The Smell of Infection:

Detecting Infectious Disease and Determining Mechanisms Underlying the Spread of Disease in Social Insects

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A thesis submitted for the degree of

Doctor of Philosophy in Biosciences

Cardiff University

September 2025

Thesis summary

Infection poses a universal challenge for animals, and many species rely on behavioural and chemical cues to limit pathogen transmission. Social immunity mitigates this risk, yet the chemical cues driving responses remain poorly understood. This thesis explores whether there is a consistent "smell of infection" in honey bees and whether such cues regulate social immunity.

Chapter 2 reviews volatile organic compounds (VOCs) associated with infection in insects, highlighting their potential for non-invasive disease surveillance. A comprehensive meta-analysis of all current studies sampling VOCs from honey bee infections (n=23) showed that infected and uninfected brood can be distinguished across major pathogens, including *Varroa destructor*, *Paenibacillus larvae*, and *Ascosphaera apis* (Chapter 3). Infection signatures were defined not by single biomarkers but by bouquets of volatiles. However, no studies focused on adult bees; an obvious gap given transmission occurs mostly between adults. This knowledge gap was addressed by sampling adult bees infected with *Vairimorpha ceranae* (Chapter 4), finding that volatile profiles shifted in detectable but transient ways. Discrimination was strongest at six and twelve days post-infection, driven by subsets of compounds whose abundances oscillated with infection stage, confirming that adult infections also alter VOCs. Whether olfactory cues mediate adult social immunity was then investigated (Chapter 5). Dyadic assays isolating volatile from low-volatility compounds showed no behavioural responses to infected bees, suggesting group context is critical. Field assays using observation hives confirmed this (Chapter 6): both infected and uninfected bees perfumed with infection scent were treated similarly when introduced into a colony; receiving heightened aggression, avoidance, and intensive grooming. This demonstrates that odour alone can trigger adult-focused social immunity, but only within the colony context.

These findings show that infection alters volatile emissions across life stages and that olfactory cues mediate colony-level responses, with implications for social immunity, pollinator health, and non-invasive disease surveillance.

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Acknowledgements

"*I am dying by inches from not having anybody to talk to about insects*"

- Charles Darwin

My obsession with insects and arthropods began in childhood. In the back garden of my family home, I found a caterpillar on a wall. Fascinated by its strange form, I couldn't resist giving it a prod, whereupon it promptly tumbled into a spider's web. The spider darted out, killed it, and began to feed. Strangely, or perhaps worryingly, I found this even more captivating than the caterpillar itself. That moment sparked a fascination with the darker side of insect life (or death) – disease, predation, and everything in between. From there, my curiosity grew into an enthusiasm that even YouTube tarantula feeding videos could not satisfy. Eventually, I wore down my mum enough to let me have a tarantula of my own. So, my first thank you goes to Mum – for tolerating the arrival of a large, venomous spider in the house.

When that same tarantula died during my undergraduate years, I wanted to preserve it. That led me to the local entomology department, where Jane Akerman taught me so much, first as a curious student, and later during my placement in her lab. It was then I realised entomology could be more than a hobby; it could be a career. Jane, thank you for opening my eyes to the extraordinary diversity of insects and for giving me the foundational skills I still use today, and will continue to use throughout my career.

To my supervisors, Carsten and Sarah – for giving me the freedom to steer this project in directions that made it truly my own, and for supporting my insect obsession. Your encouragement, kindness, and guidance have shaped me enormously as a researcher and writer. I have learned so much from you both, not only about science, but also about how to engage with and inspire students.

This project would not have been possible without Harold Williams – my bee sensei. From the very first year of my PhD, you took me under your wing with no expectation of anything in return and taught me everything I know about beekeeping. From assembling hive boxes in your workshop, to rushing out when I was convinced my

colonies were failing, to inviting me into your home to extract honey. Your generosity kept me on track. Without your guidance, I would have been completely lost.

Peter Graystock – for responding with such openness when I cold-emailed you for advice on identifying honey bee diseases. You welcomed me into your lab, taught me how to isolate spores, and showed me the best way to make honey bee cages. I dread to think how long it would have taken me to make the hundreds (upon hundreds) of cages I needed without your soldering iron trick.

Richard Ludlow and Sam Vaughaun – for all your help setting up the apiary, for bringing me coffee while I sat in my shed staring at bees all day (whether in freezing cold or sweltering heat), and for actually helping me build that very shed in the first place, without it, my observations would never have been possible. Richard, thank you especially for the time you sacrificed during my frantic pilot studies as I tried to get volatile sampling to work, and for the friendship along the way.

To my postgrad squad – from our weekly Lerkins meetings to our many nights out, having a group of friends going through the same journey kept me sane. Al Carr, for all the gaming and endless pool matches (final score 40–12 to me – you'll catch up one day). Neil Cook, for the encouragement during rough patches, our mutual love of snooker, showing me how the old-schoolers barbecue, and for traumatising me with your "rapping" skills. We laughed a lot, and I'm grateful for it.

Jordan Cuff – for welcoming me so warmly when I cornered you at my first big conference, and for our conference dinner wine traditions ever since. It's rare to find someone as equally amazed by spiders and insects as I am, and your mentorship, advice, and encouragement have meant so much. You went above and beyond to find opportunities for me, and you've been a real inspiration for the kind of researcher I aspire to be.

The Royal Entomological Society and my co-student reps, Ben Hawthorne and Vera Kaunath – for being such a brilliant team, not only in all the outreach we did together but also in the support throughout and for showing me how amazing the entomology community is.

Jess Chivers — for always being there, my buddy since Year 8. Always encouraging me and singing my praises throughout my academic journey, and for all the music, festivals, gigs, and adventures that kept me going along the way.

And last, but by no means least, Izzy – my partner through it all. You've been there every step of the way: from naming my very first tarantula after you, to sharing a house full of bug pets, to putting up with me being a perpetual student for the last ten years. You've held off on holidays because I couldn't bear to leave my bees, listened (and sometimes not) to my endless rambling about insects, and kept my life in order when all I could think about was research. You've shared in every high – jumping for joy with me when things went right – and steadied me through every low when it all felt too much. I couldn't have done any of this without your love, patience, and support. Oh, and thank you for letting me use your car to transport thousands of live, angry bees around the city.

Statement of Contributions

This PhD was funded by the Natural Environment Research Council through the GW4+ Doctoral Training Partnership (NE/S007504/1). It was also funded through our CASE Partner, Markes International and received additional funding from Bee Disease Insurance Ltd. The project was proposed by the supervisory team, Carsten T Müller and Sarah E Perkins who both contributed to execution of each manuscript/chapter in this thesis.

Chapter 1: Introduction. Writing by Ayman Asiri.

Chapter 2: The smell of infection: disease surveillance using volatile organic compounds (VOCs). Writing by Ayman Asiri.

Chapter 3: Chapter 3: Volatile organic compounds as indicators of infection in honey bees (*Apis mellifera*): A meta-analysis. Data collection, analysis, and writing by Ayman Asiri.

Chapter 4: Chapter 4: Wake Up and Smell the Infected Bees: Volatile cues of *Vairimorpha* infection in honey bees. Field and lab work, data analysis, and writing by Ayman Asiri. Richard Ludlow contributed towards GCxGC-MS volatile sample processing.

Chapter 5: Smell as a mechanism regulating the social response of honeybees to infection with *Vairimorpha ceranae*. Field and lab work, data analysis, and writing by Ayman Asiri. Peter Graystock (Imperial College London) contributed *Vairimorpha ceranae* spores and ran PCRs for species identification.

Chapter 6: Chapter 6: The smell of infection as a mechanism underlying social immunity in social insects. Field and lab work, data analysis, and writing by Ayman Asiri.

Chapter 7: General discussion. Writing by Ayman Asiri.

Preface

Publications

Chapter 2 of my thesis (*The smell of infection: disease surveillance in insects using volatile organic compounds (VOCs)*) was published as an open-access article in *Agricultural and Forest Entomology*, with co-authors by Dr Sarah E Perkins and Dr Carsten T Müller where I was invited to contribute an article to the special issue: *Advances in Insect Biomonitoring for Agriculture and Forestry* (https://doi.org/10.1111/afe.12651).

Chapter 3 (*Volatile organic compounds as indicators of infection in honey bees (Apis mellifera): A meta-analysis*) co-authored by Dr Sarah E Perkins and Dr Carsten T Müller, has also been submitted to the *International Journal for Parasitology* and is currently under review.

Chapter 4, 5, and 6 are all *in prep* for submission as manuscripts, co-authored by Dr Sarah E Perkins and Dr Carsten T Müller.

