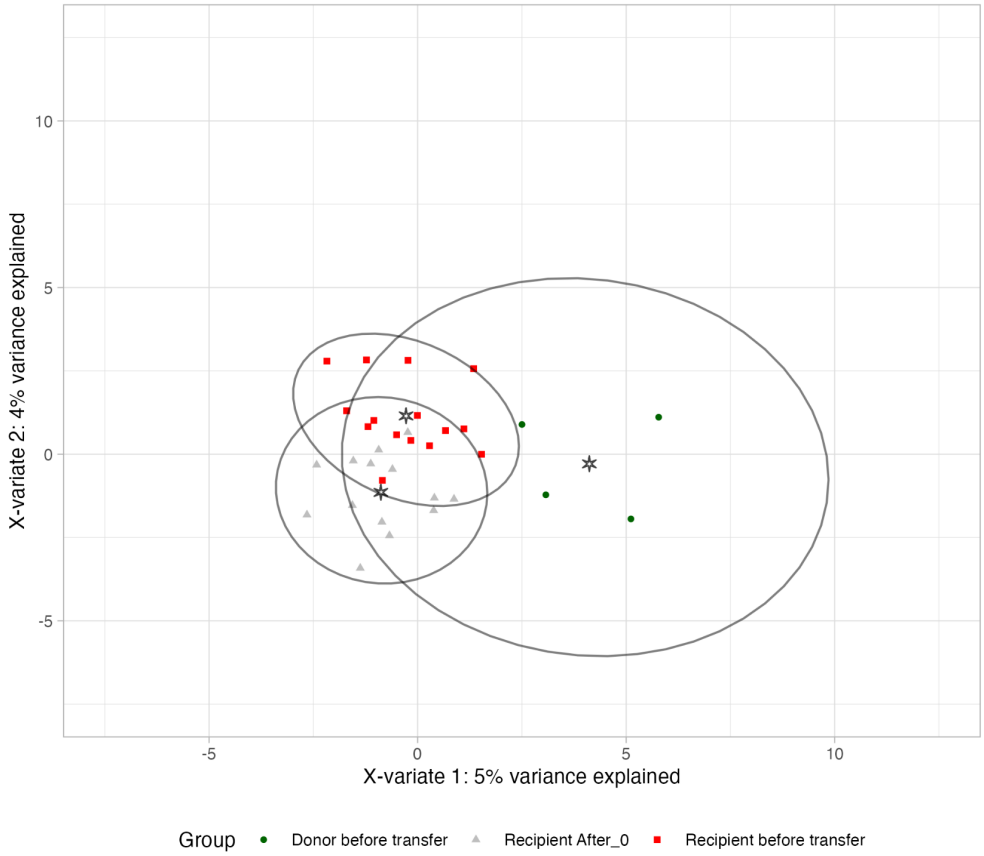

Figure 117. Sample plot visualises differences between the groups from a sparse partial least squares discriminant analysis (sPLS-DA) implemented in the MixOmics package (Rohart et al. 2017). It compares skin microbial composition (beta diversity) between the donors before transfer (green), recipients before transfer (red) and recipients immediately after transfer (light grey). Individuals are presented as small circles (donors), small triangles (recipients after transfer) or small squares (recipients before transfer). Microbiome data were pre-processed by filtering, CLR transformed and scaled (centred and standardised). The centroids, depicted as black stars, represent the average microbiome composition in the two-dimensional space on the first and second components of the analysis for each group. The enclosing ellipses (large circles) represent the 95 % confidence intervals for these groups, providing a visual estimate of the dispersion around the group means. The first component explains 5 % variance, and the second component 4 % variance, summing to a cumulative variance of 9 %.

5.04.07) Bacteria persist in the recipient skin microbiome 24 hours post-transfer

As there was no evidence of a difference in major taxa before and after transfer, I instead investigated if there is any difference in alpha diversity post-transfer that might indicate active transfer.

5.04.08) Contrasting Alpha Diversity: Comparisons Between Recipient pre and post-transfer Groups

The global differences in the skin microbiome longitudinally for recipients were explored. The Shannon diversity for recipients before and 0, 6, 24, and 72 hours after transfer are shown in Figure 118. The diagram shows the median Shannon scores appear to be reduced after transfer. As the data was not normally distributed, a Wilcoxon signed rank test with continuity correction was used to compare the alpha diversity before and after transfer with Benjamini-Hochberg correction for multiple testing. The null hypothesis was the median difference between before and after transfer was zero. There was no statistical evidence of a reduction in Shannon immediately post-transfer (median difference= -1, V=6, Adjusted p-value=0.850). There was no evidence of a reduction in alpha diversity 6 hours post-transfer (median difference= -1, V=10, Adjusted p-value=0.189), 24 hours post-transfer (median difference= -1, V=32, Adjusted p-value=0.167) or 72 hours post-transfer (median difference= -1, V=17.5, Adjusted p-value=0.784). However, this had similar limitations to the attractiveness results as control samples from the other foot were not collected to check that the participant's alpha diversity was stable over time.

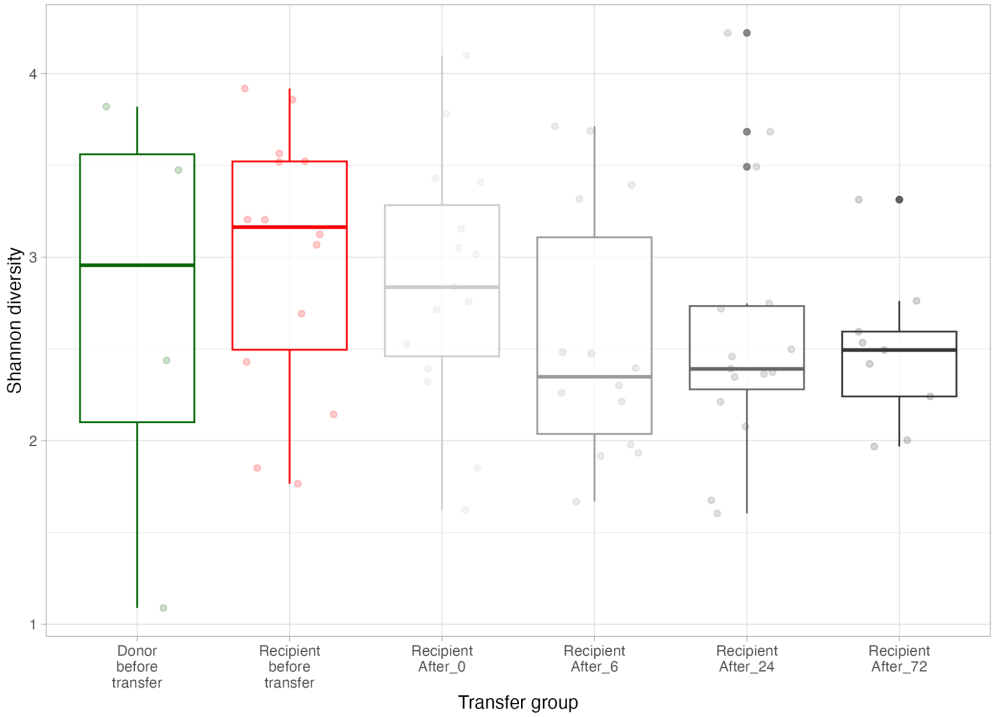

Figure 118. Boxplots with scatter comparing the distribution of alpha diversity measured with Shannon between the recipients before (red) and after the transfer (grey).

5.05) Discussion

5.05.01) Advantages and disadvantages of methods

Study design: 1-to-many vs 1-to-1 approach

In a prior MSc project I supervised, a 1-to-1 donor-recipient transfer design was used, and it appeared major taxa were transferred in some cases from donors to recipients. We could not discern the effectiveness of individual donors from differences between recipients (Potangwa 2021). Therefore, in this chapter, a 1-to-many design was chosen in which a single donor's microbiome was transferred to multiple recipients. The 1-to-many design allowed me to investigate how individual recipients responded to the same microbiome transfer from a consistent donor, emphasising recipient-specific variability. Given that each donor possesses a unique microbiome influenced by lifestyle and genetics, this design aimed to minimise potential confounding variables impacting the success of the transfer within the group of recipients that received a transfer from the same donor. In FMT trials for ulcerative colitis, 78 % of the patients that received FMT from a single donor achieved remission (7 out of 9) [277]. The major drawback of the 1-to-many design used in this chapter was the sample size of donors. By chance, may not have transferred from any good donors in this study. Increasing the sample sizes of the poorly-attractive and highly-attractive groups by screening more people would have been beneficial, but time, mosquitoes and participants available to screen were limited. There were no criteria to investigate the microbiome or metabolite profile of the donor pre-transfer. Donors were solely selected based on a low score on the foot-on-cage test. Randomly selecting donors has the risk of returning false negatives [278]. Improved outcomes for attractiveness might be possible with more strategic donor selection methods, such as identifying specific microbes or metabolites absent in the unattractive group but present in attractive individuals or utilising algorithms to differentiate between optimal and suboptimal donors [278]. Several outstanding questions remain: Can microbes be effectively transferred between unattractive and attractive people? What defines an effective donor in this context? Do unattractive individuals lack specific microbes or metabolites? To answer these questions, more extensive experimental studies with larger sample sizes and further characterisation of donors are needed.

Study design: Direction of transfer and groups

In this study, microbes were transferred solely from unattractive to attractive participants. This decision was primarily driven by a desire to understand if microbial transfers could

potentially decrease the attractiveness of individuals to mosquitoes. Based on the findings from repeat screenings in Chapter 2 and this chapter, it would have been better to conduct the transfers in the opposite direction as attractive individuals were more consistent in their attractiveness to mosquitoes than unattractive individuals. Additionally, it is important to note that in Chapter 2, individuals "unattractive" to mosquitoes rather than actively repelling them were identified. In this chapter, the cage assay was not a choice assay, so we did not compare it to a control, but the attractiveness of the donors was between 15-60 % attractive, indicating that they still attracted some mosquitoes, not that they were actively repellent. For substantial reductions in attractiveness, active repellence would likely be required in the donor or bacteriotherapy needed with specific microbes that produce repellent metabolites. Transferring microbes from attractive donors to unattractive recipients might have resulted in clearer observable behavioural changes in mosquito attraction as high attractive behaviour is more consistent than low attractiveness. For a comprehensive understanding of SMT on human attractiveness to mosquitoes, it would have also been valuable to evaluate the outcomes of microbe transfers between unattractive-to-attractive, unattractive-to-unattractive and attractive-to-attractive participants as well as attractive-to-unattractive. In future studies identifying actively repellent people to mosquitoes or transfers between alternative attractiveness groups would be interesting and may result in effects on human attractiveness to mosquitoes attributable to SMT. Likewise, utilising bacteriotherapy to introduce repellent-producing bacteria or phage therapy to selectively target and eliminate specific microbes producing attractants may result in more substantial effects.

Study design: Controls

On reflection, the experimental design would have been improved by including additional controls. A comparison with a control foot over time would have provided clarity on the efficacy of the treatment by showing the treated foot's relative attractiveness before and after the SMT application compared to the control feet that did not receive SMT. One potential concern was the unintended transfer of microbes from the SMT-treated foot to the control foot. However, this could have been mitigated by having participants wear clean socks and shoes provided by the research team for 72 hours post-transfer. Incorporating controls to study the impact of the socks on individuals not receiving the SMT would have further refined the findings. While the passive transfer of microbes might occur, it's unlikely to produce the same effect as the direct application of the SMT, which involves concentrated microbes left to dry. Minor cross-transfer might have been inconsequential, especially if the SMT yielded pronounced effects on attractiveness on the treated foot compared to the control. For sequencing controls, the Zymo controls, as detailed in Chapter 3, were utilised. In hindsight, an enhancement could have been the inclusion of a rare taxon to the SMT as

an internal control to verify the treatment's efficacy, for example, the spike in controls available from Zymo.

Study design: VOC collection

In Chapter 4, our air entrainment method required 2 hours to collect human odour. Given this duration, it was impractical to assess volatile differences between donors and recipients before and after the transfer, especially when screening numerous individuals at various time intervals throughout the day. Additionally, the GC-FID at LSHTM was inaccessible at the time as it was being used for another project. VOC collection would have provided insight into the duration and immediacy of volatile transfer after the process. Future investigations should determine if SMT influences volatile profiles and discern between passive and active transfers. Additionally, viability assays would have helped ensure the transferred material was alive and quantified the amount of bacteria applied to the recipient's foot.

Study design: Pre-treatment

Recipients were not pre-treated with antibiotics before transfer. However, doing so may have resulted in a more noticeable difference in the major taxa of the microbiome composition by removing some of the competition. Future investigations could compare outcomes with and without pre-treatment. While removing competition can aid the colonisation of transplanted bacteria and improve engraftment, antibiotics could also eliminate beneficial bacteria, potentially disrupting the native microbiome. This approach may not be advisable because attractiveness to mosquitoes is thought to be influenced by changes in bacterial ratios rather than complete removal [202]. Future work should compare with and without pre-treatment with antibiotics.

Study design: Foot-on-cage vs other methods

The foot-on-cage assay was previously developed during an MSc project. The assay was not compared against socks from the same person in a cage set-up as previously used in Chapter 2 in this chapter as *Ae. aegypti* were used instead of *An. coluzzii*. It would be interesting to investigate how it compares to collecting socks and testing attractiveness in a Y-tube. The results in Chapter 2 showed the importance of multiple replicates in ensuring that people are consistently unattractive or attractive to mosquitoes over time. In this chapter, re-screened people before applying the SMT, but more replicates would have reduced the variation, especially in the donors. The foot-on-cage assay enabled the evaluation of participants' foot body odour without subjecting them to mosquito bites. There are several ways it could have been improved. Incorporating video tracking and automated

analysis software could have produced more detailed data on landing and flight behaviour, allowing landings (attraction) and time spent on the individual (arrestment) to be measured. A limitation of the method is that we only collected attractiveness at a point in time, not over time which did not allow a more in-depth analysis of behaviour. The cage assay was not tested with CO_2, despite its known synergistic affect when combined with skin odour [21]. It would have been better to develop a method of blowing CO_2 into the cage to avoid using breath. Although in small cages Gr3 mutant *Aedes*, unable to sense CO_2, had no impairment in host-seeking in a small cage [21]. Additional improvements could have been made to enhance the odour plume by creating a blockage around the frame to guide the odour flow through the foot area. Cage-on-foot might be a better approach for odour transfer to the mosquitoes. A foot-on-cage assay should be further tested and optimised before being used in future experiments if multiple screens of a participant's foot are needed.

5.05.02) Main Discussion

This chapter delved into the feasibility of transferring skin microbiomes from individuals less attractive to *Ae. aegypti* to those more attractive, with the aim of reducing human attractiveness. The findings provide suggestive evidence for a tendency for reduced attractiveness to mosquitoes following skin microbiome transfer from a less attractive donor.

There was some statistical evidence suggesting a decrease in human attractiveness to *Aedes* at 0, 6, and 24 hours after introducing an SMT from a less attractive individual to a more attractive one, without any preliminary treatment. Although, the possibility that the attractiveness might have similarly reduced in the participant's other foot was not tested by comparing against a matched set of controls. Attractiveness to mosquitoes has been shown to be consistent for years. The reduction in attractiveness was minimal: on average, two fewer mosquitoes were attracted immediately after the transfer (out of 20), and 2.5 fewer were attracted 6 hours later (out of 20). This reduction is insufficient to shift recipients from the "attractive" to the "unattractive" category. If the observed statistical reduction was because of the transfer, it might be attributed to the transfer of VOCs or postbiotics rather than the active metabolism of skin substrate by the microbes leading to mVOCs — a factor not assessed in this study. Considering many common bacteria have short generation times of 20-60 minutes under optimum conditions and common skin microbes *Pseudomonas* and *Staphylococcus* have been shown to have doubling times of around 2 hours in the wild [279], active metabolism of microbes may not have occurred until 6 hours. There are several

outstanding questions: can SMT be applied without pre-treatment and change the major taxa, are VOCs successfully transferred using this method, and are the behavioural assay sensitive enough to detect minor shifts in attractiveness? Future studies need to incorporate additional controls and metabolomics analysis alongside 16S data for a clearer evaluation of SMT impact on attractiveness to mosquitoes.

There were differences in the skin microbiome composition between the less attractive “donors” and more attractive “recipients” before the microbiome transfer. Despite using a different mosquito species, *Aedes,* instead of *Anopheles* (studied in Chapter 3). There was a difference in skin microbiome composition between low and high-attractive individuals. For the first time, I have demonstrated that differences in the skin microbiome composition are associated with the level of attractiveness to *Aedes* mosquitoes. Similar findings were reported previously for *Anopheles* [137,209]. Prior research also revealed that changing the ratios of *Staphylococcus epidermidis, Pseudomonas aeruginosa, Corynebacterium minutissimum,* and *Brevibacterium epidermidis* selected based on their attractiveness to *Anopheles* can reduce attractiveness to *Aedes [202]*. Therefore, anticipated the observed differences in the microbiome composition of the two groups before the transfer. *Aedes* are of particular interest in developing new mosquito repellents as they are daytime outdoor biters.

Immediately post-transfer, there is no conclusive shift in recipients' microbiome composition post-transfer moving closer to donors. The recipient's profiles stay pretty much the same after the transfer, and they cannot be separated from the recipient before transfer on the sPLS-DA. No changes in major taxa appear to be due to the SMT. Therefore, did not further investigate over time as, at the gross level, there was no impact of the transfer on the microbiome of the recipient. The absence of longitudinal controls to compare temporal and sample-to-sample variation leads to a limited ability to investigate ASV transfer for lower abundant taxa transfers. Overall, it did not appear that there was an active transfer, an issue a previous study also struggled to prove [272]. Future investigations should modify the approach, potentially including pre-treatment with antibiotics, using PBS buffer, including viability checks and additional controls.

The study would have been strengthened by including additional transfers from highly attractive to poorly attractive participants and within the same attractiveness group (poorly-attractive to poorly-attractive and highly-attractive to highly-attractive). Furthermore, inactive transfers (e.g. heat treated SMT) would have helped to validate further the impact of the transfer of skin microbiome on mosquito attractiveness. To distinguish between VOC

transfers and microbiota-produced mVOC transfers (i.e., active transfer leading to the production of mVOCs impacting mosquito response), air entrainment would need to be performed before and after transfers. Including longitudinal controls from participants who did not receive a transfer could have also facilitated distinguishing whether observed differences were due to natural variation or transfer effects. Multiple collections at each time point would have further supported distinguishing these effects and collecting samples over time before the transfer. Moreover, repeated foot-on-cage screening would have increased the accuracy of attractiveness measurement. Using a pooled sample from multiple donors could have been an option [280]. This study has focused on bacteria; it has not considered other microbiome components like fungi and phage's, despite the high diversity of fungi on the feet [281]. Other variables, such as genetics and lifestyle, may influence the success of microbiome transfer but were not investigated. Additional criteria for an ideal low-attractive donor may need to be refined to select better donors in future studies.