I also published one other research paper during my PhD that was not part of my thesis:

Asiri, A. and Foster, C. (2022) 'Temporal and climatic variation in the colour forms of Adalia bipunctata and Harmonia axyridis (Coleoptera: Coccinellidae) populations in the United Kingdom', *European Journal of Entomology,* 119.

Grants

Bee Diseases Insurance Ltd (BDI) Research Grant - £11,492.60.

Royal Entomological Society Equality, Diversity and Inclusion Grant – "*What is an Entomologist?*" - £1157.56.

Royal Entomological Society Conference Participation Fund - £277.

Conference presentations

Oral presentation: “Scent profile of honey bees differs depending on infection”, Wales Ecology and Evolution Network, 2022, Centre for Alternative Technology, Machynlleth

- **Best Talk Award**

Poster presentation: “The smell of infection: is smell associated with *Melisococcus plutonius* infection in honey bee larvae?”, Royal Entomological Society Entomological Society Ento22, 2022, University of Lincoln.

Poster presentation: “The smell of infection: is smell associated with *Melisococcus plutonius* infection in honey bee larvae?”, International Union for the Study of Social Insects Winter Meeting, 2022, Natural History Museum, London.

- **Best Poster Award**

Poster presentation: “The smell of infection: is smell associated with *Melisococcus plutonius* infection in honey bee larvae?”, British Ecological Society Annual Meeting, 2022, Edinburgh.

Oral presentation: “Does infection change the smell of honey bees?”, Organisms and Environment Away Day, 2023, Cardiff University.

- **Best Talk Award**

Oral presentation: “Does infection change the smell of honey bees?”, Royal Entomological Society Entomological Society Ento23, 2023, University of Exeter, Penryn.

Poster presentation: “Smell as a mechanism regulating the social response of honey bee dyads to infection with *Vairimorpha* (*Nosema*) *ceranae*”, 2023, International Union for the Study of Social Insects Winter Meeting, University of Bristol.

Poster presentation: “Smell as a mechanism regulating the social response of honey bee dyads to infection with *Vairimorpha* (*Nosema*) *ceranae*”, 2023, Wales Ecology and Evolution Network, 2023, Centre for Alternative Technology, Machynlleth.

- **Best Poster Award**

Oral presentation: “Sniff, Detect, Reject: Honey bees use olfaction to regulate their defences against infection”, 2025, Royal Entomological Society Student Forum, Reading University.

- **Runners Up Prize for Best Talk**

Training programmes

Throughout my PhD I also attended certified postgraduate training courses to further my research skills:

Insect Chemical Ecology. ‘Impact of semiochemicals on insect behaviour’, 2023, Swedish University of Agricultural Sciences, Alnarp, Sweden.

Aarhus Comprehensive Computational Entomology Summer School (ACCESS). ‘Automated monitoring of Insects’, 2024, Mols Bjerge National Park, Aarhus, Denmark.

‘Insect Taxonomy and Field Sampling Skills’, 2025, Oxford University, Museum of Natural History.

Student supervision

Niall Fadden, 2024. Studying Disease Transmission and Behavioural Immunity in the Terrestrial Isopod *Porcellio scaber*. MSci final year project.

Outreach and engagement

Throughout the first three-years of my PhD I was a student representative for the Royal Entomological Society. I, along with two other student representatives, organised and ran the annual Student Forum conference and wrote articles for the RES magazine, Antenna.

Chapter 1: General introduction

1.1 Social immunity in Animals

Avoidance of infection across taxa

Infectious disease is a pervasive force shaping animal ecology and evolution (Wilson et al., 2019). In response, animals have evolved immunological defences against pathogens including innate and adaptive immunity (Danilova, 2006). Even before this is invoked, however, a first line of defence could be to simply avoid getting infected in the first place, and behaviour is often used to avoid infection by parasites and pathogens in many animal species (Hart, 1994; Daly and Johnson, 2011). Examples of behavioural avoidance is seen all throughout the animal kingdom, from humans (Schaller and Park, 2011) and mandrills (*Mandrillus sphinx*) (Poirotte et al., 2017), to bats (*Desmodus rotundus*) (Stockmaier et al., 2018), birds (*Carpodacus mexicanus*) (Zylberberg et al., 2013), lobsters (*Panulirus argus*) (Behringer et al., 2006), and bullfrogs (*Rana catesbeiana*) (Kiesecker et al., 1999) (see Amoroso et al., 2025 for review). By modulating social interactions, animals can reduce disease transmission through their social networks (Stroeymeyt et al., 2018).

Behavioural immune responses in eusocial insects

While many facultatively social animals reduce infection risk by altering their contact networks, behavioural disease defences are exemplified in the eusocial insects. Species such as ants, termites, and many bees and wasps form some of the most ecologically dominant animal groups (Chapman and Bourke, 2001). Their success stems from highly organised societies characterised by strict division of labour, overlapping generations, and cooperative brood care (Wilson, 1990). These traits have enabled eusocial insects to build large, resource-rich colonies that dominate terrestrial biomass and drive resource turnover across ecosystems (Chapman and Bourke, 2001). However, the very features that underpin this success also make colonies exceptionally vulnerable to infection. Living in dense, genetically similar

groups, where individuals are consistently in close proximity provides ideal conditions for pathogen transmission (Schmid-Hempel, 2021). Infection outbreaks can have severe consequences, reducing productivity, disrupting division of labour, or even causing colony collapse (Higes et al., 2009; Goblirsch et al., 2013; Olate-Olave et al., 2021). These pressures have driven the evolution of a rich repertoire of collective behaviours and adaptations that protect the colony, known as social immunity (Cremer et al., 2007).

Social immunity encompasses a broad range of services, from collective behavioural responses such as avoidance, killing, grooming, or expulsion of conspecifics, to the production of antimicrobial substances within the nest (Cremer et al., 2007). In essence, social immunity refers to any service provided by an animal that reduces the consequences of infection for its conspecifics (Cotter and Kilner, 2010a). Although not restricted to eusocial insects (e.g. antibacterial exudates produced during parental care in burying beetles (Cotter and Kilner, 2010b)), social immunity is particularly prominent in eusocial taxa. Indeed, some eusocial insects rely so heavily on behavioural immune mechanisms that they often exhibit reduced genetic capacity for innate immune responses (Evans et al., 2006; Lopez-Uribe et al., 2016; Masson et al., 2024). Well-known examples include the removal of corpses by ants (*Myrmica rubra*) (Diez et al., 2014), allogrooming and cannibalism of fungal infected termites (*Anoplotermes pacificus*) (da Silva et al., 2025), and the removal of infected brood by honey bees (*Apis mellifera*) (Spivak and Gilliam, 1998).

A central challenge social immunity, regardless of sociality, is detection. For behavioural responses to succeed, animals must be able to identify when conspecifics are infected. This raises a fundamental question: how do animals recognise infection?

1.2 Semiochemicals in Animals

Chemical communication in animals

Chemical sensing is the oldest and most widespread mode of communication in animals (Wyatt, 2014; Leonhardt et al., 2016). Many interactions between organisms are mediated by semiochemicals, defined as any chemical involved in interactions between two organisms (Wyatt, 2014). These first arise when compounds are emitted by a sender and are used as 'cues' by a receiver. Over evolutionary time, some cues become specialised signals (pheromones) that serve functional roles in communication and development (Wyatt, 2014; Leonhardt et al., 2016).

The influence of semiochemicals extends beyond communication within species, where they can also be exploited as cues by other species. Across the animal kingdom, predators use semiochemicals to locate prey (Conover, 2007), parasites use them to find hosts (González et al., 2018), and some parasites even mimic the semiochemical profiles of their host to avoid detection (Lohman et al., 2006). This diversity of uses illustrates their deep evolutionary importance in animal communication.

Volatile and non-volatile semiochemicals

Semiochemicals can be broadly grouped according to their physical properties and range of action. Larger, high molecular weight, low volatility compounds (LVCs) are typically involved in short-range communication and often serve multiple roles. A well-studied example is cuticular hydrocarbons (CHCs), which provide desiccation resistance in insects while also functioning in nestmate recognition (Drijfhout et al., 2009). By contrast, volatile organic compounds (VOCs) tend to be low-molecular weight chemicals that readily diffuse through the air and can therefore act over long distances (Cicolella, 2008).

VOCs are also tightly linked to host metabolism. In mammals, VOC profiling is becoming increasingly common in the field of metabolomics (Zhang and Raftery,

2014; Lubes and Goodarzi, 2018). Because they are continuously emitted as by-products of normal metabolism, any perturbation of metabolic processes, such as those caused by infection, can alter the VOCs released. These shifts may reflect changes in host metabolism, pathogen metabolism, or both (Hong-Geller and Adikari, 2018). Detectable changes in VOC emissions during infection have been demonstrated across diverse systems, including respiratory and urinary tract infections in humans (Sethi et al., 2013), tuberculosis in cattle (Hong-Geller and Adikari, 2018), *Pseudomonas aeruginosa* in mice (Purcaro et al., 2019), and multiple pathogens of honey bees, as reviewed in Chapter 2 (Asiri et al., 2024).