The success of SMT in altering mosquito attraction may be linked to bacterial quorum sensing on the skin. If the skin microbiota from a less attractive individual to mosquitoes exhibits a distinct microbial composition, particularly a reduced response to quorum sensing signals, this could be an advantage as knocking out the arg gene needed for quorum sensing has been shown to reduce attractiveness of *Staphylococcus epidermis* to *Aedes aegypti [204]*. Transplanting the skin microbiome could modify the skin's quorum sensing molecular profile, leading to the production of different compounds that are less appealing to mosquitoes. Moreover, the transplanted bacteria could carry quorum sensing inhibitors, disrupting the established communication system of the recipient's skin microbiome. This disruption might also affect biofilm formation, altering the skin's microenvironment in a way that reduces its attractiveness to mosquitoes. A comprehensive understanding of the bacterial species on the skin, their quorum sensing mechanisms, and the emitted signals could be used to leverage the potential of SMT for reducing mosquito attraction. Such knowledge could inform targeted interventions that selectively diminish the emission of mosquito-attracting signals, while preserving the native skin microbiome.

Several strategies could potentially enhance the efficacy of the SMT. Providing recipients with new socks and shoes post-SMT is advisable, as their pre-existing footwear likely harbours bacteria and odours that could negatively affect the outcome of the SMT. It is recommended to choose cotton socks over nylon since cotton is less conducive to bacterial proliferation. Thoroughly cleaning the recipient's foot using ethanol, antibiotics, or another disinfectant prior to the procedure could also improve results by reducing the initial microbial burden allowing the SMT to proliferate. The foot-on-cage assay could have been further

improved with video tracking and modifications previously discussed. Additionally, assessing the microbial load of the donor's sample before the SMT would provide valuable baseline data on the actual amount of microbes being applied. Conducting a larger-scale study with an increased number of microbiome transfers would further validate the findings and enhance the robustness of the research.

In conclusion, this chapter explored applying skin microbiome transfers from donors with low attractiveness to mosquitoes to recipients with high attractiveness and assessed the subsequent impact on the attractiveness to *Aedes aegypti* mosquitoes. There are differences in microbial composition and attractiveness to mosquitoes between the donors and recipients before transfer. There is some statistical evidence of a trend for reduced attractiveness to mosquitoes in recipients post-transfer. However, no significant alterations were detected in major taxa or overall microbiome composition post-transfer.

5.06) Supplementary

5.06.01) Microbiome pre-processing

Figure 119 shows the library size (number of reads) for each of the samples (blue dots), negative controls (red dots) and positive controls (green dots). Most of the negative controls have library sizes <50,000. We would expect the negative controls to have fewer reads than most of the samples (i.e. appear bottom left). Two out of the six negative controls have more reads than expected.

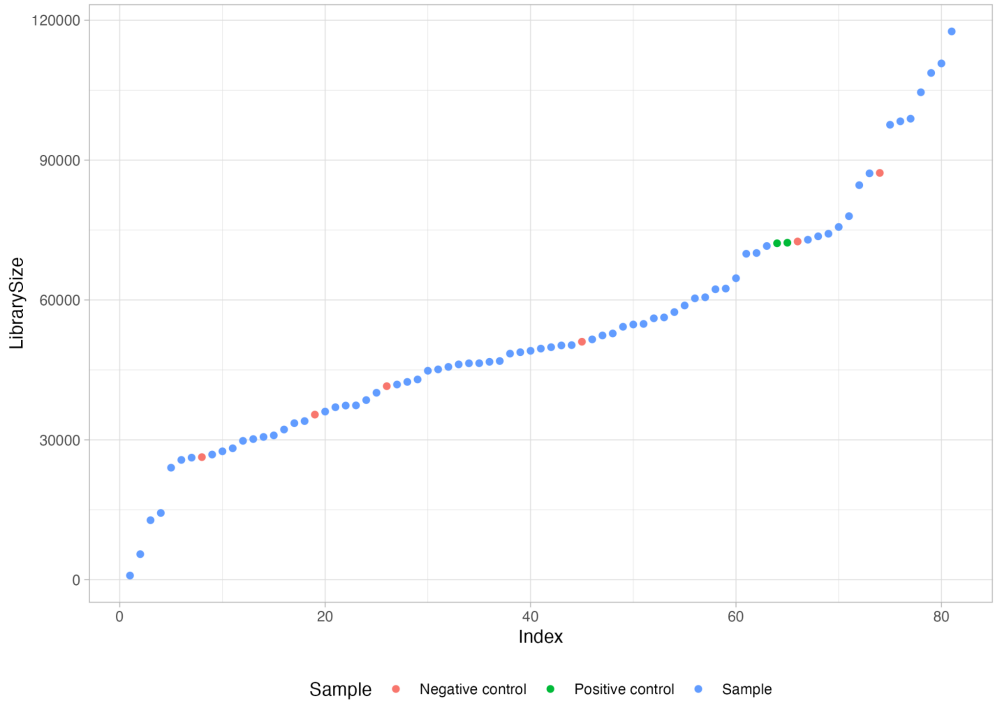

Figure 119. The library size for the samples (blue points), the negative controls (red points) and the positive controls (green points) varied.

Figure 120 shows a taxa bar plot comparing the average relative abundances at the Genus level between positive controls, negative controls and samples. There are clear differences in the relative abundances of classes between the control and samples. The samples are filtered to the top 20 most abundant taxa. Low_abundance (grey) is a combination of low abundant taxa not in the top 20 taxa. The proportion of low abundant taxa present in the samples varies between samples.

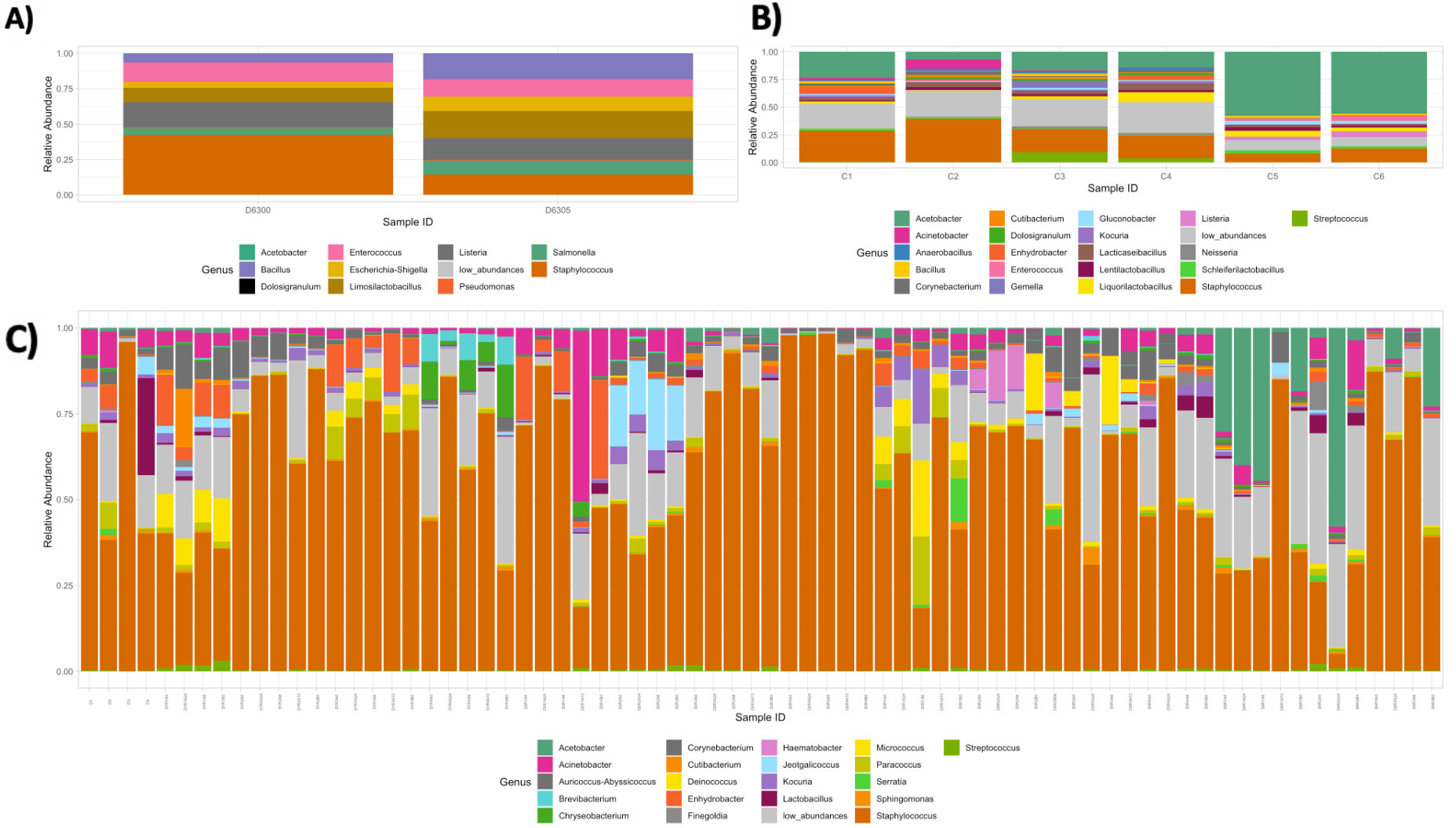

Figure 120. Summarises the taxonomy at the genus level as a taxa bar plot of relative abundances for a) positive controls, b) negative controls, and c) samples (ordered with donors on the left and recipients on the right by donor). There are clear differences in taxonomy between the samples and controls. Samples are a subset of the 20 most abundant taxa at the genera level, low_abundances is the less abundant taxa combined.

Decontam was used to remove contaminants found in the negative controls (blank swabs) [248]. A strict prevalence cut-off of 0.5 was used. 288/8320 ASVs were identified as contaminants and removed. Figure 121 compares the taxa bar plots at the genus level for 20 randomly selected samples before and after decontam, which look very similar pre and post-decontam; few highly abundant taxa have been removed. *Haematobacter* genus is removed during decontam and filtering.

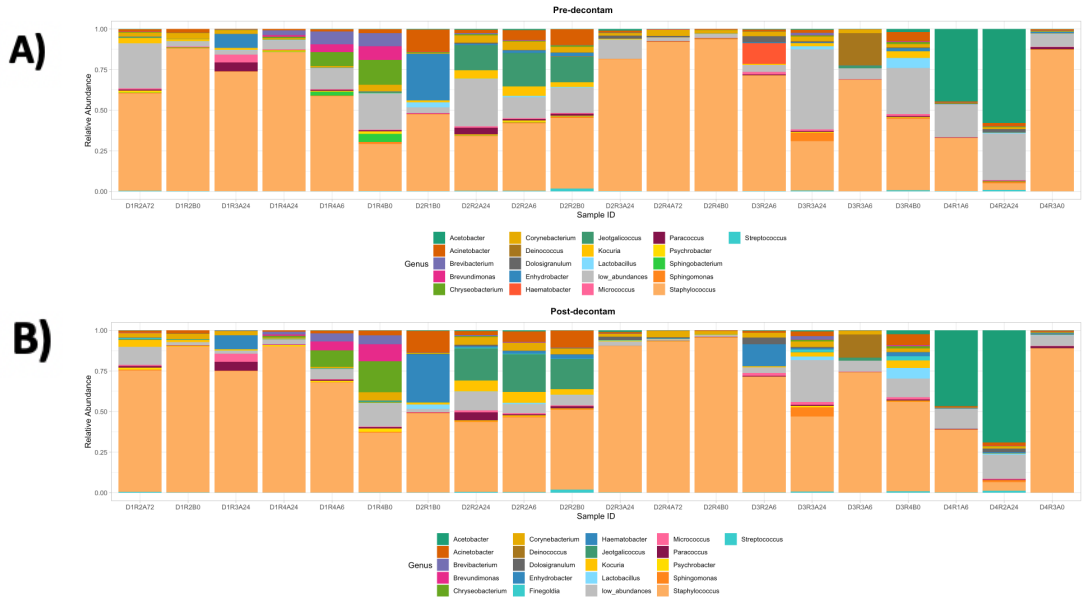

Figure 121. Comparison of 20 samples randomly selected a) Pre-decontam and b) Post-decontam. Shows the top 20 genera relative abundances grey is used for the other taxa not in the 20 most abundant.

Samples with fewer than 10 reads were filtered out. All 79 samples remained. ASVs present in one sample or less than 10 % of samples were removed. 550 ASVs remained.

Positive controls

The composition of positive controls is shown in Figure 122A as a taxa barplot. Positive controls are processed with the samples except for being excluded from the filtering step, which removes low abundance taxa not present in more than 10 % of samples, as some of the bacteria in the mock communities are not present in the samples.

Table 24 summarises the expected and actual relative abundances of the taxa in D6305 (DNA control) and D6300 (extraction control), which are expected to have equal amounts of *Pseudomonas, Eschericia, Salmonella, Lactobacillus, Enterococcus, Staphylococcus, Listeria* and *Bacillus* (12 % of each). It also includes a smaller amount (2 %) of *Saccharomyces* and *Cryptococcus,* which 16S primers cannot detect. In D6305 and D6300, there is an overabundance in the control of *Staphylococcus* and *Listeria. Staphylococcus* is

particularly overabundant in D6305. *Pseudomonas* is not present in either, which was a surprising result and suggested an issue in the sequencing of *Pseudomonas* in this run. *Eschericia* and *Salmonella* are less abundant than expected in D6305, only slightly lower than expected in D6300. *Lactobacillus, Enterococcus* are similar to expected, *Lactobacillus* is slightly more abundant than expected in D6300. *Bacillus* are slightly more abundant than expected in D6300 and lower than expected in D6305. All genera expected to be present are identifiable in both the DNA control and the extraction control, except for *Pseudomonas.*

Table 24. Summary of the expected and actual relative abundances as percentages for D6305 (DNA control) and D6300 (extraction control) for each of the genera in the positive controls.

Taxa	**Expected %**	**D6305 Actual %**	**D6300 Actual %**
Pseudomonas aeruginosa	12	0.00	0.00
Eschericia coli	12	4.16	10.28
Salmonella enterica	12	4.48	8.78
Lactobacillus fermentum	12	10.13	19.62
Enterococcus faecalis	12	13.99	12.54
Staphylococcus aureus	12	42.72	14.66
Listeria monocytogenes	12	18.06	15.53
Bacillus subtilis	12	6.45	18.60
Saccharomyces cerevisiae	2	0.00	0.00
Cryptococcus neoformans	2	0.00	0.00

6) Chapter 6: Overall discussion

Overall Discussion

Previous research has revealed that differences in the skin microbiome composition have a role in natural variation in human attractiveness to mosquitoes. In this thesis, further substantiate these findings by presenting additional evidence that indicates there is a difference in the skin microbiome composition between attractive and unattractive people to mosquitoes (Chapter 3). However, do not find evidence of differences in volatile profiles between the attractiveness groups (Chapter 4) or attractiveness to mosquitoes being strongly determined by human genetics (Chapter 2). Venturing beyond previous research, performed skin microbiome transfers (SMTs) between individuals unattractive and attractive to mosquitoes (Chapter 5).

6.01) Data synthesis, limitations and future opportunities

The findings presented in this thesis highlight several potential areas for future research in the relationship between the human skin microbiome, volatile odour profiles, and mosquito attractiveness.

Chapter 2: Differences in attractiveness to mosquitoes and the role of human genetics

Chapter 2's findings revealed variation in human attractiveness to *Anopheles coluzzii* across two cohorts, a UK cohort of female twins and a Gambian cohort of same-sex twins. Two behavioural assays were employed when assessing human attractiveness to mosquitoes: the UK cohort was evaluated using a two-choice olfactometer, and the Gambian cohort was assessed using a cage assay. Previous research has shown strong evidence that humans have consistent attractiveness to mosquitoes over time [16,141,142]. In this study, there was not a consistently low attractive group. Instead, when compared to a negative control (unworn sock), we found a group that was consistently attractive and a second group that was inconsistently attractive and which we classified as "unattractive". Attractiveness data was divided into these two groups for comparison rather than contrasting the extreme ends of attractive individuals. Whether individuals at the extreme of unattractiveness are truly "repellent" to mosquitoes remains an outstanding question. In Chapter 2, there was no evidence of the existence of a repellent group, but neither the two-choice olfactometer nor cage assays are suitable for testing repellent behaviour. Alternative assays have been developed that can test this behaviour [96,282–284] and could have been employed to determine whether any of the individuals in our cohorts actively repelled

mosquitoes. If people who are naturally repellent are identified, their skin microbiome and volatile profile should be characterised.

It is debated whether low-attractive people emit VOCs that mask attractive VOCs or actively repel mosquitoes [18]. Logan et al. (2008) found some evidence of increased amounts of some compounds in people unattractive to *Aedes aegypti* and suggested that these may be contextual repellents [25], and De Obaldia (2022) showed that low-attractive people produce less attractive carboxylic acids than highly attractive people [16]. Although they did note that they found one participant who, even though they had high carboxylic acids was poorly attractive, they did not investigate if this participant was producing a “mask” or repellent [16]. If we can understand the mechanisms that make some people less attractive to mosquitoes, we could identify new repellents.

The use of a two-choice olfactometer, a common method of choice assay, limits throughput. This is due to the necessity to equalise wind speeds between the two trap entrances and the run time of 20 minutes, which allows sufficient flight time. A higher throughput assay could have been developed while still testing mosquito choice. De Obaldia (2022) introduced an alternative choice assay that had a shorter assay time (5 minutes) and shorter length of approximately 0.91 m compared to 2 m, with the traps being nearer to the release chamber [16]. This design separated activation and choice behaviour (or attraction) more rapidly [16]. Another possibility is to develop a tube assay where mosquitoes are introduced from the centre, and the odour source emanates from both ends. The dimensions of this cylinder would need to be adjusted according to the chosen mosquito's flight behaviour. The tunnel material should also be odour-neutral, and odour releases should be combined with humidified air and supplemented with CO_2. However, such an assay would not gauge the activation phases that long-range flight attraction in a wind tunnel can measure. In summary, while the two-choice olfactometer has its limitations, and alternative methods proposed by researchers like De Obaldia offer more efficient designs, it is essential to consider factors such as the behaviours being tested and the specific requirements of the assay setup when designing the optimal mosquito choice test.

Chapter 2 primarily sought to test the hypothesis that there is no difference in the level of attractiveness to mosquitoes between MZ twins, DZ twins, and unrelated individuals. If genetics played a significant role in determining mosquito attractiveness, we would anticipate MZ twins to exhibit a higher similarity in attractiveness to mosquitoes than DZ twins. Prior research had indicated that between 62 % and 67 % of the variability in attractiveness to *Aedes aegypti* is attributable to genetics [179]. However, the results of

Chapter 2 did not support such high levels of genetic impacts on human attractiveness to *Anopheles coluzzii* mosquitoes. The reported heritability from this chapter implies that only between 13.4 % and 18.5 % of the variation in human attractiveness to mosquitoes is attributable to genetics. The wide confidence intervals gave uncertainty in these estimates. The majority of the observed variation in human attractiveness to mosquitoes can be attributed to non-genetic determinants. It is important to note that there were differences in the mosquito species, behavioural assays and participants in our experiment and those of Fernandez-Grandons. It is possible that specific rare SNPs that influence human attractiveness to mosquitoes were not identified in this study; pinpointing such SNPs would necessitate much larger numbers of participants. From the findings in Chapter 2, it is evident that future research should not prioritise genetic factors as the main contributors to mosquito attractiveness. Uncovering rare SNPs would necessitate sampling thousands of participants for a genome-wide association study (GWAS) to achieve even modest power. In summary, the evidence from Chapter 2 suggests that human attractiveness to mosquitoes is predominantly influenced by non-genetic factors, suggesting genetics play less of a role than previously reported.

Chapter 3: Microbiome

The focus of Chapter 3 was to investigate if there are differences in skin microbiome composition between attractive and unattractive groups. The results in this chapter confirm previous findings using similar molecular characterisation and analysis that there are differences in the skin microbiome composition between the attractive and unattractive groups [137]. Future research is needed to show that skin microbiome composition can be used to predict attractiveness to mosquitoes. To do this, the attractiveness to mosquitoes of a large, diverse cohort should be evaluated using bioassays, after which they should be categorised into 'attractive' and 'unattractive' groups. Concurrently, skin microbiome samples should be collected and processed from these participants. The microbiome data should then be split into training and testing datasets. The training set should be used to identify features of the attractive and unattractive groups. Finally, the model's efficacy in categorising participants based on their attractiveness should be assessed by comparing the predictions with the actual groupings from the attractiveness data. If microbiome was a predictor of attractiveness to mosquitoes, ASVs could be identified that are diagnostic of attractiveness, and it may be possible to develop a rapid diagnostic test. This could be useful to identify the individuals that are expected to receive the most bites and drive the transmission of VBDs [285]. These individuals selected based on microbiome profile could

potentially become the focus of vector control in the future to prevent them from getting VBDs.

In addition to determining differences in the skin microbiome between groups, identified differentially abundant ASVs in the attractive and unattractive groups. Employed two methodologies: the widely-used DEseq2 for differential abundance analysis and corncob which is tailored for microbiome data [236]. Analysing two twin cohorts using this combined approach increased the confidence in the identified differences. Many of the differential abundance results are inconsistent across the methods and cohorts, but they do indicate genera that could be further investigated in attractiveness to mosquitoes, some of which support the findings of Verhulst et al. (2011) [137] and experimental findings that have tested microbes in culture [23,205]. If future studies investigating attractiveness to mosquitoes are able to find larger groups of people that are consistently attractive and unattractive, it would be valuable to compare their microbiome profiles and see if there is a consistent profile of a highly attractive person with culture and molecular methods. This information would further validate the ratios tested by Lucas-Barbosa et al. (2023) in culture [202]. Consistent taxa may exist in the attractive and unattractive groups, which may go beyond the top four most abundant taxa previously indicated: *Brevibacterium epidermidis*, *Corynebacterium minutissimum*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis [23,137,202]*. In Chapter 3, *Staphylococcus* was the most abundant taxa, *Corynebacterium* consistently high abundant but *Pseudomonas* and *Brevibacterium,* although identified were not consistently in the top 10. Additionally, attractive individuals may have other distinct microbial variations, particularly associated with a high presence of *Staphylococcus*, which remains unidentified. Could there be factors other than *Pseudomonas* influencing the perceived unattractiveness in others? In Chapter 3, showed differentially abundant ASVs in both directions belonging to *Staphylococcus*. Future studies should test strains and species within this common genus to see how they impact attractiveness, as previous work with mosquitoes has largely focused on investigating differences between species belonging to different genera, Michalet et al showed differences in attractiveness and the volatile profiles of *Staphylococcus saprophyticus* and *Staphylococcus hominis* [241]. Species or strain-specific differences should be investigated in more depth experimentally through behavioural assays and volatile analysis. Some research indicates metabolic pathways and products can be conserved within a genus [286,287], while a recent study showed a difference in VOC profiles between *Staphylococcus* that impacted horsefly behaviour [89]. Further understanding of the differences between species for mosquitoes may untangle the apparently conflicting differentially abundant ASVs from the same genera identified in Chapter 3.

Alternative methods could be applied to characterise the skin microbiome beyond the 16S sequencing approach used in Chapters 3 and 5, which is proficient at identifying a broad profile of microbial communities and providing taxonomic identifications up to the genus level. For future investigations seeking a deeper, more comprehensive understanding, shotgun metagenomic sequencing is recommended. Unlike 16S rRNA sequencing, which targets a specific gene, this method covers the entirety of microbial genomes. This allows for strain-level resolution and information on fungi, which may be a missing component in the investigation of skin microbiomes in attractiveness to mosquitoes that have not received attention. Shotgun metagenomics also provides direct insights into metabolic functions through the presence of genes predicting enzymes involved in the production of VOCs [288–290]. There have been advances in the application of nanopore sequencing to skin samples, both targeted to the 16S region [291–293] and full shotgun sequencing [294]. The low biomass of skin samples complicates the extraction of sufficient microbial DNA but new protocols have optimised lysis to produce yields compatible with shotgun sequencing [294].

Recent research has highlighted concerns with sequencing-based methods; they overrepresent non-viable microbes on the skin [295]. Both 16S and shotgun sequencing detect bacterial DNA but cannot discern between live and dead bacteria. As such, they may not accurately represent microbes actively involved in VOC production, potentially skewing our understanding. An alternative is to culture microbes extracted from the skin, but this is also not without bias because it selects those taxa that are culturable. Although innovation in culturing, termed culturomics, has led to new culturing methods, especially for gut microbes, including Yeast extract, casitone and fatty acid (YCFA) media and co-culture techniques that have led to new species being cultured and identification of unknown ASVs in metagenomic studies [296,297]. Skin-microbiome techniques that encourage the growth of a core microbiome could be used to culture additional microbes through cross-feeding and shared resources but would lead to complex VOC profiles. Other options include cutting-edge methodologies like strain-level profiling of viable microbial communities by selective single-cell genome sequencing, which focuses on sequencing the genomes of only the living bacteria in a community, ignoring those that are dead [298]. There are many methods available to further investigate the role of the skin microbiome in human attractiveness to mosquitoes. The choice of method should depend on the questions to be answered.

Chapter 4: Volatiles

In Chapter 4, focused on comparing the volatile profiles of people's feet between attractiveness groups to see if there were qualitative or quantitative differences between groups. The specific components that are most relevant to mosquito attraction to humans and choice to bite are not fully understood, and no single compound has been identified that explains attractiveness to mosquitoes. An individual's attractiveness is based on blends and concentrations of odours. In this chapter, found no evidence of differences in VOC profiles between the groups. Past research has shown that highly attractive people produce more attractive carboxylic acids [16,299]. However, the methods used by De Obaldia et al. (2022) were designed to enrich highly polar acids that were not particularly volatile. Studies by Logan et al. have suggested that unattractive people have higher amounts of 6-methyl-5-hepten-2-one, geranylacetone, nonanal, and decanal [25,93], although this has not been confirmed in other studies [16,101]. Verhulst et al. found overlapping volatile profiles in their *Anopheles* study [101]. The discrepancies between studies could be attributable to the various methods used to quantify these compounds. There remains an interest in further uncovering what makes some people unattractive to mosquitoes. It would be interesting to pinpoint the specific compounds involved and decipher their mode of action, e.g. masking attractive odour or actually being repellent. With regard to attractiveness, blends of VOCs have been developed to attract mosquitoes into traps, but their effectiveness in the field is limited [136,300]. The existing attractant mixtures fall short in replicating human allure, with poor effectiveness under field conditions [88]. Specifically, the MB5 blend, which comprises ammonia, lactic acid, tetradecanoic acid, 3-methyl-1-butanol, and butan-1-amine, managed to capture less than 1 % of the released mosquitoes in field tests, as documented by Njoroge et al. (2021) [88]. If the attraction mechanism is entirely odour based, then there must be a compound missing, inadequately presented or in the wrong ratio with current methods that attempt to mimic human odour. A key gap is some of the compounds that have been detected remain unidentified, databases are not comprehensive, and some compounds do not have commercial standards. More comprehensive studies are needed with a large sample of diverse cohorts and appropriate methods to fully understand inter-individual variation in human body odour emanating from the skin.

Our method used in Chapter 4 had previously been optimised for semi-volatiles, though there are several areas for improvement. First, the collection method required a 2-hour air entrainment. In contrast, methods such as passive sampling or SPME make collecting samples in triplicate or quadruplicate practical [250,301]. While we utilised porapak for collection, there are other methods suitable for more volatile compounds, like thermal

desorption. Column selection is pivotal because the range of compounds that can be studied varies with the column's properties. For examining more polar compounds such as carboxylic acids, a more polar column would be preferable over the non-polar HP1 column that was used in Chapter 4. Collecting data over several days could isolate day-to-day variations, thereby highlighting the biologically relevant differences. There is also potential in targeted enrichment, for instance, of carboxylic acids, which have been found to be more prevalent in individuals perceived as attractive. GC-MS allows compounds to be tentatively identified, and two-dimensional GC-MS can lead to enhanced peak separation [91]. Moreover, advanced classification software options are becoming available, such as explainable artificial intelligence that relies on differences in the relative abundance of microbes (Carrieri et al., 2020).

Recent ground-breaking research in mice has integrated aspects of arbovirus infection, microbiome analysis, metabolomics, and mosquito behaviour to delve deeper into the mechanisms by which infection can alter the microbiome and subsequently modify the odour profile [174]. To further elucidate the mechanisms of skin microorganisms in producing microbial VOCs, an alternative approach would be to focus on capturing the VOCs emitted by bacteria rather than directly sampling human skin. While other researchers have used this approach (Verhulst et al. 2010), they primarily focus on commercially available microbial strains rather than selectively culturing strains from the skin. Collecting samples from skin microbes in culture will have fewer external influences, such as no environmental contamination from using soap. A headspace analysis of odour profiles is needed to identify variations between species within a single genus. This has implications, given the differential abundance of ASVs in both directions belonging to the same genera identified in Chapter 3. Such an approach could decipher whether strain-level differences significantly influence VOC profiles, which in turn affects mosquito attraction. Additionally, there is a need for improved skin microbiome models that can closely mimic real skin conditions without confounding variables [302]. This would enable testing, for instance, to determine if applying certain microbes affects attractiveness or to understand biological pathways by introducing precursors influence on VOC profiles and attraction to mosquitoes. Refining VOC collection for microbial VOCs and developing skin microbiome models will support further deciphering of the role of volatiles in human attractiveness to mosquitoes.

Chapter 5: Skin microbiome transfers as a novel tool to reduce attractiveness to mosquitoes

In Chapter 5, switched focus to *Aedes aegypti.* Investigated differences in microbiome composition between low and high-attractive groups with different mosquito species and assays (foot-on-cage). The result confirmed the previous findings of Verhulst et al. (2011) and Chapter 3 with *Anopheles*. There are differences in microbiome composition between the groups.

I conducted SMTs from the feet of "unattractive" donors to "attractive" recipients without any pre-treatment. Initial findings gave some statistical evidence of a small reduction in attractiveness 0-24 hours post-transfer, but there is no evidence of successful microbial transfer. For future research, it would be worthwhile to compare outcomes of antibiotic pre-treatment to those without it, examining if disruptive pre-treatment is essential before SMT application. Although Perin et al. (2019) reported success without pre-treatment, they could not confirm active transfers [272]. Integrating viability checks could offer insights into the quantity and activity of the transferred microbes. The foot-on-cage assay introduced in this chapter offers a faster screening alternative to the two-choice olfactometer and some evidence of consistency between two replicates. However, to fully harness this high-throughput assay in the future, it needs further refinement and should be benchmarked against other methodologies.

Although the goal of SMTs is to reduce the attractiveness of individuals to mosquitoes, to gain a comprehensive understanding of how modifications to the skin microbiome influence attractiveness to mosquitoes, transfers among various groups are needed: from unattractive to attractive, attractive to unattractive, attractive to attractive, and unattractive to unattractive. Including heat-treated SMTs would provide insight into differences between non-active microbial transfer. If transfers were made from attractive to less attractive individuals, we might have observed a more pronounced behavioural shift of the mosquitoes. For a more accurate control over time, future researchers should collect samples from a control foot at each time point rather than only prior to the transfer. Future SMT studies could also be more strategic by focusing on particular bacteria (bacteriotherapy), such as the *Staphylococcus epidermidis*, which is thought to be associated with increased attractiveness [137]. Methods like SMT are the first step towards microbiome editing. Integrating VOC analysis could be invaluable in determining whether there is VOC transfer alongside microbial transfer post-SMT.

6.02) Conclusion

In conclusion, this thesis has shown that: 1) host genetic factors do not explain as much of the variation in attractiveness to *Anopheles coluzzii* as previously suggested, and 2) the skin microbiome composition is partially responsible for differences in human attractiveness to *Anopheles coluzzii* and *Aedes aegypti*. A key strength is the largely consistent findings across the two cohorts and improved power by including all twins sampled. However, the methodology used to determine attractiveness groups is a notable limitation with few replicates and the use of GC-FID for odour analysis. Future studies on how the skin microbiome affects mosquito preferences could provide a deeper understanding of vector-host interactions. This knowledge might lead to innovative repellent strategies targeting vector-borne diseases.

6.03) Future of the field

The skin microbiome field is poised for significant advancements. I envision many products incorporating beneficial microbes or microbial actives coming to market for skin conditions (eczema, psoriasis, dandruff), wound healing and cosmetics. There have been several interesting acquisitions of microbiome companies like S-biomedic (acne treatment) and Galinee (cosmetics) this year (2023) which have brought attention to the skin microbiome field. Public awareness of the skin microbiome is growing, yet it significantly lags behind the gut microbiome.

There is potential for the development of next-generation mosquito repellents for travellers seeking extended protection against bites. Such products may contain live microbes that produce repellent volatile compounds or prebiotics designed to modify the individuals native microbiome, thereby creating a natural repellent effect. Additionally, the use of bacteriophages to selectively reduce abundance of bacteria like *Staphylococcus*, known to attract mosquitoes, may also emerge.

A long-lasting mosquito repellent may have potential in humanitarian situations where it is difficult to deliver vaccines or bed nets. Repellents offer individual protection not community so unlikely to be included in integrated control strategies. A transformative development would be a cost-effective microbiome manipulation, such as an orally administered product with prolonged impact, offering months of protection, which could be integrated into vector control if it could be delivered at a price competitive with bed-nets.

To effectively prevent the spread of vector-carrying mosquitoes, integrated vector management remains the best strategy. This approach combines environmental management and modification, such as larval source management, with chemical, biological, and physical control methods. The development and deployment of new technologies originating from within malaria-endemic regions, particularly Africa, could provide sustainable and culturally adapted solutions. These strategies should focus on long-term efficacy and affordability rather than relying solely on new insecticides, which we know lead to resistance developing.

Furthermore, the promising developments in malaria vaccines offer hope for complementary control methods that do not solely rely on the elimination of mosquitoes but rather on reducing the transmission of the malaria parasite. The integration of such vaccines into comprehensive control programs could mark a new era in the fight against this disease.

7) References

[1] WHO. Vector-borne diseases 2020. https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases (accessed December 26, 2022).

[2] Breedlove B. Deadly, Dangerous, and Decorative Creatures. Emerg Infect Dis 2022;28:495. https://doi.org/10.3201/eid2802.AC2802.

[3] WHO. Global vector control response 2017–2030 2017. https://www.who.int/publications/i/item/9789241512978 (accessed January 3, 2023).

[4] Garcia LS. Malaria. Clin Lab Med 2010;30:93–129. https://doi.org/10.1016/j.cll.2009.10.001.

[5] World Health Organization. World malaria report 2022 2022. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022 (accessed August 1, 2023).

[6] Ryan SJ, Carlson CJ, Mordecai EA, Johnson LR. Global expansion and redistribution of Aedes-borne virus transmission risk with climate change. PLoS Negl Trop Dis 2019;13:e0007213. https://doi.org/10.1371/journal.pntd.0007213.

[7] Foster WA, Takken W. Nectar-related vs. human-related volatiles: behavioural response and choice by female and male Anopheles gambiae (Diptera: Culicidae) between emergence and first feeding. Bull Entomol Res 2004;94:145–57. https://doi.org/10.1079/ber2003288.

[8] Paris V, Hardy C, Hoffmann AA, Ross PA. How often are male mosquitoes attracted to humans? bioRxiv 2023:2023.03.08.531798. https://doi.org/10.1101/2023.03.08.531798.

[9] Wooding M, Naudé Y, Rohwer E, Bouwer M. Controlling mosquitoes with semiochemicals: A review. Parasites and Vectors 2020;13:1–20. https://doi.org/10.1186/s13071-020-3960-3.

[10] Guidobaldi F, May-Concha IJ, Guerenstein PG. Morphology and physiology of the olfactory system of blood-feeding insects. J Physiol Paris 2014;108:96–111. https://doi.org/10.1016/j.jphysparis.2014.04.006.

[11] Grant AJ, Wigton BE, Aghajanian JG, O'Connell RJ. Electrophysiological responses of receptor neurons in mosquito maxillary palp sensilla to carbon dioxide. J Comp Physiol A 1995;177:389–96. https://doi.org/10.1007/BF00187475.

[12] Pitts RJ, Derryberry SL, Zhang Z, Zwiebel LJ. Variant Ionotropic Receptors in the Malaria Vector Mosquito Anopheles gambiae Tuned to Amines and Carboxylic Acids. Sci Rep 2017;7:40297. https://doi.org/10.1038/srep40297.

[13] Carey AF, Wang G, Su C-Y, Zwiebel LJ, Carlson JR. Odorant reception in the malaria mosquito Anopheles gambiae. Nature 2010;464:66–71. https://doi.org/10.1038/nature08834.

[14] Younger MA, Herre M, Goldman OV, Lu T-C, Caballero-Vidal G, Qi Y, et al. Non-Canonical Odor Coding in the Mosquito. bioRxiv 2022;185:2020.11.07.368720. https://doi.org/10.1016/j.cell.2022.07.024.