1.3 Volatiles for disease surveillance

Because VOCs are tightly linked to host metabolism and infection, they offer a unique opportunity for disease surveillance across a range of systems. Unlike traditional surveillance techniques, VOCs can be collected directly from the headspace – the air volume above a liquid/solid sample – providing a non-invasive and minimally disruptive means of monitoring (Asiri et al., 2024). This makes VOC-based approaches particularly valuable in managed systems where intensive surveillance is required. The use of VOCs for disease surveillance in managed insect populations is reviewed in detail in Chapter 2 (Asiri et al., 2024). Briefly, identifying infection-associated VOCs can enable the development of gas sensors capable of detecting patterns in VOC emissions (Bak et al., 2020; Bak et al., 2022), which are more cost-effective and accessible alternatives to molecular techniques for disease detection such as PCR, providing a scalable, and field-applicable tool (Asiri et al., 2024). As VOCs are directly linked to metabolic state, they can provide a more accurate indicator of infection than manual surveillance, which is often labour-intensive, unreliable, and disruptive to the animals (Maciel-Vergara and Ros, 2017; Chopade et al., 2021).

1.4 Social immunity using semiochemicals

Semiochemicals have the potential to trigger social immune responses by providing information that allows colonies to detect and respond to infection. This link is

particularly important in eusocial insects, where the primary mode of communication is through semiochemical signalling (Leonhardt et al., 2016). A central mechanism of chemical recognition is the use of CHCs, which mediate nestmate recognition through the distinction of self from non-self (Breed et al., 2015). Each colony develops a distinctive CHC profile, and guards assess these cues when workers attempt to enter the nest (Breed et al., 2015). Fundamentally, recognition in social insects follows a progression: cues are produced by the individual, perceived and interpreted by nestmates, and then the colony executes an appropriate response (Sherman et al., 1997).

In some cases, infected individuals actively contribute by signalling their compromised state. For eusocial insects, such honesty is beneficial because individual fitness is tied to the reproductive success of the colony (Cremer et al., 2018). Accordingly, several studies have investigated how infection alters low-volatility compounds, such as CHCs, and whether these changes are associated with social immune responses. Ant pupae (*Lasius neglectus*) infected with pathogens are destroyed by nestmates, and this response is associated with altruistic CHC signalling of infection status (Pull et al., 2018; Dawson et al., 2024). In *Solenopsis invicta*, artificially treating pupal corpses with low-volatility fatty acids associated with fungal infection accelerates necrophoresis, reducing the risk of transmission (Qiu et al., 2015). In termites, pathogen-exposed individuals are more likely to be cannibalised, and infection is linked to distinct CHC profiles (Esparza-Mora et al., 2023).

These examples show that infection is often associated with altered semiochemical cues. However, the majority of research has focused on low-volatility compounds, with far less attention given to VOCs. To date, honey bees (*Apis mellifera*) remain the only insect system in which the relationship between VOCs and infection has been explored (Asiri et al., 2024).

1.5 Study system: Honey bees (*Apis mellifera*)

Importance of Honey bees in Agriculture

Honey bees are arguably the most important agricultural pollinators worldwide. Almost half of the leading global food commodities depend on their pollination for fruit seed or fruit set (McGregor, 1976; Klein et al., 2007). Even in crops not wholly reliant on insect pollination, yields decline dramatically in the absence of pollinators. Overall, approximately 35% of global food production benefits from insect pollination (Klein et al., 2007). The global value of pollination for crops used directly for human food is estimated at €153 billion (Gallai et al., 2009). Honey bees are particularly effective because they are generalists with a large foraging range (~4.5 km radius) and can pollinate crops over an area of approximately 6360 ha (Seeley and Visscher, 1985). This allows them to service monocultures that often lack wild pollinators (Öckinger and Smith, 2007), making them essential in modern agroecosystems. As global food demand has increased, so has the need for pollination services (Aizen and Harder, 2009). Yet while pollination demand has grown by over 300% since 1961, honey bee stocks have only increased by ~45% (Aizen and Harder, 2009). This growing mismatch between pollination demand and honey bee availability has heightened concerns over colony losses and the factors contributing to them.

1.5.1 Ectoparasites and Viruses

One of the most significant drivers of annual colony losses is infection by parasites and pathogens (Smith et al., 2013). The most damaging honey bee parasite globally is the ectoparasitic mite *Varroa destructor*, a major threat to honey bee health (Rosenkranz et al., 2010; Smith et al., 2013; Warner et al., 2024). Native to Asia, *Varroa* has spread from its original host (*Apis cerana*) to every continent except Antarctica and is now nearly ubiquitous in *Apis mellifera* colonies (USDA-APHIS, 2024). The mite has two life stages: a dispersal stage on adult bees, and a reproductive stage inside capped brood cells. There, females lay eggs and offspring feed on the fat bodies of developing larvae (Rosenkranz et al., 2010; Ramsey et al.,

2019), suppressing their immune systems and increasing their vulnerability to other infections (Ramsey et al., 2019).

Varroa destructor also serves as a vector for a range of honey bee viruses, including Israeli acute paralysis virus (IAPV), deformed wing virus (DWV), Kashmir bee virus (KBV), sacbrood virus (SBV), and acute bee paralysis virus (ABPV) (Francis et al., 2013). By both suppressing immunity and enabling direct viral entry into the haemolymph, *Varroa* dramatically increases viral loads (Mondet et al., 2014). Viral infections, especially those caused by DWV, are strongly associated with increased winter mortality and colony collapse (Francis et al., 2013).

Another damaging invasive ectoparasite of honey bees is the Small hive beetle (SHB; *Aethina tumida*). Native to sub-Saharan Africa, SHB emerged as an invasive species in 1996 and has since spread to all continents except Antarctica. While it is not as widespread or ubiquitous as *V. destructor*, it poses a growing threat to apiculture and is already established in many regions. SHB is absent from several countries, including the United Kingdom, but it is listed as a notifiable disease if found in the UK (DEFRA, 2024) as its distribution continues to expand, aided by global trade and migratory beekeeping practices (Neumann et al., 2016).

In its native range, SHB is typically a minor pest, primarily affecting weakened or stressed colonies (Neumann and Elzen, 2004). Western honey bees tend to be less aggressive and strong than African honey bees, making them more prone to parasites. The larvae consume stored resources such as honey, pollen, and wax, and larval frass leads to honey fermentation, and ultimately structural collapse of the nest and bee absconding behaviour (Neumann and Elzen, 2004; Hayes et al., 2015). In addition to honey bees, SHB has been shown to parasitise bumble bee (*Bombus impatiens*) colonies, suggesting it may pose a broader threat to wild pollinators in invaded ecosystems (Spiewok and Neumann, 2006).

1.5.2 Bacterial Diseases

Honey bees are also susceptible to bacterial infections, most notably European foulbrood (EFB) and American foulbrood (AFB), both of which are highly infectious, devastating to colonies, and notifiable to the relevant authorities upon identification

(Forsgren, 2010; Matovic et al., 2023; DEFRA, 2024). EFB is caused by *Melissococcus plutonius* and leads to larval death through intestinal colonisation and nutrient competition (Forsgren, 2010; de León-Door et al., 2020). Affected larvae turn yellow and then brown, often assuming unnatural positions in the cell.

AFB is caused by the spore-forming bacterium *Paenibacillus larvae*. The bacterium proliferates in the larval midgut before invading the haemocoel, killing the host and leaving behind ropy, decaying tissue that hardens into spore-laden scales (Genersch, 2010). These spores are highly resilient, remaining infectious for over 35 years and tolerating a wide range of environmental conditions (Haseman, 1961), making the disease extremely difficult to contain and easily transmissible *via* contaminated equipment.

1.5.3 Fungal Brood Diseases

Chalkbrood, caused by *Ascosphaera apis*, is a globally distributed and virulent fungal disease of larvae (Aronstein and Murray, 2010). It is favoured by cool, humid, poorly ventilated hives and is exacerbated by practices such as over-inspection (Heath, 1982; Flores et al., 1996). Spores are transmitted orally from forager to nurse to brood (Gilliam and Vandenberg, 1997). Once ingested, they germinate in the gut, breach the peritrophic membrane, and proliferate within the larval body cavity (Aronstein and Murray, 2010). Mycelial growth eventually erupts through the larval cuticle, producing distinctive “mummies” covered in white spores (Aronstein and Murray, 2010).

Stonebrood, caused by *Aspergillus* spp., is a rarer mycosis (Lopes et al., 2015). Infection may occur *via* ingestion or cuticular entry and affects both adults and larvae. Aflatoxins produced by the fungus are thought to be the primary cause of death (Lopes et al., 2015). Though potentially damaging and outbreaks are rare, there is particular interest in reducing the prevalence of stonebrood infections due to its ability to infect humans, where aflatoxins are carcinogenic if inhaled or ingested (Jensen et al., 2013).

1.5.4 Fungal Diseases of Adults: Nosemosis

Nosemosis, a chronic and widespread infection of adult workers, is caused by two species of intracellular microsporidians: *Vairimorpha* (formerly *Nosema*) *apis* and *Vairimorpha* (formerly *Nosema*) *ceranae* (Snow, 2022). Infection occurs when spores are ingested and germinate in the midgut, invading the epithelial cells (Higes et al., 2008). A single bee will produce millions of spores during the infection cycle which are shed in faeces and orally transmitted within the hive (Smith, 2012; Li et al., 2017). While both species cause nosemosis, they differ somewhat in pathology. *V. apis* (type A) is associated with dysentery, crawling bees, reduced honey yield, and winter mortality (Fries, 1993; Malone et al., 1995), whereas *V. ceranae* (type C) often lacks visible symptoms but reduces colony size, honey production, and increases mortality (Timofeev, 2023).

Infection by either species disrupts multiple physiological processes, including the vitellogenin/juvenile hormone axis, which plays a critical role in regulating age polyethism and behavioural maturation (Snow, 2022). This disruption can accelerate ageing and induce precocious foraging, breaking down division of labour and reducing colony efficiency (Snow, 2022). Both species shorten the lifespans of bees considerably (Hassanein, 1953; Graystock et al., 2013; Snow, 2022), and cause cognitive impairment, including deficits in learning and memory (Charbonneau et al., 2016). *Vairimorpha* spp. can also infect bumble bees (*Bombus terrestris*), where it similarly reduces longevity and impairs cognition, raising concerns about spillover from managed honey bees to wild pollinators. This highlights its status as a potentially dangerous emerging infectious disease arising from apiculture (Graystock et al., 2013).

1.6 The Smell of Infection: Chemical Cues as a Window into Social Immunity in Honey Bees

Given the importance of disease in honey bees, they represent a compelling model for investigating the chemical ecology of infection. Colonies are large, experimentally tractable, and behaviourally complex, with social organisation tightly governed by chemical communication (Bortolotti and Costa, 2014). Both CHCs and VOCs play

central roles in regulating foraging, reproduction, defensive behaviour, and nestmate interactions (Bortolotti and Costa, 2014).

Semiochemical cues of infection in honey bees

A growing body of work shows that infection alters chemical cues. Associations between changes in CHC profiles and infection have been demonstrated across a range of honey bee pathogens, including *V. destructor* infestation (Masterman et al., 2000; Cappa et al., 2016; Wagoner et al., 2019; Wagoner et al., 2020), European foulbrood infection (Kathe et al., 2021), and *Vairimorpha* spp. infection (McDonnell et al., 2013; Murray et al., 2015). For some of these infections, this is also true of their associated VOCs: Chapter 2 (Asiri et al., 2024) reviews current knowledge of VOC-based infection signatures in honey bees and their potential as non-invasive biomarkers of infection, while Chapter 3 synthesises the VOCs reported in association with honey bee infections in a meta-analysis.

Importantly, honey bees not only produce infection-related semiochemicals but also appear capable of responding to them. Workers detect changes in brood scent and initiate hygienic removal (Swanson et al., 2009; Cappa et al., 2016; Wagoner et al., 2020). Adults increase their social distance from nestmates infested with *V. destructor* (Pusceddu et al., 2021) and modify social interactions during viral infections (Geffre et al., 2020). These responses occur across multiple contexts and are potentially mediated by olfaction.

While infection-related changes in semiochemicals have been documented, their behavioural consequences remain supported mostly by correlational evidence. In brood, applying CHCs associated with *Varroa* infestation can induce removal (Wagoner et al., 2020), but equivalent experiments have not been carried out with other pathogens or on adults. Furthermore, studies on adults have been limited to documenting associations with CHCs (McDonnell et al., 2013; Murray et al., 2015; Geffre et al., 2020), and no VOCs have yet been identified in association with adult infections (Chapter 3). In the context of adult infections caused by *Vairimorpha* spp., social immune responses are inconsistent: two studies found no response (McDonnell et al., 2013; Murray et al., 2015), while another reported increased

grooming and killing of *Vairimorpha*-infected adults (Biganski et al., 2018). Taken together, this highlights critical gaps in the literature: the absence of studies on adult VOCs associated with infection, a lack of consistent evidence for social immunity towards *Vairimorpha*-infected adults, and the absence of direct tests isolating olfaction as the mechanism underlying social immune responses to adults. These gaps provide the foundation for the objectives of this thesis.

1.7 Thesis overview

The overarching aim of this thesis is to determine whether there is a detectable and functionally significant “smell of infection” in honey bees. It explores whether such semiochemical cues underlie social immune responses and whether semiochemicals could be co-opted for disease surveillance. Specifically, it examines whether volatile organic compounds (VOCs) are associated with infection in honey bees, focussing on adults and their *Vairimorpha* infections as model systems. It aims to determine the following questions: (i) to what extent are VOCs associated with honey bee infections, and does this extend to adult infections as well as brood; (ii) at what point during infection are such changes detectable in adults; (iii) does olfaction mediate social immune responses between adult bees.

1.7.1 Chapter outlines

- **Chapter 2** reviews VOCs associated with infection in honey bees, current surveillance techniques and potential applications of VOC-based monitoring, highlighting their promise as non-invasive, cost-effective tools for disease surveillance in managed insect systems.
- **Chapter 3** presents a meta-analysis of VOCs associated with honey bee infections across 23 studies. Using network analysis, it identifies distinct communities of VOCs linked to different pathogens and highlights major gaps in current knowledge, particularly the lack of studies on adult bee infections, including *Vairimorpha* spp.

- **Chapter 4** uses comprehensive two-dimensional gas chromatography (GCxGC-MS) to characterise VOC emissions associated with *Vairimorpha* spp. infection. This chapter examines how infection alters host VOC profiles over a 14-day time series and explores the implications of these changes for the detection of infection.
- **Chapter 5** investigates whether *Vairimorpha ceranae* infection influences social interactions in adult bees under laboratory conditions. By testing dyads with and without physical contact, it addresses whether volatile cues alone are sufficient to mediate behavioural responses between nestmates
- **Chapter 6** examines social immunity in ecologically realistic settings using observation hives. It assesses how colonies respond to infected individuals and whether bees perfumed with infection-related semiochemicals elicit similar behavioural responses to truly infected bees, providing insight into the role of odour cues in mediating social defences at the colony level.
- **Chapter 7** synthesises the overall findings of the thesis in relation to social immunity and disease surveillance. It discusses the implications of VOCs for colony-level defences and transmission beyond the colony, and outlines how these results can guide future research on chemical communication and pollinator health.

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Chapter 2: The smell of infection: disease surveillance in insects using volatile organic compounds (VOCs)

2.1 Abstract

Insects play crucial roles in nearly every ecosystem and provide a wide array of ecosystem services. However, both managed and wild insect populations face threats from parasites and pathogens which require surveillance to mitigate. Current infectious disease surveillance methods for insects often involve invasive, time-consuming, and occasionally destructive techniques, such as manual inspections and molecular detection. Volatile organic compound (VOC) surveillance provides a real-time, accurate, and non-invasive alternative for disease detection and has been well-established in humans and livestock. Recent advances in sensor technology now allow for the development of in-field VOC surveillance devices. This review explores the need for disease surveillance in insects and highlights recent advances of using VOCs for this purpose, focussing on honey bees as an example. We outline potential applications, challenges, and future prospects of using VOCs for insect disease surveillance, providing examples of how this technology could be globally applied to mitigate the impacts of disease in a range of insect systems.

2.2 Introduction

Outnumbering any other taxa in terms of species diversity, insects provide crucial ecosystem services as pollinators, decomposers, soil aerators, and nutrient cyclers (Schowalter, 2013). They are critical food sources for various taxa including humans, and also include numerous pest species that cause significant global economic losses (Losey and Vaughan, 2006; Schowalter, 2013). Insects face threats from a diverse range of parasites and pathogens, further exacerbated by the intensive farming of insects for pollination and protein, putting key ecosystem services and global food security at risk (Mennerat et al., 2010; Manley et al., 2015). Yet, despite

their importance, disease surveillance is mostly focused on insects that vector diseases (Kalluri et al., 2007; Kading et al., 2018) and key pathogens in agricultural species (Lee et al., 2015). In particular, infections threaten apiculture (Forsgren, 2010; Genersch, 2010; Noël et al., 2020; Pasho et al., 2021), sericulture (Chopade et al., 2021) and insects cultured for food and feed (Eilenberg et al., 2015; Maciel-Vergara and Ros, 2017) used to meet a growing demand for protein (Specht et al., 2019).