[15] Konopka JK, Task D, Afify A, Raji J, Deibel K, Maguire S, et al. Olfaction in Anopheles mosquitoes. Chem Senses 2021:1–10. https://doi.org/10.1093/chemse/bjab021.

[16] De Obaldia ME, Morita T, Dedmon LC, Boehmler DJ, Jiang CS, Zeledon EV, et al. Differential mosquito attraction to humans is associated with skin-derived carboxylic acid levels. Cell 2022;185:4099–116.e13. https://doi.org/10.1016/j.cell.2022.09.034.

[17] Vale GA. The flight of tsetse flies (Diptera : Glossinidae) to and from a stationary ox. Bull Entomol Res 1977;67:297–303. https://doi.org/10.1017/S0007485300011111.

[18] Costantini C, Birkett MA, Gibson G, Ziesmann J, Sagnon N 'f, Mohammed HA, et al. Electroantennogram and behavioural responses of the malaria vector Anopheles gambiae to human-specific sweat components. Med Vet Entomol 2001;15:259–66. https://doi.org/10.1046/j.0269-283X.2001.00297.x.

[19] Marinković ŽJ, Hackenberger BK, Merdić E. Maximum radius of carbon dioxide baited trap impact in woodland: Implications for host-finding by mosquitoes. Biologia 2014;69:522–9. https://doi.org/10.2478/s11756-014-0330-7.
[20] Cooperband MF, Cardé RT. Comparison of plume structures of carbon dioxide emitted from different mosquito traps. Med Vet Entomol 2006;20:1–10. https://doi.org/10.1111/j.1365-2915.2006.00614.x.
[21] McMeniman CJ, Corfas RA, Matthews BJ, Ritchie SA, Vosshall LB. Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell 2014;156:1060–71. https://doi.org/10.1016/j.cell.2013.12.044.
[22] Takken W, Knols BGJ. Olfaction in vector-host interactions. vol. 2. 2010. https://doi.org/10.3920/978-90-8686-698-4.
[23] Verhulst NO, Andriessen R, Groenhagen U, Kiss GB, Schulz S, Takken W, et al. Differential attraction of malaria mosquitoes to volatile blends produced by human skin bacteria. PLoS One 2010;5. https://doi.org/10.1371/journal.pone.0015829.
[24] Webster B, Lacey ES, Cardé RT. Waiting with bated breath: opportunistic orientation to human odor in the malaria mosquito, Anopheles gambiae, is modulated by minute changes in carbon dioxide concentration. J Chem Ecol 2015;41:59–66. https://doi.org/10.1007/s10886-014-0542-x.
[25] Logan JG, Birkett MA, Clark SJ, Powers S, Seal NJ, Wadhams LJ, et al. Identification of Human-Derived Volatile Chemicals that Interfere with Attraction of Aedes aegypti Mosquitoes 2008:308–22. https://doi.org/10.1007/s10886-008-9436-0.
[26] Bernier UR, Kline DL, Posey KH, Booth MM, Yost RA, Barnard DR. Synergistic attraction of Aedes aegypti (L.) to binary blends of L-lactic acid and acetone, dichloromethane, or dimethyl disulfide. J Med Entomol 2003;40:653–6. https://doi.org/10.1603/0022-2585-40.5.653.
[27] Braks MAH, Meijerink J, Takken W. The response of the malaria mosquito, Anopheles gambiae, to two components of human sweat, ammonia and L-lactic acid, in an olfactometer. Physiol Entomol 2001;26:142–8. https://doi.org/10.1046/j.1365-3032.2001.00227.x.
[28] Majeed S, Hill SR, Birgersson G, Ignell R. Detection and perception of generic host volatiles by mosquitoes modulate host preference: Context dependence of (R)-1-octen-3-ol. Royal Society Open Science 2016;3. https://doi.org/10.1098/rsos.160467.
[29] Giraldo D, Rankin-Turner S, Corver A, Tauxe GM, Gao AL, Jackson DM, et al. Human scent guides mosquito thermotaxis and host selection under naturalistic conditions. Curr Biol 2023. https://doi.org/10.1016/j.cub.2023.04.050.
[30] Becker N, Petrić D, Zgomba M, Boase C, Madon MB, Dahl C, et al. Mosquitoes: Identification, Ecology and Control. Springer International Publishing; 2020.
[31] Gibson G, Torr SJ. Visual and olfactory responses of haematophagous Diptera to host stimuli. Med Vet Entomol 1999;13:2–23. https://doi.org/10.1046/j.1365-2915.1999.00163.x.
[32] Land MF, Gibson G, Horwood J. Mosquito eye design: Conical rhabdoms are matched to wide aperture lenses. Proceedings of the Royal Society B: Biological Sciences 1997;264:1183–7. https://doi.org/10.1098/rspb.1997.0163.
[33] Bowen M. The Sensory Physiology Of Host-Seeking Behavior In Mosquitos. Annu Rev Entomol 1991;36:139–58. https://doi.org/10.1146/annurev.ento.36.1.139.
[34] Takken W, Knols BGJ, Otten H. Interactions between physical and olfactory cues in the host-seeking behaviour of mosquitoes: The role of relative humidity. Ann Trop Med Parasitol 1997;91:19–21. https://doi.org/10.1080/00034989761427.
[35] Carnaghi M, Belmain SR, Hopkins RJ, Hawkes FM. Multimodal synergisms in host stimuli drive landing response in malaria mosquitoes. Sci Rep 2021;11:7379. https://doi.org/10.1038/s41598-021-86772-4.
[36] Sumner BD, Cardé RT. Primacy of Human Odors Over Visual and Heat Cues in Inducing Landing in Female Aedes aegypti Mosquitoes. J Insect Behav 2022;35:31–43. https://doi.org/10.1007/s10905-022-09796-2.
[37] Hawkes F, Gibson G. Seeing is believing: The nocturnal malarial mosquito Anopheles

coluzzii responds to visual host-cues when odour indicates a host is nearby. Parasites and Vectors 2016;9:1–13. https://doi.org/10.1186/s13071-016-1609-z.
[38] Qiu YT, Smallegange RC, Van Loon JJA, Ter Braak CJF, Takken W. Interindividual variation in the attractiveness of human odours to the malaria mosquito Anopheles gambiae s. s. Med Vet Entomol 2006;20:280–7. https://doi.org/10.1111/j.1365-2915.2006.00627.x.
[39] Pates HV, Takken W, Stuke K, Curtis CF. Differential behaviour of Anopheles gambiae sensu stricto (Diptera: Culicidae) to human and cow odours in the laboratory. Bull Entomol Res 2001;91:289–96. https://doi.org/10.1079/ber200198.
[40] Kröber T, Kessler S, Frei J, Bourquin M, Guerin PM. An In Vitro Assay for Testing Mosquito Repellents Employing a Warm Body and Carbon Dioxide as a Behavioral Activator. J Am Mosq Control Assoc 2010;26:381–6. https://doi.org/10.2987/10-6044.1.
[41] Spitzen J, Smallegange RC, Takken W. Effect of human odours and positioning of CO2 release point on trap catches of the malaria mosquito Anopheles gambiae sensu stricto in an olfactometer. Physiol Entomol 2008;33:116–22. https://doi.org/10.1111/j.1365-3032.2008.00612.x.
[42] Takken W, Dekker T, Wijnholds YG. Odor-mediated flight behavior of Anopheles gambiae giles Sensu stricto and An. stephensi liston in response to Co2, acetone, and 1-octen-3-ol (Diptera: Culicidae). J Insect Behav 1997;10:395–407. https://doi.org/10.1007/BF02765606.
[43] Thongsripong P, Hyman JM, Kapan DD, Bennett SN. Human-Mosquito Contact: A Missing Link in Our Understanding of Mosquito-Borne Disease Transmission Dynamics. Ann Entomol Soc Am 2021;114:397–414. https://doi.org/10.1093/aesa/saab011.
[44] Gikonyo NK, Hassanali A, Njagi PGN, Saini RK. Behaviour of Glossina morsitans morsitans Westwood (Diptera: Glossinidae) on waterbuck Kobus defassa Ruppel and feeding membranes smeared with waterbuck sebum indicates the presence of allomones. Acta Trop 2000;77:295–303. https://doi.org/10.1016/S0001-706X(00)00153-4.
[45] Lehane M. Chapter 4: Location of the host. The Biology of Blood-Sucking in Insects, Cambridge University Press; 2005, p. 27–55.
[46] Peterson DG, Brown AWA. Studies of the Responses of the Female Aëdes Mosquito. Part III. The Response of Aëdes aegypti (L.) to a Warm Body and its Radiation. Bull Entomol Res 1951;42:535–41. https://doi.org/10.1017/S0007485300028935.
[47] Wilson AL, Chen-Hussey V, Logan JG, Lindsay SW. Are topical insect repellents effective against malaria in endemic populations? A systematic review and meta-analysis. Malar J 2014;13:446. https://doi.org/10.1186/1475-2875-13-446.
[48] Londono-Renteria B, Patel JC, Vaughn M, Funkhauser S, Ponnusamy L, Grippin C, et al. Long-Lasting Permethrin-Impregnated Clothing Protects Against Mosquito Bites in Outdoor Workers. Am J Trop Med Hyg 2015;93:869–74. https://doi.org/10.4269/ajtmh.15-0130.
[49] Lengeler C. Insecticide-treated bednets and curtains for preventing malaria. Cochrane Database Syst Rev 2000:CD000363. https://doi.org/10.1002/14651858.CD000363.
[50] Tusting LS, Thwing J, Sinclair D, Fillinger U, Gimnig J, Bonner KE, et al. Mosquito larval source management for controlling malaria. Cochrane Database Syst Rev 2013;2013:CD008923. https://doi.org/10.1002/14651858.CD008923.pub2.
[51] Satchidanandam V. Japanese Encephalitis Vaccines. Curr Treat Options Infect Dis 2020;12:375–86. https://doi.org/10.1007/s40506-020-00242-5.
[52] Curtis CF. Should the use of DDT be revived for malaria vector control? Biomedica 2002;22:455–61. https://doi.org/10.7705/biomedica.v22i4.1171.
[53] Sadasivaiah S, Tozan Y, Breman JG. Dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying in Africa: How can it be used for malaria control? Am J Trop Med Hyg 2007;77:249–63. https://doi.org/10.4269/ajtmh.2007.77.249.

[54] Tangena JAA, Hendriks CMJ, Devine M, Tammaro M, Trett AE, Williams I, et al. Indoor residual spraying for malaria control in sub-Saharan Africa 1997 to 2017: An adjusted retrospective analysis. Malar J 2020;19:1–15. https://doi.org/10.1186/s12936-020-03216-6.
[55] WHO. Global strategic framework for integrated vector management 2004. https://apps.who.int/iris/handle/10665/68624 (accessed October 10, 2019).
[56] WHO. Multisectoral Approach to the Prevention and Control of Vector-Borne Diseases 2020. https://apps.who.int/iris/handle/10665/331861 (accessed May 3, 2022).
[57] WHO. World Malaria Report 2019 2019. https://www.who.int/publications/i/item/9789241565721 (accessed June 14, 2020).
[58] Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature 2015;526:207–11. https://doi.org/10.1038/nature15535.
[59] Kline DL. Traps and trapping techniques for adult mosquito control. J Am Mosq Control Assoc 2006;22:490–6. https://doi.org/10.2987/8756-971X(2006)22[490:TATTFA]2.0.CO;2.
[60] Kline DL. Introduction to symposium on attractants for mosquito surveillance and control. Journal of the American Mosquito Control Association-Mosquito News 1994;10:253–7.
[61] Jourdain F, Samy AM, Hamidi A, Bouattour A, Alten B, Faraj C, et al. Towards harmonisation of entomological surveillance in the Mediterranean area. PLoS Negl Trop Dis 2019;13:e0007314. https://doi.org/10.1371/journal.pntd.0007314.
[62] Killeen GF, Tatarsky A, Diabate A, Chaccour CJ, Marshall JM, Okumu FO, et al. Developing an expanded vector control toolbox for malaria elimination. BMJ Global Health 2017;2:1–8. https://doi.org/10.1136/bmjgh-2016-000211.
[63] Yoon JK, Kim K-C, Cho Y, Gwon Y-D, Cho HS, Heo Y, et al. Comparison of Repellency Effect of Mosquito Repellents for DEET, Citronella, and Fennel Oil. J Parasitol Res 2015;2015:361021. https://doi.org/10.1155/2015/361021.
[64] Maia MF, Kliner M, Richardson M, Lengeler C, Moore SJ. Mosquito repellents for malaria prevention. Cochrane Database Syst Rev 2018;2018. https://doi.org/10.1002/14651858.CD011595.pub2.
[65] Msoffe R, Hewitt M, Masalu JP, Finda M, Kavishe DR, Okumu FO, et al. Participatory development of practical, affordable, insecticide-treated mosquito proofing for a range of housing designs in rural southern Tanzania. Malar J 2022;21:318. https://doi.org/10.1186/s12936-022-04333-0.
[66] Ranson H, Lissenden N. Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. Trends Parasitol 2016;32:187–96. https://doi.org/10.1016/j.pt.2015.11.010.
[67] Gatton ML, Chitnis N, Churcher T, Donnelly MJ, Ghani AC, Godfray HCJ, et al. The importance of mosquito behavioural adaptations to malaria control in Africa. Evolution 2013;67:1218–30. https://doi.org/10.1111/evo.12063.
[68] Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African anopheline mosquitoes: What are the implications for malaria control? Trends Parasitol 2011;27:91–8. https://doi.org/10.1016/j.pt.2010.08.004.
[69] IVCC. Insecticide Resistance Management (IRM). IVCC 2022. https://www.ivcc.com/vector-control/irm/ (accessed December 27, 2022).
[70] Chanda-Kapata P, Kapata N, Zumla A. COVID-19 and malaria: A symptom screening challenge for malaria endemic countries. Int J Infect Dis 2020;94:151–3. https://doi.org/10.1016/j.ijid.2020.04.007.
[71] Nghochuzie NN, Olwal CO, Udoakang AJ, Amenga-Etego LNK, Amambua-Ngwa A. Pausing the Fight Against Malaria to Combat the COVID-19 Pandemic in Africa: Is the Future of Malaria Bleak? Front Microbiol 2020;11:1–5. https://doi.org/10.3389/fmicb.2020.01476.
[72] Killeen GF. Characterizing, controlling and eliminating residual malaria transmission.

Malar J 2014;13:1–22. https://doi.org/10.1186/1475-2875-13-330.
[73] Sherrard-Smith E, Skarp JE, Beale AD, Fornadel C, Norris LC, Moore SJ, et al. Mosquito feeding behavior and how it influences residual malaria transmission across Africa. Proc Natl Acad Sci U S A 2019;116:15086–96. https://doi.org/10.1073/pnas.1820646116.
[74] Govella NJ, Ferguson H. Why use of interventions targeting outdoor biting mosquitoes will be necessary to achieve malaria elimination. Front Physiol 2012;3 JUN:1–5. https://doi.org/10.3389/fphys.2012.00199.
[75] Gleave K, Lissenden N, Chaplin M, Choi L, Ranson H. Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. Cochrane Database Syst Rev 2021;2021:1–3. https://doi.org/10.1002/14651858.CD012776.pub3.
[76] Portwood NM, Shayo MF, Tungu PK, Mbewe NJ, Mlay G, Small G, et al. Multi-centre discriminating concentration determination of broflanilide and potential for cross-resistance to other public health insecticides in Anopheles vector populations. Sci Rep 2022;12:22359. https://doi.org/10.1038/s41598-022-26990-6.
[77] Webber R. Communicable Diseases A Global Perspective. 6th ed. 2019.
[78] WHO. First malaria vaccine in Africa: A potential new tool for child health and improved malaria control 2018. https://www.who.int/malaria/publications/atoz/first-malaria-vaccine/en/ (accessed September 1, 2023).
[79] WHO. WHO recommends groundbreaking malaria vaccine for children at risk 2021. https://www.who.int/news/item/06-10-2021-who-recommends-groundbreaking-malaria-vaccine-for-children-at-risk (accessed January 13, 2023).
[80] Alonso PL. Malaria: a problem to be solved and a time to be bold. Nat Med 2021. https://doi.org/10.1038/s41591-021-01492-6.
[81] Datoo MS, Natama HM, Somé A, Bellamy D, Traoré O, Rouamba T, et al. Efficacy and immunogenicity of R21/Matrix-M vaccine against clinical malaria after 2 years' follow-up in children in Burkina Faso: a phase 1/2b randomised controlled trial. Lancet Infect Dis 2022;22:1728–36. https://doi.org/10.1016/S1473-3099(22)00442-X.
[82] The Lancet Infectious Diseases. The dengue vaccine dilemma. Lancet Infect Dis 2018;18:123. https://doi.org/10.1016/S1473-3099(18)30023-9.
[83] Mallapaty S. Dengue vaccine poised for roll-out but safety concerns linger 2022. https://www.nature.com/articles/d41586-022-03546-2.pdf (accessed December 29, 2022).
[84] McLean KJ, Jacobs-Lorena M. Genetic Control Of Malaria Mosquitoes. Trends Parasitol 2016;32:174–6. https://doi.org/10.1016/j.pt.2016.01.002.
[85] Gao H, Bai L, Jiang Y, Huang W, Wang L, Li S, et al. A natural symbiotic bacterium drives mosquito refractoriness to Plasmodium infection via secretion of an antimalarial lipase. Nature Microbiology 2021;6:806–17. https://doi.org/10.1038/s41564-021-00899-8.
[86] Ahmad NA, Mancini M-V, Ant TH, Martinez J, Kamarul GMR, Nazni WA, et al. Wolbachia strain wAlbB maintains high density and dengue inhibition following introduction into a field population of Aedes aegypti. Philos Trans R Soc Lond B Biol Sci 2021;376:20190809. https://doi.org/10.1098/rstb.2019.0809.
[87] Menger DJ, Otieno B, De Rijk M, Mukabana WR, Van Loon JJ, Takken W. A push-pull system to reduce house entry of malaria mosquitoes. Malar J 2014;13:1–8. https://doi.org/10.1186/1475-2875-13-119.
[88] Njoroge MM, Fillinger U, Saddler A, Moore S, Takken W, van Loon JJA, et al. Evaluating putative repellent "push" and attractive "pull" components for manipulating the odour orientation of host-seeking malaria vectors in the peri-domestic space. Parasites and Vectors 2021;14:1–21. https://doi.org/10.1186/s13071-020-04556-7.
[89] Nayani SA, Meraj S, Mohr E, Gries R, Kovacs E, Devireddy A, et al. Staphylococcus microbes in the bovine skin microbiome attract blood-feeding stable flies. Front Ecol Evol 2023;11. https://doi.org/10.3389/fevo.2023.1212222.
[90] Lucas-Barbosa D, DeGennaro M, Mathis A, Verhulst NO. Skin bacterial volatiles:

propelling the future of vector control. Trends Parasitol 2022;38:15–22. https://doi.org/10.1016/j.pt.2021.08.010.
[91] De Lacy Costello B, Amann A, Al-Kateb H, Flynn C, Filipiak W, Khalid T, et al. A review of the volatiles from the healthy human body. J Breath Res 2014;8. https://doi.org/10.1088/1752-7155/8/1/014001.
[92] Schmidt R, Cordovez V, de Boer W, Raaijmakers J, Garbeva P. Volatile affairs in microbial interactions. ISME J 2015;9:2329–35. https://doi.org/10.1038/ismej.2015.42.
[93] Logan JG, Stanczyk NM, Hassanali A, Kemei J, Santana AEG, Ribeiro KAL, et al. Arm-in-cage testing of natural human-derived mosquito repellents. Malar J 2010;9:239. https://doi.org/10.1186/1475-2875-9-239.
[94] Ruel DM, Bohbot JD. The molecular and neural determinants of olfactory behaviour in mosquitoes. Sensory ecology of disease vectors, 2022, p. 71–115. https://doi.org/10.3920/978-90-8686-932-9_3.
[95] Dormont L, Bessière JM, Cohuet A. Human Skin Volatiles: A Review. J Chem Ecol 2013;39:569–78. https://doi.org/10.1007/s10886-013-0286-z.
[96] Menger DJ, Van Loon JJA, Takken W. Assessing the efficacy of candidate mosquito repellents against the background of an attractive source that mimics a human host. Med Vet Entomol 2014;28:407–13. https://doi.org/10.1111/mve.12061.
[97] Tian J, Mao J, Yu B, Fouad H, Ga'al H, Mao G, et al. Laboratory and Field Evaluation of Multiple Compound Attractants to Culex pipiens pallens. J Med Entomol 2018;55:787–94. https://doi.org/10.1093/jme/tjy015.
[98] Tchouassi DP, Jacob JW, Ogola EO, Sang R, Torto B. Aedes vector-host olfactory interactions in sylvatic and domestic dengue transmission environments. Proc Biol Sci 2019;286:20192136. https://doi.org/10.1098/rspb.2019.2136.
[99] Ruzsanyi V, Mochalski P, Schmid A, Wiesenhofer H, Klieber M, Hinterhuber H, et al. Ion mobility spectrometry for detection of skin volatiles. J Chromatogr B Analyt Technol Biomed Life Sci 2012;911:84–92. https://doi.org/10.1016/j.jchromb.2012.10.028.
[100] Leal HM, Hwang JK, Tan K, Leal WS. Attraction of Culex mosquitoes to aldehydes from human emanations. Sci Rep 2017;7:1–10. https://doi.org/10.1038/s41598-017-18406-7.
[101] Verhulst NO, Beijleveld H, Qiu YT, Maliepaard C, Verduyn W, Haasnoot GW, et al. Relation between HLA genes, human skin volatiles and attractiveness of humans to malaria mosquitoes. Infect Genet Evol 2013;18:87–93. https://doi.org/10.1016/j.meegid.2013.05.009.
[102] Duffy E, Morrin A. Endogenous and microbial volatile organic compounds in cutaneous health and disease. Trends Analyt Chem 2019;111:163–72. https://doi.org/10.1016/j.trac.2018.12.012.
[103] Zou Z, Yang X. Volatile organic compound emissions from the human body: Decoupling and comparison between whole-body skin and breath emissions. Build Environ 2022;226:109713. https://doi.org/10.1016/j.buildenv.2022.109713.
[104] Filipiak W, Mochalski P, Filipiak A, Ager C, Cumeras R, Davis CE, et al. A Compendium of Volatile Organic Compounds (VOCs) Released By Human Cell Lines. Curr Med Chem 2016;23:2112–31. https://doi.org/10.2174/0929867323666160510122913.
[105] VanderGiessen M, Tallon AK, Damico B, Lahondère C, Vinauger C. Soap application alters mosquito-host interactions. iScience 2023;0. https://doi.org/10.1016/j.isci.2023.106667.
[106] Wisthaler A, Weschler CJ. Reactions of ozone with human skin lipids: sources of carbonyls, dicarbonyls, and hydroxycarbonyls in indoor air. Proc Natl Acad Sci U S A 2010;107:6568–75. https://doi.org/10.1073/pnas.0904498106.
[107] Picardo M, Ottaviani M, Camera E, Mastrofrancesco A. Sebaceous gland lipids. Dermatoendocrinol 2009;1:68–71. https://doi.org/10.4161/derm.1.2.8472.
[108] Fruekilde P, Hjorth J, Jensen NR, Kotzias D, Larsen B. Ozonolysis at vegetation

surfaces. a source of acetone, 4-oxopentanal, 6-methyl-5-hepten-2-one, and geranyl acetone in the troposphere. Atmos Environ 1998;32:1893–902. https://doi.org/10.1016/S1352-2310(97)00485-8.
[109] James AG, Hyliands D, Johnston H. Generation of volatile fatty acids by axillary bacteria. Int J Cosmet Sci 2004;26:149–56. https://doi.org/10.1111/j.1467-2494.2004.00214.x.
[110] James AG, Casey J, Hyliands D, Mycock G. Fatty acid metabolism by cutaneous bacteria and its role in axillary malodour. World J Microbiol Biotechnol 2004;20:787–93. https://doi.org/10.1007/s11274-004-5843-8.
[111] Ara K, Hama M, Akiba S, Koike K, Okisaka K, Hagura T, et al. Foot odor due to microbial metabolism and its control. Can J Microbiol 2006;52:357–64. https://doi.org/10.1139/W05-130.
[112] Grice EA, Segre JA. The skin microbiome. Nat Rev Microbiol 2011;9:244–53. https://doi.org/10.1038/nrmicro2537.
[113] Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R. Bacterial Community Variation in Human Body Habitats Across Space and Time. Science 2009;326:1694–7. https://doi.org/10.1093/mind/XC.357.61.
[114] Si J, Lee S, Park JM, Sung J, Ko GP. Genetic associations and shared environmental effects on the skin microbiome of Korean twins. BMC Genomics 2015;16:1–11. https://doi.org/10.1186/s12864-015-2131-y.
[115] Takken W, Verhulst NO. Chemical signaling in mosquito–host interactions: the role of human skin microbiota. Current Opinion in Insect Science 2017;20:68–74. https://doi.org/10.1016/j.cois.2017.03.011.
[116] Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A 2010;107:11971–5. https://doi.org/10.1073/pnas.1002601107.
[117] Gaitanis G, Tsiouri G, Spyridonos P, Stefos T, Stamatas GN, Velegraki A, et al. Variation of cultured skin microbiota in mothers and their infants during the first year postpartum. Pediatr Dermatol 2019;36:460–5. https://doi.org/10.1111/pde.13829.
[118] Oh J, Byrd Allyson L, Park M, NISC Comparative sequencing program, Kong HH, Segre JA. Temproal stability of the human skin microbiome. Physiol Behav 2017;176:139–48. https://doi.org/10.1016/j.physbeh.2017.03.040.
[119] Grice EA. The intersection of microbiome and host at the skin interface: Genomic- and metagenomic-based insights. Genome Res 2015;25:1514–20. https://doi.org/10.1101/gr.191320.115.
[120] Ross AA, Rodrigues Hoffmann A, Neufeld JD. The skin microbiome of vertebrates. Microbiome 2019;7:79. https://doi.org/10.1186/s40168-019-0694-6.
[121] Smallegange RC, Verhulst NO, Takken W. Sweaty skin: An invitation to bite? Trends Parasitol 2011;27:143–8. https://doi.org/10.1016/j.pt.2010.12.009.
[122] Verhulst NO, Takken W, Dicke M, Schraa G, Smallegange RC. Chemical ecology of interactions between human skin microbiota and mosquitoes. FEMS Microbiol Ecol 2010;74:1–9. https://doi.org/10.1111/j.1574-6941.2010.00908.x.
[123] Jha SK. Characterization of human body odor and identification of aldehydes using chemical sensor. Rev Anal Chem 2017;36. https://doi.org/10.1515/revac-2016-0028.
[124] Smallegange RC, Qiu YT, van Loon JJA, Takken W. Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito Anopheles gambiae sensu stricto (Diptera: Culicidae). Chem Senses 2005;30:145–52. https://doi.org/10.1093/chemse/bji010.
[125] Shelley WB, Hurley HJ Jr, Nichols AC. Axillary odor; experimental study of the role of bacteria, apocrine sweat, and deodorants. AMA Arch Derm Syphilol 1953;68:430–46. https://doi.org/10.1001/archderm.1953.01540100070012.
[126] Shreck CE, James J. Broth cultures of bacteria that attract female mosquitoes 1968:33–8.
[127] Braks MAH, Takken W. Incubated human sweat but not fresh sweat attracts the

malaria mosquito Anopheles gambiae sensu stricto. J Chem Ecol 1999;25:663–72. https://doi.org/10.1023/A:1020970307748.
[128] Dekker T, Takken W. Differential responses of mosquito sibling species Anopheles arabiensis and An. quadriannulatus to carbon dioxide, a man or a calf. Med Vet Entomol 1998;12:136–40. https://doi.org/10.1046/j.1365-2915.1998.00073.x.
[129] Braack L, Hunt R, Koekemoer LL, Gericke A, Munhenga G, Haddow AD, et al. Biting behaviour of African malaria vectors:1. Where do the main vector species bite on the human body? Parasites and Vectors 2015;8:1–10. https://doi.org/10.1186/s13071-015-0677-9.
[130] De Jong R, Knols BGJ. Selection of biting sites on man by two malaria mosquito species. Experientia 1995;51:80–4. https://doi.org/10.1007/BF01964925.
[131] Verhulst NO, Weldegergis BT, Menger D, Takken W. Attractiveness of volatiles from different body parts to the malaria mosquito Anopheles coluzzii is affected by deodorant compounds. Sci Rep 2016;6:1–9. https://doi.org/10.1038/srep27141.
[132] Nicolaides N. Skin lipids: Their biochemical uniqueness. Science 1974;186:19–26. https://doi.org/10.1126/science.186.4158.19.
[133] Marshall J, Holland KT, Gribbon EM. A comparative study of the cutaneous microflora of normal feet with low and high levels of odour. J Appl Bacteriol 1988;65:61–8. https://doi.org/10.1111/j.1365-2672.1988.tb04318.x.
[134] Knols BGJ, De Jong R. Limburger cheese as an attractant for the malaria mosquito Anopheles gambiae s.s. Parasitol Today 1996;12:159–61. https://doi.org/10.1016/0169-4758(96)10002-8.
[135] Kobayashi S. [Relationship between an offensive smell given off from human foot and Staphylococcus epidermidis]. Nihon Saikingaku Zasshi 1990;45:797–800. https://doi.org/10.3412/jsb.45.797.
[136] Verhulst NO, Beijleveld H, Knols BG, Takken W, Schraa G, Bouwmeester HJ, et al. Cultured skin microbiota attracts malaria mosquitoes. Malar J 2009;8:1–12. https://doi.org/10.1186/1475-2875-8-302.
[137] Verhulst NO, Qiu YT, Beijleveld H, Maliepaard C, Knights D, Schulz S, et al. Composition of human skin microbiota affects attractiveness to malaria mosquitoes. PLoS One 2011;6. https://doi.org/10.1371/journal.pone.0028991.
[138] Arıkan Ş, İpek M, Pırlak L, Eşitken A. Chapter 2 - Physiological and molecular mechanisms in improving salinity stress tolerance by beneficial microorganisms in plants. In: Kumar A, Droby S, editors. Microbial Management of Plant Stresses, Woodhead Publishing; 2021, p. 13–43. https://doi.org/10.1016/B978-0-323-85193-0.00006-1.
[139] Obaldia MED, Morita T, Dedmon LC, Boehmler J, Jiang CS, Zeledon EV, et al. Differential mosquito attraction to humans is associated with skin-derived carboxylic acid levels 2022:1–50.
[140] Schreck CE, Kline DL, Carlson DA. Mosquito attraction to substances from the skin of different humans. J Am Mosq Control Assoc 1990;6:406–10.
[141] Lindsay SW, Adiamah JH, Miller JE, Pleass RJ, Armstrong JR. Variation in attractiveness of human subjects to malaria mosquitoes (Diptera: Culicidae) in The Gambia. J Med Entomol 1993;30:368–73. https://doi.org/10.1093/jmedent/30.2.368.
[142] Knols BG, de Jong R, Takken W. Differential attractiveness of isolated humans to mosquitoes in Tanzania. Trans R Soc Trop Med Hyg 1995;89:604–6. https://doi.org/10.1016/0035-9203(95)90406-9.
[143] Brady J, Costantini C, Sagnon N, Gibson G, Coluzzi M, Costantini C, et al. The role of body odours in the relative attractiveness of different men to malarial vectors in Burkina Faso 1997;4983:20–2. https://doi.org/10.1080/00034983.1997.11813252.
[144] Rudolfs W. Chemotropism of mosquitoes. vol. 367. New Jersey Agricultural Experiment Stations; 1922.
[145] Smallegange RC, Qiu YT, Bukovinszkiné-Kiss G, Van Loon JJA, Takken W. The effect of aliphatic carboxylic acids on olfaction-based host-seeking of the malaria mosquito Anopheles gambiae sensu stricto. J Chem Ecol 2009;35:933–43.

https://doi.org/10.1007/s10886-009-9668-7.
[146] Qiu YT. Sensory and behavioural responses of the malaria mosquito Anopheles gambiae to human odours. PhD. Wageningen Universiteit, 2005.
[147] Sriharsha M DSA, Krupa SN NRK. Role of Diet in Dermatological Conditions. J Nutr Food Sci 2015;05. https://doi.org/10.4172/2155-9600.1000400.
[148] Paskewitz S, Irwin P, Konwinski N, Larson S. Impact of Consumption of Bananas on Attraction of Anopheles stephensi to Humans. Insects 2018;9. https://doi.org/10.3390/insects9040129.
[149] Lefèvre T, Gouagna LC, Dabiré KR, Elguero E, Fontenille D, Renaud F, et al. Beer consumption increases human attractiveness to malaria mosquitoes. PLoS One 2010;5:1–8. https://doi.org/10.1371/journal.pone.0009546.
[150] Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, et al. Topographical and temporal diversity of the human skin microbiome. Science 2009;324:1190–2. https://doi.org/10.1126/science.1171700.
[151] McInnes P, Cutting M. Manual of Procedures for Human Microbiome Project 2010. https://doi.org/10.1891/9781617050992.0067.
[152] Kong HH, Andersson B, Clavel T, Common JE, Jackson SA, Olson ND, et al. Performing Skin Microbiome Research: A Method to the Madness. J Invest Dermatol 2017;137:561–8. https://doi.org/10.1016/j.jid.2016.10.033.
[153] Oh J, Conlan S, Polley EC, Segre JA, Kong HH. Shifts in human skin and nares microbiota of healthy children and adults. Genome Med 2012;4:77. https://doi.org/10.1186/gm378.
[154] Ying S, Zeng DN, Chi L, Tan Y, Galzote C, Cardona C, et al. The influence of age and gender on skin-associated microbial communities in urban and rural human populations. PLoS One 2015;10:1–16. https://doi.org/10.1371/journal.pone.0141842.
[155] Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D, et al. Cohabiting family members share microbiota with one another and with their dogs. Elife 2013;2013:1–22. https://doi.org/10.7554/eLife.00458.
[156] Meadow JF, Bateman AC, Herkert KM, O'Connor TK, Green JL. Significant changes in the skin microbiome mediated by the sport of roller derby. PeerJ 2013;2013:1–17. https://doi.org/10.7717/peerj.53.
[157] Fierer N, Hamady M, Lauber CL, Knight R. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. Proc Natl Acad Sci U S A 2008;105:17994–9. https://doi.org/10.1073/pnas.0807920105.
[158] Port GR, Boreham PFL, Bryan JH. The relationship of host size to feeding by mosquitoes of the Anopheles gambiae Giles complex (Diptera: Culicidae). Bull Entomol Res 1980;70:133–44. https://doi.org/10.1017/S0007485300009834.
[159] Smith CN, Smith N, Gouck HK, Weidhaas DE, Gilbert IH, Mayer MS, et al. L-lactic acid as a factor in the attraction of Aedes aegypti (Diptera: Culicidae) to human hosts. Ann Entomol Soc Am 1970;63:760–70. https://doi.org/10.1093/aesa/63.3.760.
[160] Muirhead-Thomson RC. The distribution of anopheline mosquito bites among different age groups. Br Med J 1951;1:1114–7. https://doi.org/10.1136/bmj.1.4715.1114.
[161] Kirk KM, Eaves LJ, Meyer JM, Saul A, Martin NG. Twin study of adolescent genetic susceptibility to mosquito bites using ordinal and comparative rating data. Genet Epidemiol 2000;19:178–90. https://doi.org/10.1002/1098-2272(200009)19:2<178::AID-GEPI5>3.0.CO;2-3.
[162] Lindsay S, Ansell J, Selman C, Cox V, Hamilton K, Walraven G. Effect of pregnancy on exposure to malaria mosquitoes. Lancet 2000;355:1972. https://doi.org/10.1016/S0140-6736(00)02334-5.
[163] Menendez C. Malaria during pregnancy: A priority area of malaria research and control. Parasitol Today 1995;11:178–83. https://doi.org/10.1016/0169-4758(95)80151-0.
[164] Ansell J, Hamilton KA, Pindeti M, Walraven GEL. Short-range atttractiveness of pregnant women to An. gam mosquitoes 2002. https://doi.org/10.1016/s0035-