Detection and analysis of Volatile Organic Compounds (VOCs) offers a promising avenue for surveillance of insect diseases, providing an opportunity to develop an innovative approach to non-invasive surveillance. VOCs serve as the chemical language of communication and thus are commonly emitted by insects (Ali and Morgan, 1990). Advances in analytical techniques have resulted in rapid detection and high sensitivity (as low as 1 part per trillion) sufficient to detect changes in the VOC signatures of individuals (Liu et al., 2012; Misra, 2021).

This review explores the need for disease surveillance, current knowledge, and suitable approaches for VOC disease surveillance in insects. Throughout, we use the western honey bee (*Apis mellifera*) as an example which, with respect to VOC disease surveillance, is the most widely researched of all insect species, to date. This review seeks to explore the potential applications, challenges, and future prospects of utilising VOCs as a diagnostic tool for infectious disease surveillance in insects, enabling effective management strategies.

2.3 Why monitor infection in insects?

Around 35% of all global food production benefits from insect pollination, with an estimated value of €153 billion (McGregor, 1976; Klein et al., 2007; Gallai et al., 2009). Managed insects, such as honey (*Apis* spp.) and mason bees (*Osmia* spp.), play a crucial role in pollination, especially in monocultures with lower native bee diversity (Vides-Borrell et al., 2019).

Honey bees, integral to pollination, host a diverse array of parasites and pathogens which directly and indirectly lead to colony collapse (Pasho et al., 2021). Six of these are ‘listed diseases’ by the World Organisation for Animal Health (WOAH); defined

as those that could cause serious impact on global health and/or adversely affect wildlife conservation (WOAH, 2023), including Acarapisosis (*Acarapis woodi*), American foulbrood (*Paenibacillus larvae,* AFB), European foulbrood (*Melissococcus plutonius,* EFB), Varroosis (*Varroa destructor*), Small hive beetle (*Aethina tumida*), and Tropilaelaps mite (*Tropilaelaps* spp.). Migratory beekeeping i.e. seasonally transporting hives between different crops, is a major contributor to global parasite dispersal that has sparked debates over the potential ban of this practice to curb the spread of emerging parasites (Schäfer et al., 2019; Martinez-Lopez et al., 2022). Similarly, the importation and international trade of pollinators, such as bumble bees, often introduces parasites and pathogens that threaten native bees (Graystock et al., 2013; Figueroa et al., 2023).

Beyond pollination services, insects can be farmed for the products that they produce, such as domestic silk moths (*Bombyx mori*) for sericulture which benefits economies worldwide by generating income for farmers through silk and mulberry plant (*Morus* spp.) production (Van Huis, 2020; Ssemugenze et al., 2021). Insect farming also includes the practice of raising insects as a protein source, and is expanding in attempts to improve future food security (Van Huis, 2020). The international trade of insects for food and feed has seen substantial growth, with major producers like Thailand, France, South Africa, China, Canada, and the United States contributing to a projected market value of $16.39 billion USD by 2032 (Rowe, 2020). Major insect taxa already used in mass edible insect farming include Coleoptera (*Tenebrio molitor,* mealworm), Orthoptera (*Acheta domesticus,* house cricket), Lepidoptera (domestic silk moth)*, Galleria mellonella* (greater wax moth) and Diptera (*Hermetia illucens,* black soldier fly) (Van Huis et al., 2013). However, intensive insect farming, whether for food sources or farmed products, faces a myriad of parasites and pathogens impacting their survival, reproduction, and behaviour, which could have cascading effects in ecosystems and threaten food security.

Insect farming comprises dense collections of beehives or large insect colonies, which comes with the risk of disease outbreaks (see Mennerat et al., 2010; Eilenberg et al., 2015 for review). For example, densoviruses are associated with high mortalities in commercial *T. molitor* farms (Armien et al., 2023). The same pathogen caused such severe mortality in cricket farming that suppliers went into bankruptcy

(Szelei et al., 2011; Weissman et al., 2012). Fungal and viral pathogens, causing diseases such as grasserie (Baculoviridae), muscardine (*Beauveria* spp.), and Pebrine (*Nosema bombycis*) increase mortality and decrease silk production in sericulture (Chopade et al., 2021). Many pathogens/parasites infect multiple orders of insect, and could impact both native and managed insect populations through spillover events into other arthropods (Manley et al., 2015; Nanetti et al., 2021). Therefore, effective disease surveillance in insects is not just vital for agriculture and insect farming, but for conserving arthropods in general. Furthermore, many insects vector devastating diseases themselves, causing agricultural losses by transmitting diseases of plants (Butter, 2018), livestock (Narladkar, 2018), and humans (Asenso-Okyere et al., 2011). Detecting the pathogens they harbour as early as possible is vital to reducing the damage caused by vector-borne diseases (Dorea et al., 2016; Parnell et al., 2017).

Traditional disease surveillance in insects

Traditionally, disease surveillance involves visually inspecting insects for symptoms of disease and/or mortality (Chopade et al., 2021; FAO et al., 2021). For example, detecting pebrine disease in domestic silk moths relies on manual inspections of moths, larvae, and eggs (Chopade et al., 2021). However, manual inspections are labour-intensive, impractical for large-scale applications, and are challenging to perform reliably due to the need for specific expertise in identifying infections due to the similarities in symptoms between diseases (Chopade et al., 2021). Moreover, relying on visual inspection alone has limitations, as by the time clinical symptoms become apparent it is often too late to treat, meaning infected colonies must be destroyed, as observed in American foulbrood infection in honey bees (Locke et al., 2019). Therefore, early detection of sub-clinical symptoms is crucial for minimising losses during outbreaks (Locke et al., 2019)

Molecular techniques, such as PCR, offer a potential solution for early detection, but their effectiveness for surveillance can come at a cost if the pathogen requires destructive sampling for detection, which could impact yield (Evans et al., 2013; Maciel-Vergara and Ros, 2017). Non-destructive molecular screening can be achieved using faecal sampling by placing individuals in containers until defecation

occurs before returning them to the colony (Evans et al., 2013). However, this non-destructive approach will only detect those pathogens that are faecal-oral transmitted, and only if they are being shed in the faeces at the time of sampling. It should be noted that non-destructive does not equal non-invasive, as faecal sampling can involve the disturbance of a colony. Disturbance during winter, particularly in apiculture, is potentially harmful due to the risk of cold stress (FAO et al., 2021). Whilst non-invasive and non-destructive molecular screening of dead individuals is possible, the reliability of detection depends on the samples being fresh; RNA, for example, degrades rapidly after death, which can lead to false-negative results in the case of some viruses (Evans et al., 2013). Therefore, there is a pressing need to develop non-invasive methods that are effective for large scale in-field disease surveillance of insects.

Non-invasive disease surveillance

Non-invasive surveillance of disease has received much attention in apiculture with the advent of precision beekeeping and smart monitoring of hives. Various metrics, such as temperature, video, weight, humidity, and sound have been employed to assess the state of the colony, including forager activity, nectar flow and swarming (Meikle and Holst, 2015; Zacepins et al., 2015). While these metrics offer insights into colony health, they have limitations as an indirect marker for disease. For instance, honey bee infections influence colony temperature through the social fever response, which is detectable using temperature loggers (Goblirsch et al., 2020). However, these changes could also indicate responses to general stress, immune stimulation, or hypermetabolism resulting from artificial feeding with sucrose (Nieh et al., 2006; Goblirsch et al., 2020). VOC surveillance is advantageous in this regard, as VOC emissions are directly linked to changes in metabolism (Calcagnile et al., 2019; Gaude et al., 2019), which may act as more accurate measures of infection, either through signalling changes in the hosts metabolism in response to the infection or by detecting metabolites produced by the microbe itself. Consequently, VOC surveillance provides a promising avenue for disease surveillance.

2.4 What are VOCs and how are they monitored?

VOCs are defined as carbon-based chemicals abundant in the air above a sample (i.e. insects), known as the headspace, due to their high vapour pressure at room temperature (Cicolella, 2008; Turner, 2016). Both parasites/pathogens and host naturally produce VOCs as metabolic by-products or signalling molecules that may serve as biomarkers of infection (Shirasu and Touhara, 2011). In the case of insects, capturing the headspace of an entire colony could allow for real-time, accurate, and non-invasive detection of infection, a capability not achievable with traditional surveillance techniques.