9203(02)90271-3.
[165] Himeidan YE, Elbashir MI, Adam I. Attractiveness of pregnant women to the malaria vector, Anopheles arabiensis, in Sudan. Ann Trop Med Parasitol 2004;98:631–3. https://doi.org/10.1179/000349804225021307.
[166] Cornet S, Nicot A, Rivero A, Gandon S. Malaria infection increases bird attractiveness to uninfected mosquitoes. Ecol Lett 2013;16:323–9. https://doi.org/10.1111/ele.12041.
[167] Lacroix R, Mukabana WR, Gouagna LC, Koella JC. Malaria infection increases attractiveness of humans to mosquitoes. PLoS Biol 2005;3:1590–3. https://doi.org/10.1371/journal.pbio.0030298.
[168] Day JF, Edman JD. Malaria renders mice susceptible to mosquito feeding when gametocytes are most infective. J Parasitol 1983;69:163–70. https://doi.org/10.2307/3281292.
[169] De Moraes CM, Stanczyk NM, Betz HS, Pulido H, Sim DG, Read AF, et al. Malaria-induced changes in host odors enhance mosquito attraction. Proc Natl Acad Sci U S A 2014;111:11079–84. https://doi.org/10.1073/pnas.1405617111.
[170] De Boer JG, Robinson A, Powers SJ, Burgers SLGE, Caulfield JC, Birkett MA, et al. Odours of Plasmodium falciparum-infected participants influence mosquito-host interactions. Sci Rep 2017;7:1–9. https://doi.org/10.1038/s41598-017-08978-9.
[171] Batista EPA, Costa EFM, Silva AA. Anopheles darlingi (Diptera: Culicidae) displays increased attractiveness to infected individuals with Plasmodium vivax gametocytes. Parasites and Vectors 2014;7:1–4. https://doi.org/10.1186/1756-3305-7-251.
[172] Robinson A, Busula AO, Voets MA, Beshir KB, Caulfield JC, Powers SJ, et al. Plasmodium-associated changes in human odor attract mosquitoes. Proc Natl Acad Sci U S A 2018;115:E4209–18. https://doi.org/10.1073/pnas.1721610115.
[173] Emami SN, Lindberg BG, Hua S, Hill SR, Mozuraitis R, Lehmann P, et al. A key malaria metabolite modulates vector blood seeking, feeding, and susceptibility to infection. Science 2017;355:1076–80. https://doi.org/10.1126/science.aah4563.
[174] Zhang H, Zhu Y, Liu Z, Peng Y, Peng W, Tong L, et al. A volatile from the skin microbiota of flavivirus-infected hosts promotes mosquito attractiveness. Cell 2022:2510–22. https://doi.org/10.1016/j.cell.2022.05.016.
[175] Hepper PG. The discrimination of human odour by the dog. Perception 1988;17:549–54. https://doi.org/10.1068/p170549.
[176] Roberts SC, Gosling LM, Spector TD, Miller P, Penn DJ, Petrie M. Body odor similarity in noncohabiting twins. Chem Senses 2005;30:651–6. https://doi.org/10.1093/chemse/bji058.
[177] Kuhn F, Natsch A. Body odour of monozygotic human twins: A common pattern of odorant carboxylic acids released by a bacterial aminoacylase from axilla secretions contributing to an inherited body odour type. J R Soc Interface 2009;6:377–92. https://doi.org/10.1098/rsif.2008.0223.
[178] Logan JG, Cook JI, Stanczyk NM, Weeks EN, Welham SJ, Mordue AJ. To bite or not to bite! A questionnaire-based survey assessing why some people are bitten more than others by midges. BMC Public Health 2010;10. https://doi.org/10.1186/1471-2458-10-275.
[179] Fernández-Grandon GM, Gezan SA, Armour JAL, Pickett JA, Logan JG. Heritability of attractiveness to mosquitoes. PLoS One 2015;10:1–10. https://doi.org/10.1371/journal.pone.0122716.
[180] Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature 1974;248:701–2. https://doi.org/10.1038/248701a0.
[181] Wedekind C, Füri S. Body odour preferences in men and women: do they aim for specific MHC combinations or simply heterozygosity? Proceedings of the Royal Society B: Biological Sciences 1997;264:1471. https://doi.org/10.1098/RSPB.1997.0204.

[182] Wedekind C, Seebeck T, Bettens F, Paepke AJ. MHC-dependent mate preferences in humans. Proc Biol Sci 1995;260:245–9. https://doi.org/10.1098/rspb.1995.0087.
[183] Roberts SC, Gosling LM, Carter V, Petrie M. MHC-correlated odour preferences in humans and the use of oral contraceptives. Proc Biol Sci 2008;275:2715–22. https://doi.org/10.1098/rspb.2008.0825.
[184] Ober C, Weitkamp LR, Cox N, Dytch H, Kostyu D, Elias S. HLA and mate choice in humans. Am J Hum Genet 1997;61:497–504. https://doi.org/10.1086/515511.
[185] Natsch A, Kuhn F, Tiercy JM. Lack of evidence for HLA-linked patterns of odorous carboxylic acids released from glutamine conjugates secreted in the human axilla. J Chem Ecol 2010;36:837–46. https://doi.org/10.1007/s10886-010-9826-y.
[186] Kutsuwada Y, Yokota K, Yoshida K, Tsuda H, Watanabe K, Matsumoto A, et al. Association of HLA-DPB1, NLRP10, OVOL1, and ABCC11 with the axillary microbiome in a Japanese population. J Dermatol Sci 2022;105:98–104. https://doi.org/10.1016/j.jdermsci.2022.01.003.
[187] Penn D, Potts W. How Do Major Histocompatibility Complex Genes Influence Odor and Mating Preferences? In: Dixon FJ, editor. Advances in Immunology, vol. 69, Academic Press; 1998, p. 411–36. https://doi.org/10.1016/S0065-2776(08)60612-4.
[188] Savelev SU, Antony-Babu S, Roberts SC, Wang H, Clare AS, Gosling LM, et al. Individual variation in 3-methylbutanal: A putative link between human leukocyte antigen and skin microflora. J Chem Ecol 2008;34:1253–7. https://doi.org/10.1007/s10886-008-9524-1.
[189] Callewaert C, Knödlseder N, Karoglan A, Güell M, Paetzold B. Skin microbiome transplantation and manipulation: Current state of the art. Comput Struct Biotechnol J 2021. https://doi.org/10.1016/j.csbj.2021.01.001.
[190] Logan JG. Why do mosquitoes “choose” to bite some people more than others? Outlooks on Pest Management 2008;19:280–3. https://doi.org/10.1564/19dec14.
[191] Ellwanger JH, Cardoso J da C, Chies JAB. Variability in human attractiveness to mosquitoes. Current Research in Parasitology & Vector-Borne Diseases 2021;1:100058. https://doi.org/10.1016/j.crpvbd.2021.100058.
[192] Knols BGJ, DE Jong R, Takken W. Trapping system for testing olfactory responses of the malaria mosquito Anopheles gambiae in a wind tunnel. Med Vet Entomol 1994;8:386–8. https://doi.org/10.1111/j.1365-2915.1994.tb00104.x.
[193] Wilson AJ, Réale D, Clements MN, Morrissey MM, Postma E, Walling CA, et al. An ecologist’s guide to the animal model. J Anim Ecol 2010;79:13–26. https://doi.org/10.1111/j.1365-2656.2009.01639.x.
[194] Gilmour AR, Gogel BJ, Cullis BR, Welham SJ, Thompson R. ASReml User Guide 2015. https://asreml.kb.vsni.co.uk/wp-content/uploads/sites/3/2018/02/ASReml-4.1-Functional-Specification.pdf (accessed March 6, 2020).
[195] Lynch M, Walsh B. Genetics and Analysis of Quantitative Traits. OUP USA; 1998.
[196] Visscher PM, Hill WG, Wray NR. Heritability in the genomics era - Concepts and misconceptions. Nat Rev Genet 2008;9:255–66. https://doi.org/10.1038/nrg2322.
[197] Silventoinen K, Sammalisto S, Perola M, Boomsma DI, Cornes BK, Davis C, et al. Heritability of Adult Body Height: A Comparative Study of Twin Cohorts in Eight Countries. Twin Res 2003;6:399–408. https://doi.org/10.1375/136905203770326402.
[198] Goor RM, Forman Neall L, Hoffman D, Sherry ST. A mathematical approach to the analysis of multiplex DNA profiles. Bull Math Biol 2011;73:1909–31. https://doi.org/10.1007/s11538-010-9598-0.
[199] Jones AV, Tilley M, Gutteridge A, Hyde C, Nagle M, Ziemek D, et al. GWAS of self-reported mosquito bite size, itch intensity and attractiveness to mosquitoes implicates immune-related predisposition loci. Hum Mol Genet 2017;26:1391–406. https://doi.org/10.1093/HMG/DDX036.
[200] Lam TH, Verzotto D, Brahma P, Ng AHQ, Hu P, Schnell D, et al. Understanding the microbial basis of body odor in pre-pubescent children and teenagers. Microbiome 2018;6:213. https://doi.org/10.1186/s40168-018-0588-z.

[201] Baker TC, Linn CE. Wind Tunnels in Pheromone Research. In: Hummel HE, Miller TA, editors. Techniques in Pheromone Research, New York, NY: Springer New York; 1984, p. 75–110. https://doi.org/10.1007/978-1-4612-5220-7_3.
[202] Lucas-Barbosa D, Balvers C, Bellantuono AJ, Castillo JS, Costa-da-Silva AL, De Moraes CM, et al. Competition matters: using in vitro community models to study the impact of human skin bacteria on mosquito attraction. Frontiers in Ecology and Evolution 2023;11. https://doi.org/10.3389/fevo.2023.1156311.
[203] Verhulst NO, Mukabana WR, Takken W, Smallegange RC. Human skin microbiota and their volatiles as odour baits for the malaria mosquito Anopheles gambiae s.s. Entomol Exp Appl 2011;139:170–9. https://doi.org/10.1111/j.1570-7458.2011.01119.x.
[204] Zhang X, Crippen TL, Coates CJ, Wood TK, Tomberlin JK. Effect of quorum sensing by Staphylococcus epidermidis on the attraction response of female adult yellow fever mosquitoes, Aedes aegypti aegypti (linnaeus) (diptera: Culicidae), to a blood-feeding source. PLoS One 2015;10:1–15. https://doi.org/10.1371/journal.pone.0143950.
[205] Busula AO, Takken W, de Boer JG, Mukabana WR, Verhulst NO. Variation in host preferences of malaria mosquitoes is mediated by skin bacterial volatiles. Med Vet Entomol 2017;31:320–6. https://doi.org/10.1111/mve.12242.
[206] Chen H, Zhao Q, Zhong Q, Duan C, Krutmann J, Wang J, et al. Skin Microbiome, Metabolome and Skin Phenome, from the Perspectives of Skin as an Ecosystem. Phenomics 2022;2:363–82. https://doi.org/10.1007/s43657-022-00073-y.
[207] Meijerink J, Braks MAH, Brack AA, Adam W, Dekker T, Posthumus MA, et al. Identification of olfactory stimulants for Anopheles gambiae from human sweat samples. J Chem Ecol 2000;26:1367–82. https://doi.org/10.1023/A:1005475422978.
[208] Abdill RJ, Adamowicz EM, Blekhman R. Public human microbiome data dominated by highly developed countries. bioRxiv 2021:2021.09.02.458641. https://doi.org/10.1371/journal.pbio.3001536.
[209] Showering A, Martinez J, Benavente ED, Gezan SA, Jones RT, Oke C, et al. Skin microbiome alters attractiveness to Anopheles mosquitoes. BMC Microbiol 2022:1–14. https://doi.org/10.1186/s12866-022-02502-4.
[210] Hospodsky D, Pickering AJ, Julian TR, Miller D, Gorthala S, Boehm AB, et al. Hand bacterial communities vary across two different human populations. Microbiology 2014;160:1144–52. https://doi.org/10.1099/mic.0.075390-0.
[211] Harker M, Carvell A-M, Marti VPJ, Riazanskaia S, Kelso H, Taylor D, et al. Functional characterisation of a SNP in the ABCC11 allele - effects on axillary skin metabolism, odour generation and associated behaviours. J Dermatol Sci 2014;73:23–30. https://doi.org/10.1016/j.jdermsci.2013.08.016.
[212] McCall LI, Callewaert C, Zhu Q, Song SJ, Bouslimani A, Minich JJ, et al. Home chemical and microbial transitions across urbanization. Nature Microbiology 2020;5:108–15. https://doi.org/10.1038/s41564-019-0593-4.
[213] Ruiz-Calderon JF, Cavallin H, Song SJ, Novoselac A, Pericchi LR, Hernandez JN, et al. Walls talk: Microbial biogeography of homes spanning urbanization. Science Advances 2016;2:e1501061. https://doi.org/10.1126/sciadv.1501061.
[214] Callewaert C, Ravard Helffer K, Lebaron P. Skin Microbiome and its Interplay with the Environment. Am J Clin Dermatol 2020;21:4–11. https://doi.org/10.1007/s40257-020-00551-x.
[215] Soininen L, Roslund MI, Nurminen N, Puhakka R, Laitinen OH, Hyöty H, et al. Indoor green wall affects health-associated commensal skin microbiota and enhances immune regulation: a randomized trial among urban office workers. Sci Rep 2022;12:6518. https://doi.org/10.1038/s41598-022-10432-4.
[216] Lehtimäki J, Karkman A, Laatikainen T, Paalanen L, von Hertzen L, Haahtela T, et al. Patterns in the skin microbiota differ in children and teenagers between rural and urban environments. Sci Rep 2017;7:45651. https://doi.org/10.1038/srep45651.
[217] Grice EA, Kong HH, Renaud G, Young AC, Bouffard GG, Blakesley RW, et al. A

diversity profile of the human skin microbiota. Genome Res 2008;18:1043–50. https://doi.org/10.1101/gr.075549.107.
[218] Aagaard K, Petrosino J, Keitel W, Watson M, Katancik J, Garcia N, et al. The Human Microbiome Project strategy for comprehensive sampling of the human microbiome and why it matters. FASEB J 2013;27:1012–22. https://doi.org/10.1096/fj.12-220806.
[219] Castelino M, Eyre S, Moat J, Fox G, Martin P, Ho P, et al. Optimisation of methods for bacterial skin microbiome investigation: Primer selection and comparison of the 454 versus MiSeq platform. BMC Microbiol 2017;17:1–12. https://doi.org/10.1186/s12866-017-0927-4.
[220] Klymiuk I, Bambach I, Patra V, Trajanoski S, Wolf P. 16S based microbiome analysis from healthy subjects' skin swabs stored for different storage periods reveal phylum to genus level changes. Front Microbiol 2016;7:1–12. https://doi.org/10.3389/fmicb.2016.02012.
[221] Cuthbertson L, Rogers GB, Walker AW, Oliver A, Hoffman LR, Carroll MP, et al. Implications of multiple freeze-thawing on respiratory samples for culture-independent analyses. J Cyst Fibros 2015;14:464–7. https://doi.org/10.1016/j.jcf.2014.10.004.
[222] Invitrogen. Qubit® dsDNA HS Assay Kits 2010. https://tools.thermofisher.com/content/sfs/manuals/Qubit_dsDNA_HS_Assay_UG.pdf (accessed November 21, 2019).
[223] Meisel JS, Hannigan GD, Tyldsley AS, SanMiguel AJ, Hodkinson BP, Zheng Q, et al. Skin microbiome surveys are strongly influenced by experimental design. SpringerBriefs in Applied Sciences and Technology 2017;136:17–39. https://doi.org/10.1007/978-3-319-56964-2_2.
[224] Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. 16S ribosomal RNA gene PCR primers evaluation. Nucleic Acids Res 2013;41:1–11. https://doi.org/10.1093/nar/gks808.
[225] Illumina. 16S Metagenomic Sequencing Library. Illumina.com 2013:1–28.
[226] Sinha R, Abnet CC, White O, Knight R, Huttenhower C. The microbiome quality control project: Baseline study design and future directions. Genome Biol 2015;16:1–6. https://doi.org/10.1186/s13059-015-0841-8.
[227] Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol 2014;12:1–12. https://doi.org/10.1186/s12915-014-0087-z.
[228] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 2016;13:581–3. https://doi.org/10.1038/nmeth.3869.
[229] Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41:D590–6. https://doi.org/10.1093/nar/gks1219.
[230] McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 2013;8. https://doi.org/10.1371/journal.pone.0061217.
[231] Chiller K, Selkin BA, Murakawa GJ. Skin microflora and bacterial infections of the skin. J Investig Dermatol Symp Proc 2001;6:170–4. https://doi.org/10.1046/j.0022-202x.2001.00043.x.
[232] Kandi V, Palange P, Vaish R, Bhatti AB, Kale V, Kandi MR, et al. Emerging Bacterial Infection: Identification and Clinical Significance of Kocuria Species. Cureus 2016;8:e731. https://doi.org/10.7759/cureus.731.
[233] Berlau J, Aucken H, Malnick H, Pitt T. Distribution of Acinetobacter species on skin of healthy humans. Eur J Clin Microbiol Infect Dis 1999;18:179–83. https://doi.org/10.1007/s100960050254.
[234] Khayyira AS, Rosdina AE, Irianti MI, Malik A. Simultaneous profiling and cultivation of the skin microbiome of healthy young adult skin for the development of therapeutic agents. Heliyon 2020;6:e03700. https://doi.org/10.1016/j.heliyon.2020.e03700.