Actively sampling headspace VOCs involves pumping gas over an adsorbent, while passive sampling relies on diffusion (Kumar and Víden, 2007). Following collection of headspace VOCs, a gas chromatograph (GC) coupled with a detector, such as a mass spectrometer (MS), is employed to separate, identify, and quantify the VOCs in the sample – specific VOCs can then, in theory, be identified as biomarkers of disease (Figure 2.1a). For in-field applications, highly selective semiconductor sensors, such as metal oxide semiconductor (MOS) sensors, could be utilised to detect target VOCs (Schütze et al., 2017) (Figure 2.1b). As gasses interact with the metal oxides present, the conductivity of the sensor increases and an electrical circuit can convert that change in conductivity into a signal that indicates the gas concentration (Bak et al., 2023). These sensors are cost-effective and portable that can be highly sensitive to specific VOCs, making them particularly well suited to in-field mass applications (Schütze et al., 2017). If key VOCs are associated with a given disease, linking sensor technology with smartphone technology could provide a real-time and non-invasive disease detection tool (Figure 2.1b).

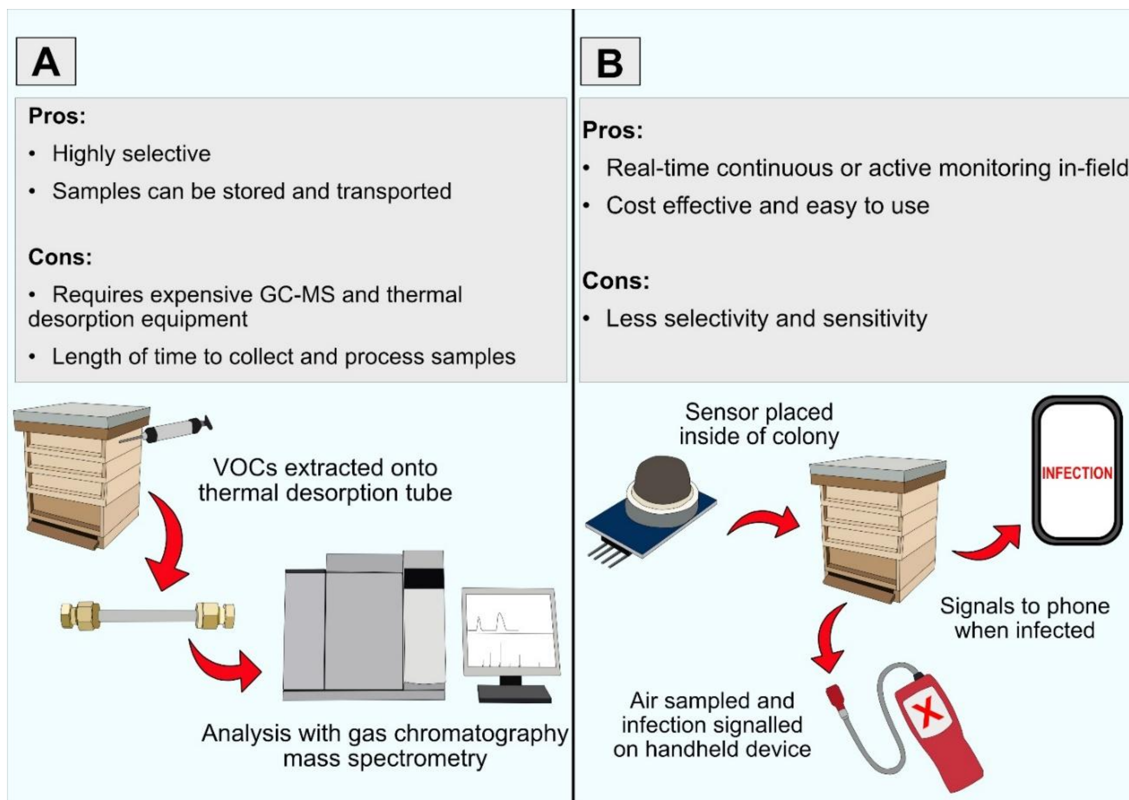

Figure 2.1: Current and future methods for disease surveillance using VOCs. (A) Current methods for analysing VOCs using gas-chromatography and thermal desorption tubes and mass spectrometry. (B) Potential future applications for detecting disease with VOCs. Handheld devices could be used to identify disease in-field. Gas sensors could signal alerts to phones, allowing for real-time remote surveillance.

Disease surveillance using VOCs.

In humans, VOC surveillance is an established field for detecting respiratory, urinary tract, and gastrointestinal infections (Sethi et al., 2013), and was utilised for SARS CoV-2 surveillance during the 2021 pandemic (Sharma et al., 2023). VOC surveillance has been extensively studied for detecting the insects themselves, especially those assessed to be pests, for example, wood borer beetles (*Semanotus bifasciatus* and *Phloeosinus auebi*) (Wang et al., 2020), stink bugs (*Chinavia hilaris* and *Nezara viridula*) (Henderson et al., 2010), flour and grain beetles (*Tribolium castaneum* and *Cryptolestes ferrugineus,* respectively) (Senthilkumar et al., 2012), and bark beetles (Scolytinae spp.) (Amin et al., 2013; Berg et al., 2013; Paczkowski et al., 2021). To the best of our knowledge, the only insect system that VOC disease

surveillance has been applied to is apiculture. This proof of concept in honey bees, however, provides valuable insight for expanding disease surveillance to other insect species.

Previous empirical evidence suggests disease-indicating VOC profiles can either contain compounds specific to the etiological agent or be represented by changes in compounds commonly emitted by honey bees due to the presence of a pathogen/parasite, or potentially a combination of both scenarios. American foulbrood (AFB) infection *in vivo*, for example, is characterised by unique emissions of propionic acid, valeric acid, 2,5-dimethyl pyrazine, acetamide, isobutyramide, methyl 3-methyl-2-oxopentanoate, and 2-nonanone (Lee et al., 2020; Bikaun et al., 2022), alongside a range of volatile sulphides and acids (Gochnauer and Margetts, 1981; Gochnauer and Shearer, 1981). These biomarkers of AFB infection are recognised as metabolites released by the bacteria genus *Paenibacillus,* during feeding, amino acid metabolism, and as compounds produced to suppress the growth of competing microbes (Verginer et al., 2010; Rybakova et al., 2016; Bikaun et al., 2022). Similarly, chalkbrood (*Ascosphaera apis*) infection (a fungal brood parasite) emits over 10 VOCs only found during infection, consisting of several lactones, phenethyl alcohol and its derivatives (Swanson et al., 2009; Finstrom et al., 2023). These are metabolites known to be commonly produced by other fungal species (Romero-Guido et al., 2011; Finstrom et al., 2023). By contrast, some VOCs associated with infection are compounds whose concentrations may vary in response to infection but are also released by healthy colonies, meaning they are not specific to infection. For example, levels of β-ocimene, a commonly emitted brood pheromone used in social regulation (Maisonnasse et al., 2010), are elevated in dead and Varroa infested bees (Mondet et al., 2016; McAfee et al., 2017), whereas reduced concentrations are linked with AFB infection (Lee et al., 2020; Bikaun et al., 2022). Additionally, brood parasitised by *V. destructor* emit pentadecene, which is believed to trigger the removal of infested brood from the hive (Nazzi et al., 2004). While this compound may signal infection, it is also released by unhealthy brood not necessarily infected, that are signalling for removal (Wagoner et al., 2020).

Developing sensors specific to VOCs that are associated directly with an etiological agent could clearly offer effective surveillance. VOCs that are non-specific, however, present challenges. Concentrations of VOCs may vary with the natural fluctuations in

colony populations over the year (Seeley, 2014). Changes in the number of individuals would therefore make it necessary to design sensors that can consistently adapt to varying population densities. Furthermore, infection and mortality can alter VOC production in conflicting ways, further complicating the surveillance of non-specific VOC biomarkers. For example, β-ocimene concentrations are lower in live bees infected with AFB but also increase when brood die from any cause (McAfee et al., 2017; Lee et al., 2020). As AFB infection progresses and leads to an increased number of dead bees in a colony, the reduced β-ocimene concentration associated with AFB infection in live bees may be masked by higher emissions in dead bees from both AFB-induced death, and uninfected dead bees that have died from other causes. An approach to monitoring VOC disease biomarkers could be to examine entire VOC profiles before and after infection. This method aims to identify shifts in VOC profiles, rather than single compounds, and focus on subsets of those VOCs that remain diagnostic of the disease. Sensor arrays sensitive to both host and pathogen derived VOCs could be used to monitor for diagnostic patterns of VOCs that could be distinguished from natural fluctuations.

Therefore, VOC disease surveillance can be achieved by: 1) detecting unique VOC biomarkers associated with an infection, or 2) detecting infection-induced changes in concentrations host-derived VOCs, such as brood pheromones. Monitoring VOCs associated directly with the etiological agent is advantageous as they are not only simpler to detect in-field, but biomarkers linked to pathogen metabolism are particularly interesting as a disease surveillance targets as they could act as indicators of infection across multiple host species. However, currently the research focussing on pathogens with broad host ranges is lacking; AFB and other bee diseases (Varroa and chalkbrood) only infect one host species, limiting cross-species inference. Only one study has investigated the VOCs associated with a multi-host pathogen, namely Sacbrood virus (SBV), which infects both social and solitary bees, as well as Lepidoptera (*Galleria mellonela*), Coleoptera (*Aethina tumida*), and wasps (*Vespula vulgaris* and *Polistes metricus*) (Manley et al., 2015; Gisder and Genersch, 2017; Bikaun et al., 2022). Sacbrood virus itself, however, has not been associated with unique VOC biomarkers; rather, emissions are associated with compounds

released by honey bees during decomposition (Bikaun et al., 2022) meaning disease-induced mortality cannot be easily discerned from other drivers.

There are multiple honey bee viruses that pose threats as emerging diseases to wild pollinators (Manley et al., 2015), however, in the main, the VOCs associated with these infections have not been studied. Furthermore, VOCs of many common infections of honey bees, such as nosemosis (*Vairimorpha* spp.) and European foulbrood (*M. plutonius*), as well as infections of other insect systems have not, to date, been identified. It is unknown if, in the face of disease, whether these etiological agents and/or the host emit VOC biomarkers. If unique VOC biomarkers of an etiological agent are not present, detecting changes in host VOCs from pre-post infection could provide biomarkers. However, these would have to be identified on individual host-pathogen cases, as they would likely consist of pheromones or other species-specific VOCs. These biomarkers may prove more challenging to define and adapt to detection with sensors due to their susceptibility to alteration by confounding factors, such as natural colony fluctuations.

Other detectable changes in insect chemical profiles

Whilst there is a current lack of data focusing on VOC markers for disease in any insects other than honey bees, research has shown that other chemical profiles, specifically cuticular hydrocarbons (CHCs) shift in response to infection. CHCs are non-volatile compounds comprising long-chain alkanes and alkenes, serving essential functions in insect physiology, particularly in moisture retention and nest-mate recognition (Drijfhout et al., 2009). Detectable alterations in CHC profiles have been observed in multiple ant species, for example *Megaponera analis* infected by soil pathogens (*Burkholderia* sp. and *Pseudomonas aeruginosa*; Frank et al., 2023), *Leptothorax nylanderi* parasitised by tapeworms (*Anomotaenia brevis*; Trabalon et al., 2000), and *Lasius neglectus* pupae infected by fungi (*Metarhizium brunneum*; Pull et al., 2018). Entomopathogenic fungi, such as *Beuvaria bassiana*, target and breakdown the CHCs of multiple insect orders, which can directly change the CHC profiles during infection (Pedrini et al., 2007; Pedrini et al., 2013). Similarly, distinct CHC profiles have also been observed in paper wasps (*Polistes ferreri*) parasitised by *Xenos* sp. (Torres et al., 2016).

CHCs tend not to be volatile, and thus are not ideal for passive field monitoring of infection, but their modulation in response to infection suggests a potential parallel shift in VOCs could occur. This supposition is supported in studies of honey bees where various infections that induce changes in CHC profiles were also associated with alterations in VOCs (Wagoner et al., 2019; Lee et al., 2020; Wagoner et al., 2020; Wagoner et al., 2021). Therefore, it is likely that VOCs also shift in response to infection in insects other than honey bees, warranting further investigation.

2.5 The future of insect disease surveillance

Currently, insect VOC disease surveillance requires access to expensive GC-MS equipment capable of processing VOC samples, followed by specialist knowledge to interpret the outputs produced (Figure 2.1a). However, once the VOC biomarkers associated with a given disease is known it can lead to in-field sensors that form multi-sensor arrays or 'E-noses', that react to the identified VOCs (Bak et al., 2023). Ongoing trials in apiculture have been exploring the in-field application of insect VOC disease surveillance using MOS sensors. Laboratory and field trials have successfully identified *V. destructor* infestations and promising results have also been seen in efforts to detect AFB infection (Szczurek et al., 2019; Bak et al., 2020; König, 2021; Bak et al., 2022). While sensors focused on the most important known and prevalent diseases offer valuable in-field surveillance, novel and emerging diseases will be missed by this approach. E-noses, however, also offer some promise for surveillance here, as they could be capable of detecting an unhealthy colony. DL-pantolactone, for example, is associated with decomposing honey bee larvae (Bikaun et al., 2022) and could serve as a biomarker of poor health. Although not a pathogen/parasite-specific VOC, sensors detecting high DL-pantolactone concentrations could indicate elevated larval mortality, serving as a warning system for novel emerging infectious diseases.

The food industry has already developed wireless, portable sensors capable of signalling food spoilage using VOCs (Ma et al., 2018; Xing et al., 2023), and similar devices could be developed for insect disease surveillance. These could offer cost-effective, continuous, non-invasive surveillance of diseases in insect systems. As sensor technology advances, integrating VOC disease surveillance into insect

farming seems plausible, enabling sensors to relay colony health directly to an app or database (Figure 2.1b). This approach would eliminate the need for manual inspections, ensuring early detection and reducing losses (Zacepins et al., 2015; Figure 2.1b). Furthermore, VOC disease surveillance could extend to handheld sensors (Figure 2.1b), actively sampling in the field to screen for insect diseases during transportation and importation. With the rise in international insect trade, this screening could facilitate safe trade by detecting insect diseases at borders.

Sensors also have potential for widespread application to disease surveillance in agriculture. Insect pollination is vital for agriculture and agroforestry with the majority of global crops susceptible to production losses if pollinators are limited (Klein et al., 2007). Both wild and managed pollinators play crucial roles in pollinating a wide range of crops globally (Klein et al., 2007). For certain crops, wild pollinators can be just as, and often more effective for pollination than honey bees (Garibaldi et al., 2013; Esquivel et al., 2020). Furthermore, the presence of wild pollinators on crops, such as sunflowers (*Helianthus annuus*), enhances honey bee pollination efficiency up to 5-fold (Greenleaf and Kremen, 2006), and have also been seen to increase honey bee movements between crops, enhancing pollination effectiveness (Brittain et al., 2013). However, the emergence of pollinator pathogens in managed pollinator populations, such as the honey bee viruses capable of infecting multiple orders of insect pollinators (Manley et al., 2015) and *Crithidia bombi* spillover in bumble bees, poses potential dangers to wild pollinator populations and is thought to be a contributing factor for wild pollinator declines (Otterstatter and Thomson, 2008).

Flower sharing is major avenue of disease transmission among both managed and unmanaged pollinators. (Graystock et al., 2015; Manley et al., 2015). As infected pollinators drink from the same nectar source, rub against and defecate on flowers, they deposit pathogens that can survive and transmit orally to the next pollinator that visits the flower (Graystock et al., 2015). Bumblebees have demonstrated the ability to avoid flowers heavily contaminated with a pathogen, suggesting there must be a detectable signal associated with pathogen contamination (Fouks and Lattorff, 2011). Therefore, it seems likely that VOC biomarkers of pathogens could be detectable on flowers. Recent studies have shown that nectar microbes can influence the VOC profile of flowers, attracting pollinators to enhance their own dispersal (Crowley-Gall et al., 2021; Sobhy and Berry, 2024). Should this phenomenon extend to pollinator

pathogens, VOC surveillance could be used to pinpoint hotspots of pollinator pathogens. A similar technique is already developing in agricultural and forestry settings for detecting crop diseases, where E-nose technologies have been used to diagnose diseases caused by phytopathogenic microbes (Wilson, 2013). Similar methods could be applied to monitor for pollinator pathogens harboured on flowers by collecting headspace samples from flowers in agricultural settings. Beekeepers could use these data to avoid transporting managed colonies to contaminated areas, thereby reducing the distribution of pathogens between cropland caused by migratory beekeeping (Martinez-Lopez et al., 2022). Additionally, as wild pollinator diversity is evidently beneficial to crop yields (Greenleaf and Kremen, 2006; Brittain et al., 2013; Garibaldi et al., 2013; Esquivel et al., 2020), farmers could be encouraged to survey crops for pollinator pathogens with handheld gas sensors (Figure 2.1b) by sampling flower heads for VOCs. Control measures could then be applied, such as the timed application of fungicides or introducing microbial antagonists of the detected pathogen (Heydari and Pessarakli, 2010). This kind of VOC disease surveillance and control could reduce the impact of pathogen spillover on wild pollinator diversity in croplands, thereby enhancing pollination effectiveness and crop yields.