[235] Love M, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. 2014. https://doi.org/10.1101/002832.
[236] Martin BD, Witten D, Willis AD. MODELING MICROBIAL ABUNDANCES AND DYSBIOSIS WITH BETA-BINOMIAL REGRESSION. Ann Appl Stat 2020;14:94–115. https://doi.org/10.1214/19-aoas1283.
[237] van Rensburg JJ, Lin H, Gao X, Toh E, Fortney KR, Ellinger S, et al. The Human Skin Microbiome Associates with the Outcome of and Is Influenced by Bacterial Infection. MBio 2015;6:e01315–15. https://doi.org/10.1128/mBio.01315-15.
[238] Timm CM, Loomis K, Stone W, Mehoke T, Brensinger B, Pellicore M, et al. Isolation and characterization of diverse microbial representatives from the human skin microbiome. Microbiome 2020;8:58. https://doi.org/10.1186/s40168-020-00831-y.
[239] Tomczak H, Bilska-Stokłosa J, Osmola K, Marcinkowski M, Błażejewska W, Myczko K, et al. Rothia mucilaginosa, rarely isolated pathogen as an etiological factor of infection of soft tissues in young, healthy woman. Postepy Hig Med Dosw 2013;67:1–5. https://doi.org/10.5604/17322693.1028763.
[240] Ara K, Hama M, Akiba S, Koike K, Okisaka K, Hagura T, et al. Foot odor due to microbial metabolism and its control. Can J Microbiol 2006;52:357–64. https://doi.org/10.1139/w05-130.
[241] Michalet S, Minard G, Chevalier W, Meiffren G, Saucereau Y, Tran Van V, et al. Identification of human skin bacteria attractive to the Asian Tiger mosquito. Environ Microbiol 2019;21:4662–74. https://doi.org/10.1111/1462-2920.14793.
[242] Van Rossum T, Ferretti P, Maistrenko OM, Bork P. Diversity within species: interpreting strains in microbiomes. Nat Rev Microbiol 2020;18:491–506. https://doi.org/10.1038/s41579-020-0368-1.
[243] Feltman H, Schulert G, Khan S, Jain M, Peterson L, Hauser AR. Prevalence of type III secretion genes in clinical and environmental isolates of Pseudomonas aeruginosa. Microbiology 2001;147:2659–69. https://doi.org/10.1099/00221287-147-10-2659.
[244] Jamal QMS, Ahmad V. Lysinibacilli: A Biological Factories Intended for Bio-Insecticidal, Bio-Control, and Bioremediation Activities. J Fungi (Basel) 2022;8. https://doi.org/10.3390/jof8121288.
[245] Li J, Gao R, Chen Y, Xue D, Han J, Wang J, et al. Isolation and Identification of Microvirga thermotolerans HR1, a Novel Thermo-Tolerant Bacterium, and Comparative Genomics among Microvirga Species. Microorganisms 2020;8. https://doi.org/10.3390/microorganisms8010101.
[246] Liu CM, Aziz M, Kachur S, Hsueh P-R, Huang Y-T, Keim P, et al. BactQuant: an enhanced broad-coverage bacterial quantitative real-time PCR assay. BMC Microbiol 2012;12:56. https://doi.org/10.1186/1471-2180-12-56.
[247] Smythe P, Wilkinson HN. The Skin Microbiome: Current Landscape and Future Opportunities. Int J Mol Sci 2023;24. https://doi.org/10.3390/ijms24043950.
[248] Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 2018;6:1–14. https://doi.org/10.1186/s40168-018-0605-2.
[249] Coutinho-Abreu IV, Riffell JA, Akbari OS. Human attractive cues and mosquito host-seeking behavior. Trends Parasitol 2022;38:246–64. https://doi.org/10.1016/j.pt.2021.09.012.
[250] Wooding M, Rohwer ER, Naudé Y. Chemical profiling of the human skin surface for malaria vector control via a non-invasive sorptive sampler with GC×GC-TOFMS. Anal Bioanal Chem 2020;412:5759–77. https://doi.org/10.1007/s00216-020-02799-y.
[251] Peñuelas J, Asensio D, Tholl D, Wenke K, Rosenkranz M, Piechulla B, et al. Biogenic volatile emissions from the soil. Plant Cell Environ 2014;37:1866–91. https://doi.org/10.1111/pce.12340.
[252] Menger DJ, Omusula P, Wouters K, Oketch C, Carreira AS, Durka M, et al. Eave

screening and push-pull tactics to reduce house entry by vectors of Malaria. Am J Trop Med Hyg 2016;94:868–78. https://doi.org/10.4269/ajtmh.15-0632.
[253] Colón-Crespo LJ, Herrera-Hernández D, Holness H, Furton KG. Determination of VOC marker combinations for the classification of individuals by gender and race/ethnicity. Forensic Sci Int 2017;270:193–9. https://doi.org/10.1016/j.forsciint.2016.09.011.
[254] Prokop-Prigge KA, Greene K, Varallo L, Wysocki CJ, Preti G. The Effect of Ethnicity on Human Axillary Odorant Production. J Chem Ecol 2016;42:33–9. https://doi.org/10.1007/s10886-015-0657-8.
[255] Bernier UR, Kline DL, Barnard DR, Schreck CE, Yost RA. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for the yellow fever mosquito (Aedes aegypti). Anal Chem 2000;72:747–56. https://doi.org/10.1021/ac990963k.
[256] Ottensmann M, Stoffel MA, Nichols HJ, Hoffman JI. GCalignR: An R package for aligning gas-chromatography data for ecological and evolutionary studies. PLoS One 2018;13:e0198311. https://doi.org/10.1371/journal.pone.0198311.
[257] Cork A, Park KC. Identification of electrophysiologically-active compounds for the malaria mosquito, Anopheles gambiae, in human sweat extracts. Med Vet Entomol 1996;10:269–76. https://doi.org/10.1111/J.1365-2915.1996.TB00742.X.
[258] Lemfack MC, Gohlke BO, Toguem SMT, Preissner S, Piechulla B, Preissner R. MVOC 2.0: A database of microbial volatiles. Nucleic Acids Res 2018;46:D1261–5. https://doi.org/10.1093/nar/gkx1016.
[259] James AG, Cox D, Worrall K. Microbiological and biochemical origins of human foot malodour. Flavour Fragr J 2013;28:231–7. https://doi.org/10.1002/ffj.3136.
[260] Dormont L, Bessiere JM, McKey D, Cohuet A. New methods for field collection of human skin volatiles and perspectives for their application in the chemical ecology of human-pathogen-vector interactions. J Exp Biol 2013;216:2783–8. https://doi.org/10.1242/jeb.085936.
[261] Nyamundanda G, Brennan L, Gormley IC. Probabilistic principal component analysis for metabolomic data. BMC Bioinformatics 2010;11:571. https://doi.org/10.1186/1471-2105-11-571.
[262] Jolliffe IT, Cadima J. Principal component analysis: a review and recent developments. Philos Trans A Math Phys Eng Sci 2016;374:20150202. https://doi.org/10.1098/rsta.2015.0202.
[263] Grant AJ, O'Connell RJ. Electrophysiological responses from receptor neurons in mosquito maxillary palp sensilla. Ciba Found Symp 1996;200:233–48; discussion 248–53, 281–4. https://doi.org/10.1002/9780470514948.ch17.
[264] Rios-Navarro A, Gonzalez M, Carazzone C, Celis Ramírez AM. Learning about microbial language: possible interactions mediated by microbial volatile organic compounds (VOCs) and relevance to understanding Malassezia spp. metabolism. Metabolomics 2021;17:39. https://doi.org/10.1007/s11306-021-01786-3.
[265] Cuadros-Rodríguez L, Gámiz-Gracia L, Almansa-López E, Laso-Sánchez J. Calibration in chemical measurement processes: I. A metrological approach. Trends Analyt Chem 2001;20:195–206. https://doi.org/10.1016/S0165-9936(00)00093-5.
[266] Leyden JJ, McGinley KJ, Mills OH, Kligman AM. Propionibacterium levels in patients with and without acne vulgaris. J Invest Dermatol 1975;65:382–4. https://doi.org/10.1111/1523-1747.ep12607634.
[267] Leyden JJ, Marples RR, Kligman AM. Staphylococcus aureus in the lesions of atopic dermatitis. Br J Dermatol 1974;90:525–30. https://doi.org/10.1111/j.1365-2133.1974.tb06447.x.
[268] Saxena R, Mittal P, Clavaud C, Dhakan DB, Hegde P, Veeranagaiah MM, et al. Comparison of Healthy and Dandruff Scalp Microbiome Reveals the Role of Commensals in Scalp Health. Front Cell Infect Microbiol 2018;8:346. https://doi.org/10.3389/fcimb.2018.00346.
[269] Paetzold B, Willis JR, Pereira De Lima J, Knödlseder N, Brüggemann H, Quist SR,

et al. Skin microbiome modulation induced by probiotic solutions. Microbiome 2019;7:1–9. https://doi.org/10.1186/s40168-019-0709-3.
[270] Adamu BO, Lawley TD. Bacteriotherapy for the treatment of intestinal dysbiosis caused by Clostridium difficile infection. Curr Opin Microbiol 2013;16:596–601. https://doi.org/10.1016/j.mib.2013.06.009.
[271] El-Salhy M, Hatlebakk JG, Gilja OH, Bråthen Kristoffersen A, Hausken T. Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. Gut 2020;69:859–67. https://doi.org/10.1136/gutjnl-2019-319630.
[272] Perin B, Addetia A, Qin X. Transfer of skin microbiota between two dissimilar autologous microenvironments: A pilot study. PLoS One 2019;14:1–17. https://doi.org/10.1371/journal.pone.0226857.
[273] Callewaert C, Lambert J, Van de Wiele T. Towards a bacterial treatment for armpit malodour. Exp Dermatol 2017;26:388–91. https://doi.org/10.1111/exd.13259.
[274] Morris BEL, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. FEMS Microbiol Rev 2013;37:384–406. https://doi.org/10.1111/1574-6976.12019.
[275] Dormont L, Mulatier M, Carrasco D, Cohuet A. Mosquito Attractants. J Chem Ecol 2021;47:351–93. https://doi.org/10.1007/s10886-021-01261-2.
[276] Potangwa H. Can the skin microbiome reduce Aedes aegypti bites? MSc in Medical Microbiology. London School of Hygiene and Tropical Medicine, 2021.
[277] Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, et al. Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. Gastroenterology 2015;149:102–9.e6. https://doi.org/10.1053/j.gastro.2015.04.001.
[278] Duvallet C, Zellmer C, Panchal P, Budree S, Osman M, Alm EJ. Framework for rational donor selection in fecal microbiota transplant clinical trials. PLoS One 2019;14:e0222881. https://doi.org/10.1371/journal.pone.0222881.
[279] Gibson B, Wilson DJ, Feil E, Eyre-Walker A. The distribution of bacterial doubling times in the wild. Proc Biol Sci 2018;285. https://doi.org/10.1098/rspb.2018.0789.
[280] Bubier JA, Chesler EJ, Weinstock GM. Host genetic control of gut microbiome composition. Mamm Genome 2021;32:263–81. https://doi.org/10.1007/s00335-021-09884-2.
[281] Steglińska A, Jachowicz A, Szulc J, Adamiak J, Otlewska A, Pielech-Przybylska K, et al. Factors influencing microbiological biodiversity of human foot skin. Int J Environ Res Public Health 2019;16. https://doi.org/10.3390/ijerph16183503.
[282] Dogan EB, Ayres JW, Rossignol PA. Behavioural mode of action of deet: inhibition of lactic acid attraction. Med Vet Entomol 1999;13:97–100. https://doi.org/10.1046/j.1365-2915.1999.00145.x.
[283] Verhulst NO, Cavegn JC, Mathis A. Spatial repellency and vapour toxicity of transfluthrin against the biting midges Culicoides nubeculosus and C. sonorensis (Ceratopogonidae). Curr Res Insect Sci 2021;1:100002. https://doi.org/10.1016/j.cris.2020.100002.
[284] Wu W, Yang Y, Feng Y, Ren X, Li Y, Li W, et al. Study of the Repellent Activity of 60 Essential Oils and Their Main Constituents against Aedes albopictus, and Nano-Formulation Development. Insects 2022;13. https://doi.org/10.3390/insects13121077.
[285] Woolhouse ME, Dye C, Etard JF, Smith T, Charlwood JD, Garnett GP, et al. Heterogeneities in the transmission of infectious agents: implications for the design of control programs. Proc Natl Acad Sci U S A 1997;94:338–42. https://doi.org/10.1073/pnas.94.1.338.
[286] Krieger CJ, Zhang P, Mueller LA, Wang A, Paley S, Arnaud M, et al. MetaCyc: a multiorganism database of metabolic pathways and enzymes. Nucleic Acids Res 2004;32:D438–42. https://doi.org/10.1093/nar/gkh100.
[287] Noble WC, McEwan Jenkinson D, Holland KT, McBride ME, Leyden JJ, McGinley KJ, et al. The Skin Microflora and Microbial Skin Disease. Cambridge University

Press; 1993. https://doi.org/10.1017/CBO9780511527012.
[288] Nam NN, Do HDK, Loan Trinh KT, Lee NY. Metagenomics: An Effective Approach for Exploring Microbial Diversity and Functions. Foods 2023;12. https://doi.org/10.3390/foods12112140.
[289] Srinivas M, O'Sullivan O, Cotter PD, van Sinderen D, Kenny JG. The Application of Metagenomics to Study Microbial Communities and Develop Desirable Traits in Fermented Foods. Foods 2022;11. https://doi.org/10.3390/foods11203297.
[290] Ferrocino I, Bellio A, Giordano M, Macori G, Romano A, Rantsiou K, et al. Shotgun Metagenomics and Volatilome Profile of the Microbiota of Fermented Sausages. Appl Environ Microbiol 2018;84. https://doi.org/10.1128/AEM.02120-17.
[291] Cuscó A, Catozzi C, Viñes J, Sanchez A, Francino O. Microbiota profiling with long amplicons using Nanopore sequencing: Full-length 16S rRNA gene and the 16S-ITS-23S of the rrn operon. F1000Res 2019;7:1–29. https://doi.org/10.12688/f1000research.16817.2.
[292] Cuscó A, Viñes J, D'Andreano S, Riva F, Casellas J, Sánchez A, et al. Using MinION™ to characterize dog skin microbiota through full-length 16S rRNA gene sequencing approach. bioRxiv 2017:1–20. https://doi.org/10.1101/167015.
[293] Rozas M, Brillet F, Callewaert C, Paetzold B. MinION™ Nanopore Sequencing of Skin Microbiome 16S and 16S-23S rRNA Gene Amplicons. Front Cell Infect Microbiol 2022;11:1–9. https://doi.org/10.3389/fcimb.2021.806476.
[294] Serghiou IR, Baker D, Evans R, Dalby MJ, Kiu R, Trampari E, et al. An efficient method for high molecular weight bacterial DNA extraction suitable for shotgun metagenomics from skin swabs. Microbial Genomics 2023;9:001058. https://doi.org/10.1099/mgen.0.001058.
[295] Acosta EM, Little KA, Bratton BP, Lopez JG, Mao X, Payne A, et al. Bacterial DNA on the skin surface overrepresents the viable skin microbiome 2023. https://doi.org/10.7554/elife.87192.1.
[296] Tidjani Alou M, Naud S, Khelaifia S, Bonnet M, Lagier J-C, Raoult D. State of the Art in the Culture of the Human Microbiota: New Interests and Strategies. Clin Microbiol Rev 2020;34. https://doi.org/10.1128/CMR.00129-19.
[297] Forster SC, Kumar N, Anonye BO, Almeida A, Viciani E, Stares MD, et al. A human gut bacterial genome and culture collection for improved metagenomic analyses. Nat Biotechnol 2019;37:186–92. https://doi.org/10.1038/s41587-018-0009-7.
[298] Hosokawa M, Endoh T, Kamata K, Arikawa K, Nishikawa Y, Kogawa M, et al. Strain-level profiling of viable microbial community by selective single-cell genome sequencing. Sci Rep 2022;12:4443. https://doi.org/10.1038/s41598-022-08401-y.
[299] McBride CS, Baier F, Omondi AB, Spitzer SA, Lutomiah J, Sang R, et al. Evolution of mosquito preference for humans linked to an odorant receptor. Nature 2014;515:222–7. https://doi.org/10.1038/nature13964.
[300] Hiscox A, Otieno B, Kibet A, Mweresa CK, Omusula P, Geier M, et al. Development and optimization of the Suna trap as a tool for mosquito monitoring and control. Malar J 2014;13:1–14. https://doi.org/10.1186/1475-2875-13-257.
[301] Gallagher M, Wysocki CJ, Leyden JJ, Spielman AI, Sun X, Preti G. Analyses of volatile organic compounds from human skin. Br J Dermatol 2008;159:780–91. https://doi.org/10.1111/j.1365-2133.2008.08748.x.
[302] Larson PJ, Chong D, Fleming E, Oh J. Challenges in Developing a Human Model System for Skin Microbiome Research. J Invest Dermatol 2021;141:228–31.e4. https://doi.org/10.1016/j.jid.2020.05.096.

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Dr Julien Martinez
Research Fellow
Department of Disease Control (DCD)
Infectious and Tropical Diseases (ITD)
LSHTM

13 December 2017

Dear Julien

Study Title: Genetics of human attractiveness to malaria mosquitoes

LSHTM Ethics Ref: 14500

Thank you for responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Investigator CV	CV_Julien Martinez	27/08/2017	v1
Investigator CV	CV_Catherine Oke	27/08/2017	v1
Investigator CV	CV_James Logan	27/08/2017	v1
Investigator CV	CV_John Armour_Sept2017	27/08/2017	v1
Investigator CV	CV_Steve Lindsay	27/08/2017	v1
Investigator CV	CV_Rachel Allen	27/08/2017	v1
Protocol / Proposal	Study Protocol	22/09/2017	v1
Protocol / Proposal	Questionnaire	22/09/2017	v1
Investigator CV	CV_John Pickett	27/09/2017	v1
Covering Letter	Cover Letter	05/12/2017	v1
Information Sheet	PARTICIPANT INFORMATION SHEET_v2	06/12/2017	v2
Information Sheet	Consent Form	06/12/2017	v1
Advertisements	Email template_v2	06/12/2017	v2
Local Approval	TwinsUK approval	06/12/2017	v1

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

Professor John DH Porter
Chair

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

Faculty of Infectious and Tropical Disease
Department of Disease Control
London School of Hygiene & Tropical Medicine
Keppel Street
London School of Hygiene & Tropical Medicine WC1E 7HT
Phone: 02076127813
Email: julien.martinez@lshtm.ac.uk

PARTICIPANT INFORMATION SHEET

1. Research project title: Why do some people attract mosquitoes more than others?

2. Invitation

You are being invited to take part in an experiment to investigate the genetic basis of your level of attractiveness to mosquitoes. Before you decide, it is important for you to understand why the test is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

3. What is the purpose of this experiment?

There is strong evidence that some people attract mosquitoes more than others. Understanding why attractiveness varies according to people is a first step towards developing new ways to protect against biting insects as well as identifying populations that are more at risk of being bitten. This is important for disease control since the frequency of biting is a major determinant of mosquito-borne diseases transmission such as malaria.

In previous studies we have shown that people who are less attractive to mosquitoes produce natural repellent volatiles through their skin and that this is determined by your genetic make-up. Several other studies also pointed out that bacteria living on your skin can influence the biting behaviour of mosquitoes. This new study has been designed to investigate further the genetic basis of attractiveness to mosquitoes and examine to what extent skin bacteria are involved.

4. Why have I been chosen?

You have been chosen as someone who may be interested in assisting in research associated with mosquitoes. Moreover, you are among the twins for which TwinsUK has already collected genetic data. Such data will be extremely useful to identify genes that influence attractiveness to mosquitoes.

5. Do I have to take part?

It is up to you to decide whether or not to take part in this study. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time before the tests start without giving a reason.

Faculty of Infectious and Tropical Disease
Department of Disease Control
London School of Hygiene & Tropical Medicine
Keppel Street
London School of Hygiene & Tropical Medicine WC1E 7HT
Phone: 02076127813
Email: julien.martinez@lshtm.ac.uk

6. What will happen to me if I take part?

Firstly, you will be given a full explanation of the study and what will be involved. You will then be shown a list of inclusion criteria (things you must have or do) and exclusion (things you must not have or not do) to make sure you are eligible to take part.

7. What do I have to do?

Those who proceed to take part in the study will need to provide written consent that they agree to participate after all their questions have been answered.

Once consented, you will be requested to follow these rules for 24 hours before experiment:

- No alcohol or spicy food i.e. curries, chilli, garlic to be consumed prior to experiments, keep your amount of exercise to a minimum – no excessive exercise.

- Wash using hot water and unperfumed soap (provided, Simple soap) only – no other soap, shampoo, deodorant, perfumes, cosmetics, etc please.

- After washing, wear the nylon stockings for 7-8 hours (including overnight) before your visit to LSHTM.

You will be asked to attend the insectary at LSHTM along with your twin. There you will be asked to sit for 2 hours on a chair during which the experiment to collect your body odour will be conducted. The experiment will be carried out in a concealed room. You will be asked to place one foot into a collection bag with the assistance of a member of the research team who will seal the bags. Tubes will be attached to the bag, leading to air pumps which will slowly push purified air into the bag, and pulled out at an even slower rate into collection tubes. This allows the air to circulate around the foot before being removed. Air will then be passed through a tube containing filters which removes chemicals from the air. Your heart rate will be taken by counting pulse and body temperature will be taken immediately before, periodically throughout and immediately after the experiment. The body temperature will be taken using a Braun ThermoScan Instant Ear Thermometer.

At the end of the experiment, we will also collect a sample of the bacteria living on the skin of your hands and feet by gently rubbing a premoistened swab on the skin surface. This non-invasive collection technique will allow us to identify the type of bacteria that may affect your attractiveness to mosquitoes.

The odour samples will be stored at -20°C and used at a later date for gas-chromatography, electrophysiological techniques and mass spectrometry to identify volatile chemicals from your body and find which ones affect mosquito behaviour. The stockings will also be used later in behavioural studies with mosquitoes.

Faculty of Infectious and Tropical Disease
Department of Disease Control
London School of Hygiene & Tropical Medicine
Keppel Street
London School of Hygiene & Tropical Medicine WC1E 7HT
Phone: 02076127813
Email: julien.martinez@lshtm.ac.uk

Finally, an analysis combining the above measurements and your genetic data provided by TwinsUK will be conducted to identify potential genes underlying attractiveness to mosquitoes.

Reasonable travel expenses to attend your visit at the School will be reimbursed. These expenses will need to be justified and supported by the relevant receipts.

8. What are the side effects of taking part?

There is very little risk involved to those taking part in the study, and we have undertaken several similar experiments over the last 12 years. Your movement will be restricted for 2 hours, as you will be required to keep one foot in the odour collection bag but we will make sure that you sit comfortably. It is possible, though unlikely, that you may experience distress or discomfort. If this happens you must inform the experiment operator and the experiment will be stopped immediately at your request. Although as a participant you are not expected to have any contact with mosquitoes, there is a small risk you may be bitten when passing through the laboratories. All insects are free from any diseases or viruses. If you experience any adverse reaction during the experiment you must inform the investigators and you will be referred directly to your own GP or the GP associated with the London School of Hygiene and Tropical Medicine.

9. What are the possible benefits of taking part?

There is no direct benefit to you, however, the information we get from this study may provide essential materials that will aid in the identification of effective control methods of vector insects. In particular, a greater understanding of the biology of the human body and the mosquito *Anopheles gambiae*, the most important vector of malaria in humans, will be obtained. It is hoped that this information will contribute to achieving adequate control of this and many other pathogen-vectoring species. If achieved, this will benefit many people who live in developing countries and those that visit, by reducing their risk of contracting these diseases.

10. What happens when the experiment finishes?

Results from this experiment will be analysed and could be published in a scientific journal. Names and details of volunteers will not be reported and will be kept confidential.

11. What if something goes wrong?

The London School of Hygiene & Tropical Medicine holds insurance policies which apply to this study. If you experience harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that the School is at fault. This does not affect your legal rights to seek compensation.

Faculty of Infectious and Tropical Disease
Department of Disease Control
London School of Hygiene & Tropical Medicine
Keppel Street
London School of Hygiene & Tropical Medicine WC1E 7HT
Phone: 02076127813
Email: julien.martinez@lshtm.ac.uk

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the investigator.

12. Will my taking part in this study be kept confidential?

Yes. Your name will not be disclosed outside the London School of Hygiene and Tropical Medicine. You will not be identified in any report or publication.

Contact for further information

Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine
Dr Julien Martinez, Research Fellow ; E-mail: julien.martinez@lshtm.ac.uk ; Tel: 02076127813
Catherine Oke, Research Assistant ; E-mail: Catherine.oke@lshtm.ac.uk ; Tel: 02076127814
Prof James Logan, Chief Investigator ; E-mail: james.logan@lshtm.ac.uk ; Tel: 02079272008

Thank you for taking part in this study.

Faculty of Infectious and Tropical Disease
Department of Disease Control
London School of Hygiene & Tropical Medicine
Keppel Street
London School of Hygiene & Tropical Medicine WC1E 7HT
Phone: 02076127813
Email: julien.martinez@lshtm.ac.uk

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Consent Form

Consent by volunteer to participate in: Why do some people attract mosquitoes more than others?

Name of Volunteer: ……

Volunteer Code: ……

Chief/Principle Investigator Name: …….

I have read the volunteer information sheet on the above study and have had the opportunity to discuss the details with Dr Julien Martinez and ask questions. Dr Martinez has explained to me the nature and purpose of the tests to be undertaken. I understand fully what is to be done.

I have agreed to take part in the study as it has been outlined to me, but I understand that I am completely free to withdraw from the study or any part of the study at any time I wish.

I hereby fully and freely consent to participate in the study which has been explained to me.

Signature of Volunteer: ……

Date: …….

I confirm that I have explained to the volunteer named above, the nature and the purpose of the tests to be undertaken.

Signature of Investigator: …….

Date: …….

C/o MRC Unit: The Gambia @ LSHTM, Fajara
P.O. Box 273, Banjul
The Gambia, West Africa
Fax: +220 – 4495919 or 4496513
Tel: +220 – 4495442-6 Ext. 2308
Email: ethics@mrc.gm

The Gambia Government/MRC Joint

ETHICS COMMITTEE

Professor James Logan

London School of Hygiene & Tropical Medicine

24 September 2019

Dear Professor Logan

GG/MRCG Ethics Ref: 17537: The mechanisms underlying the production of natural mosquito repellents by human beings (The Gambia)

Thank you for responding to the Ethics Committee's request for further information on the above research. The further information has been considered by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Other	Certificate for working with human tissue assessment	28/05/2019	1
Protocol / Proposal	Study Protocol Gambia	28/05/2019	1.0
Other	Working with Human Tissue_Human Tissue online training Certificate	30/05/2019	1
Investigator CV	James Logan	04/06/2019	1
Investigator CV	John Armour	04/06/2019	1
Investigator CV	John Pickett	04/06/2019	1
Investigator CV	Rachel Allen	04/06/2019	1
Covering Letter	Ethics_coverletter	14/08/2019	1.0
Information Sheet	Participant Infomation Sheet and Consent Form V1.1	14/08/2019	1.1

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: https://leo.lshtm.ac.uk. Additional information is available at: www.lshtm.ac.uk/ethics.

Yours sincerely,

Dr. Mohammadou Kabir Cham
Chair, Gambia Government/MRCG Joint Ethics Committee

C/O MRC Unit The Gambia at LSHTM
PO Box 273 Banjul, The Gambia
West Africa
Switchboard (+220) 4495442/6 Ext 2308
Fax (+220) 4495919/4496513
E-mail: scc@mrc.gm
Intranet: http://mrcportal/Committees/SCC/SitePages/Home.aspx
Webpage:https://mrcportal.mrc.gm/Committees/SCC/SitePages/Home.aspx

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Miss Alicia Showering
LSHTM

15 January 2020

Dear Alicia,

Study Title: The relative contributions of the skin microbiome, genetics and Plasmodium infection in human attractiveness to malaria mosquitoes

LSHTM Ethics Ref: 18044

Thank you for responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Other	Research ethics certificate	20/11/2019	1.0
Protocol / Proposal	Study Protocol Gambia_v3.0 nontracked	20/11/2019	3.0
Protocol / Proposal	Study protocol UK	20/11/2019	1.0
Investigator CV	James Logan CV_2page_2017	20/11/2019	1.0
Investigator CV	New Academic CV Current2	20/11/2019	1.0
Consent form	Consent Form UK	20/11/2019	1.0
Consent form	Participant Infomation Sheet and Consent Form_v3.0	20/11/2019	1.0
Consent form	PARTICIPANT INFORMATION SHEET_v3	20/11/2019	3.0
Other	GCP 15 March 2018 JL	14/01/2020	1
Covering Letter	Ethics changes2	14/01/2020	2

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

Professor Jimmy Whitworth
Chair

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Miss Alicia Showering
LSHTM

19 January 2022

Dear Miss Alicia Showering

Study Title: Aedes SMT 2.0: Can skin microbiome transfers reduce Aedes mosquito bites?

LSHTM Ethics Ref: 26659

Thank you for responding to the Interventions Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Protocol / Proposal	Participant Information Sheet	15/11/2021	1
Protocol / Proposal	AedesSMTConsentForm	15/11/2021	1
Investigator CV	James Logan CV_2page_2017	16/11/2021	1
Investigator CV	academic CV	16/11/2021	1
Other	goodclinicalpractice2021	16/11/2021	1
Other	ResearchEthics2021	16/11/2021	1
Information Sheet	Participant Information Sheet	16/11/2021	1
Information Sheet	AedesSMTConsentForm	16/11/2021	1
Advertisements	Email	16/11/2021	1
Other	human tissue	16/11/2021	1
Sponsor Letter	2021-KEP-719 Sponsor Confirmation	22/11/2021	1
Protocol / Proposal	Final SOP	23/11/2021	1
Protocol / Proposal	Questionnaire	23/11/2021	1
Protocol / Proposal	Study Protocol ASV2	23/11/2021	1
Covering Letter	Ethics cover letter AS SMT2.0	12/01/2022	1

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

Professor Jimmy Whitworth
Chair

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

Participant Information Sheet

Title of project: Can the skin microbiome reduce *Aedes* mosquito bites?

Introduction

We would like to invite you to take part in our research study. Joining the study is entirely up to you, before you decide we would like you to understand why the research is being done and what it would involve. One of our team will go through this information sheet with you and answer any questions you may have. Please feel free to talk to others about the study if you wish and take time to decide whether or not to take part.

What is the purpose of the study?

The London School of Hygiene and Tropical Medicine (LSHTM) are conducting research into the skin microbiome to investigate why certain microbes on human skin are attractive to *Aedes* mosquitoes, and whether skin microbiome transplantation can reduce mosquito bites. Human skin microbiome consists of a variety of microorganisms such as bacteria, viruses and fungi, the composition varies between people which results in your unique skin microbiome. Understanding which microbes are different between highly and poorly attractive people to mosquitoes will allow us to explore alternative protective strategies to reduce the number of mosquito bites and therefore protect from mosquito-borne diseases such as dengue, Zika and chikungunya.

Do I have to take part?

No. It is up to you to decide if you would like to take part or not. If you do not want to take part, that is ok.

We will discuss the study together and give you a copy of this information sheet. If you agree to take part, we will then ask you to sign a consent form.

What will happen to me if I take part?

During your first visit to the LSHTM laboratory we will conduct a foot on cage assay. You will be asked to remove your sock from your right foot and place it on

top of a cage containing 20 female *Aedes* mosquitoes for 2 minutes. You will not be bitten. To prevent bites there will a safe gap between the foot and the cage. Cage assays are a common method used in behavioural studies with mosquitoes to screen people for how attractive they are to mosquitoes. The data collected from the screening will allow us to classify your attractiveness to mosquitoes. Figure 1 shows the cage assay we will be using to screen attractiveness to *Aedes* mosquitoes.

Figure 1: Foot on cage assay, the participants foot is placed on top of a cage containing 20 female mosquitoes, the plastic grid prevents the participant from being bitten.

If you are selected as a highly or poorly attractive individual you will be asked to return for a second visit. During this visit if you are in the poorly attractive group we will ask you to sit for a few minutes whilst we take skin swabs from the sole of your feet. This will be done aseptically using a sterile premoistened swab. The procedure itself should take 2-3 minutes. We may re-screen you for attractiveness to mosquitoes using the foot on cage assay. We may culture some bacteria from the swabs we collect from the foot.

If you are selected as the highly attractive group, you will be asked to sit while a skin microbiome sample is put on your foot using a plastic spreader, the procedure itself should take 30 seconds. We will also collect skin swabs from the sole of your feet before and after the transfer. This will be done aseptically using a sterile premoistened swab. The procedure itself should take 2-3 minutes. If you are in this group, you will be invited back for three further visits where we will collect a skin

microbiome sample from both the foot that received the skin microbiome transplant and the control foot using a swab as described above and check your attractiveness to mosquitoes using the foot on cage test. We will ask you to avoid washing between these sampling points which will be 6 and 24 hours post the transfer. There is an optional final time point at 72 hours.

You will not be paid for participating in this study.

If the research study needs to be stopped, you will be informed as soon as possible.

What will I have to do?

You will be asked to adhere to the following protocol for 24 hours prior to each visit for sample collection:

- No alcohol
- No spicy or strong-smelling foods
- No bathing with antibacterial soaps prior to test (we can provide odour free soap on request)
- No scented soaps, moisturisers, perfumes, cosmetics etc

What harm or discomfort can you expect in the study?

Your movement will be restricted during the procedure, as you will be required to keep one foot on top of the cage containing mosquitoes for a few minutes, but we will ensure that you sit comfortably, during both the foot on cage assay and during the skin swab. During the foot on cage test the mosquitoes will not be able to bite you through the cage. However, if you experience any discomfort, we will end the sample collection.

There will be a low risk of transferring infections (e.g., verruca's, athlete's foot), but this will be reduced by the screening (questionnaire). Only participants who do not have/ had skin condition will be chosen for this study and during the sample collection we will look for visible signs of skin infection.

To further reduce the risk of transmission of potential skin infections the square frame on which participants place their foot on will be cleaned between visits.

What are the possible benefits?

We hope that the data collected during this study will help our knowledge and understanding of skin microbiome transfers and could be a method of protecting from deadly vector borne diseases like dengue in the future.

What if something goes wrong?

The London School of Hygiene and Tropical Medicine holds insurance policies which apply to this study. If you experience harm or injury as a result of taking part in this study, you may be eligible to claim compensation.

If you have a concern about any aspect of this study, you should ask to speak to the researcher who will do their best to answer your questions alicia.showering1@lshtm.ac.uk. If you remain unhappy and wish to complain formally, you can do this by contacting Professor James Logan at james.logan@lshtm.ac.uk .

Can I change my mind about taking part?

Yes. You can withdraw from the study at any time without giving a reason. You just need to let us know that you do not want to be part of the study anymore.

If you decide to withdraw your participation during the study, any information already generated from the samples until the time of withdrawal will be used and samples already collected, for which you have given consent, will also be analysed and data used.

What will happen to information collected about me?

All information that is collected about you in the course of the study will be kept strictly confidential. Your personal information will only be available to the study team members and might be seen by some members of the Ethics Committee, Government authorities and sponsor (LSHTM).

Your samples will be stored at the lab for up to two years and if you agree, we may use some of the samples collected for future studies. These will be anonymised when stored, and all future research using these samples will be reviewed by an independent ethics committee.

What are your choices about how your information is used?

You have the option to stop being part of the study at any time, without giving a reason, but we will keep information about you that we have already collected.

Where can you find out more about how your information is used?

You can find out more about how we use your information.

- At https://www.lshtm.ac.uk/files/research-participant-privacy-notice.pdf
- By asking one of the research team via email or during a visit.

What will happen to the results of this study?

The study results will be published in a scientific journal and made available to the scientific community. Your personal information will not be included in the study report and there is no way that you can be identified from it.

Who is organising and funding this study?

London School of Hygiene & Tropical Medicine is the sponsor for the research, and they have full responsibility for the project including the collection, storage and analysis of your data. This means we are responsible for looking after your information and using it properly.

Who has reviewed this study?

All research involving human participants is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by The London School of Hygiene and Tropical Medicine Ethics Committee.

Further information and contact details.

Thank you for taking time to read this information sheet. If you think you will take part in the study, please read and sign the consent form.

If you would like any further information the contact details are below.

Contact details:

alicia.showering1@lshtm.ac.uk

Phone number:

020 7927 2161

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Questionnaire

Note: This form was converted into ODK format for data collection

Title of project: Can the skin microbiome reduce *Aedes* mosquito bites?

Section 1: About you

1. How old are you (age in years)?

_____years

2. What is your sex?

☐ Female

☐ Male

☐ Other

3. How tall are you?

_____

4. How much do you weigh?

_____

5. What is your ethnic group?

White

☐ English, Welsh, Scottish, Northern Irish or British

☐ Irish

☐ Gypsy or Irish Traveller

☐ Any other White background

Mixed or Multiple ethnic groups

☐ White and Black Caribbean

☐ White and Black African

☐ White and Asian

☐ Any other Mixed or Multiple ethnic background

Asian or Asian British

☐ Indian

☐ Pakistani

☐ Bangladeshi

☐ Chinese

☐ Any other Asian background

Black, African, Caribbean or Black British

☐ African

☐ Caribbean

☐ Any other Black, African or Caribbean background

Other ethnic group

☐ Arab

☐ Any other ethnic group

Section 2: Your health

6. Have you taken any antibiotics in the last 6 months?

☐ Yes
☐ No

b) If so, what were they?

_____

7. a) Do you or have any skin conditions?

☐ Yes

☐ No

b) If yes, what condition?

_____

c) If yes, have you taken any over-the-counter medicine or cream or been prescribed any drugs for your skin condition?

☐ Yes

☐ No

d) If yes, which?

_____

8) Are you taking any medication?

☐ Yes

☐ No

b) If so, what medication or type of medication?

_____

Thank you for taking the time to fill out this questionnaire.

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Participant Consent Form

Title of project: Can the skin microbiome reduce *Aedes* mosquito bites?

Name of participant	
Participant ID	
Date	

Statement	**Tick option**
I confirm that I have read and understand the Participant information sheet for the above-named study. I have had the opportunity to consider the information, ask questions and have these answered satisfactorily.	Yes ☐ No ☐
I understand and agree that my consent is voluntary and that I am free to withdraw this consent at any time without giving any reason and without my/the participant's medical care or legal rights being affected.	Yes ☐ No ☐
I understand and agree that relevant sections of my/ the participant's data collected during the study may be looked at by authorised individuals, where it is relevant to my/ the participant's taking part in this research. I give permission for these individuals to have access to these records.	Yes ☐ No ☐
I understand and agree that data about/ from me/ the participant will be shared via a public data repository or be shared directly with other researchers, and that I will not be identifiable from this information.	Yes ☐ No ☐
I understand and agree that the tissue sample collected from me/ the participant will be used to support other research in the future and may be shared anonymously with other researchers for their ethically approved projects.	Yes ☐ No ☐
I agree to / the participant agrees to taking part in the above-named study.	Yes ☐ No ☐

Signature of participant	

Name of the person obtaining consent	**Signature of person obtaining consent**	**Date**