Another interesting potential application for insect VOC disease surveillance in agriculture is to track the effectiveness of entomopathogenic biocontrol. The use of entomopathogens to control pest insects has long been established (Lacey et al., 2015). Entomopathogenic fungi, such as *Beauveria bassiana,* are widely applied to control for a range of pests including various wasp, ants, and bark beetle pest species (Singh et al., 2017). One setback with the commercialisation and development of entomopathogens is assessing their effectiveness in-field, as their persistence and efficacy vary among insect species (Singh et al., 2017). VOC disease surveillance could be employed in these systems to quantify the efficacy of entomopathogens applied to large pest infestations. Pest species are often characterised by phases of extremely high population densities, as seen in the epidemic phase of bark beetle outbreaks (Hlásny et al., 2021). During these phases the volume of VOCs emitted by the pests could be detected by gas sensors placed in the field (Figure 2.1b). Host-specific VOC biomarkers would allow for the identification of how effectively the biocontrol is impacting the target species.

Conclusions

Insects, vital for ecosystem services and global food security, face threats from infections impacting agriculture and insect farming. VOCs are an exciting, novel method for non-invasive surveillance of infectious diseases in insects. Integrating VOC surveillance into insect farming and international trade could revolutionise disease surveillance by facilitating swift treatment and minimising losses, with potential applications extending broadly to agriculture and agroforestry. In the future, handheld sensors could be used to monitor for pathogens in the field, allowing for rapid measures to be implemented to control pollinator pathogen levels in agricultural settings. This would enhance both managed and wild pollinator populations. Additionally, VOC disease surveillance may have applications in monitoring the efficacy of entomopathogenic biocontrols. However, while the future looks promising for honey bee disease surveillance, it is important to acknowledge that many pathogens remain understudied. Specifically, there is a critical knowledge gap concerning which VOCs are associated with infection in other insect systems. Addressing this gap is crucial before sensor development can progress effectively in these systems. By deciphering the volatile signatures emitted during infection, we are poised to unlock a new era in the surveillance and management of insect diseases.

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Chapter 3: Volatile organic compounds as indicators of infection in honey bees (*Apis mellifera*): A meta-analysis

3.1 Abstract

Honey bees (*Apis mellifera*) are critical pollinators facing intensifying threats from parasites and pathogens. However, current disease surveillance methods are labour-intensive, disruptive, and often detect infections too late for effective intervention. Volatile organic compounds (VOCs) emitted during infection offer a promising, non-invasive alternative, yet no study has synthesised VOC data across the literature to define a comprehensive "smell of infection" in honey bees. Here, we conducted a meta-analysis of 23 articles (from 1190 screened) that used headspace sampling to identify VOCs associated with honey bee infections. We applied a novel network analysis approach using presence/absence data to identify communities of VOCs that consistently co-occurred across different parasite treatments, including Varroa mites, American foulbrood (AFB), Sacbrood virus, Chalkbrood, and uninfected controls. Our analysis revealed eight distinct VOC communities, six of which were composed exclusively of compounds emitted by infected treatments. These infection-specific communities were associated with Varroa mite, AFB, and Chalkbrood, and featured pathogen-derived volatiles (e.g. microbial pyrazines, esters, and lactones) as well as host stress signals (e.g. (S)-2-heptanol, ethyl hexanoate). While compound clusters were highly distinct at the individual VOC level, there was notable overlap at the broader chemical class level. Some VOCs were unique to specific pathogens and may serve as reliable biomarkers for non-invasive biosensing. However, many compounds commonly occurred across both infected and uninfected treatments, highlighting that compound co-occurrence patterns, rather than single biomarkers, may better define the smell of infection. This work provides the first synthesis of VOC profiles associated with honey bee disease, offering new directions for developing gas sensor technologies and behavioural assays to support early detection and enhance disease resistance in insects. These

findings also have broader implications for understanding chemical communication in social immunity and for disease monitoring in other animal systems.

3.2 Introduction

Honey bees (*Apis mellifera*) are one of the most important agricultural pollinators worldwide. Almost half of the leading global food commodities rely on animal pollination (McGregor, 1976; Klein et al., 2007), and pollinating insects enhance fruit or seed set in 39 of the 57 major crops (Klein et al., 2007). The economic value of honey bee pollination reflects this vital ecosystem service, with global estimates reaching €153 billion (Gallai et al., 2009).

Despite the high monetary value of honey bees, Europe, South America, and North America have been facing unmitigated long-term declines in honey bee colony numbers (VanEngelsdorp et al., 2008; Potts et al., 2010; Meixner, 2010; Maggi et al., 2016). Over the past two decades, annual colony mortality rates have been alarmingly high in the United states; the acceptable rate of colony loss during a beekeeping season (10%) (Laurent et al., 2016), has not been achieved in any year since records began (Aurell et al., 2024; Giacobino et al., 2025). Multiple factors contribute to honey bee declines, including habitat loss, pesticide use and the increasing pressure of infectious diseases (Goulson et al., 2015). Among these stressors, infectious diseases pose one of the major threats and are posited to be a key driver in the rising rates of colony loss (Potts et al., 2010; Goulson et al., 2015; Maggi et al., 2016; Hristov et al., 2021).

Honey bee diseases

Honey bees are host to a wide diversity of parasites/pathogens (hereafter referred to as ‘parasites’) that infect both brood and adult life stages (Pasho et al., 2021). Whilst the virulence of these parasites varies from lethal to sub-lethal the latter contribute to reducing the colony’s chances of surviving stressful events, such as winter (Ulgezen et al., 2021). American and European foulbrood (AFB and EFB, respectively) are common acute colony bacterial infections with high mortality and panzootic distributions (Forsgren, 2010; Genersch, 2010; Boncristiani et al., 2020). Similarly

widespread is the ectoparasitic Varroa mite, *Varroa destructor*. This invasive mite has spread from Asia to every habitable continent, and recently (2022) established in Australia, making it globally distributed (Rosenkranz et al., 2010; Iwasaki et al., 2015; Boncristiani et al., 2020; Le Breton et al., 2025). At a country scale, Varroa is similarly widely distributed; in the US, surveys of Varroa prevalence between 2009-2023 show a country-wide prevalence of 87% (USDA-APHIS National Honey Bee Survey, 2024). Varroa mite vectors a range of viruses, such as Israeli Acute Paralysis Virus (IAPV) and Deformed Wing Virus (DWV) (See Chen and Siede, 2007; Pasho et al., 2021 for review) that are a leading cause of mortality in honey bees (Francis et al., 2013). Honey bees are also host to multiple fungal infections, such as chalkbrood disease, which is a globally distributed mycosis caused by *Ascosphaera apis,* that infects and kills honey bee brood (Aronstein and Murray, 2010). Mycoses are also present in the adults, including nosemosis, a disease caused by microsporidians in the genus *Vairimorpha* (*Nosema*). There are two types of nosemosis; type A and type C, caused by *Vairimorpha apis* and *Vairimorpha cerenae,* respectively (See Higes et al., 2008; Pasca et al., 2019 for review). Nosemosis type A causes dysentery and reduces worker lifespan whilst type C is often asymptomatic but reduces colony size and brood rearing (Pasca et al., 2019). While infection does not always lead to colony collapse (Invernizzi et al., 2009) both types of nosemosis weaken hives, and infection has been linked to higher winter mortality (Higes et al., 2008; Higes et al., 2010).

Current methods to detect and control disease

Currently, beekeepers perform routine weekly inspections to monitor visually for signs of infection (FAO, 2021). This is an invasive process that involves manually inspecting the frames of the hive (Cramp, 2008). Considering many commercial beekeepers have up to a hundred hives or more, this can also be an incredibly labour-intensive process and at the point when the infection, or signs of it (e.g. bee deaths), become visible, treatment is often not effective (Waite et al., 2003). Furthermore, frequent visual inspections can stress the colony by disturbing the temperature equilibrium within the hive, which can exacerbate certain diseases, such as chalkbrood, by chilling the brood (Flores et al., 1996). As a result, winter

inspections are not recommended when the temperature is low, as this risks disturbing the winter cluster, which is vital for colonies to survive cold temperatures (Stabentheiner et al., 2003; FAO, 2021). That, however, creates an issue for disease surveillance as at the advanced stages of infection, treatment is not possible. Often, beekeepers must resort to burning the infected and surrounding hives to quell the infection (Locke et al., 2019). Therefore, it is vital to detect disease as early as possible while using non-invasive methods in order to improve the prognosis of the hive.

The smell of infection

Every organism emits volatile organic compounds (VOCs), the signature of which can be quantified into a 'smell'. VOCs are directly linked to metabolic processes (Calcagnile et al., 2019; Gaude et al., 2019) so can provide insights to the health of an organism. Previous studies have demonstrated that shifts in VOCs accompany both infectious and non-infectious diseases in humans and livestock, for example, bovine tuberculosis and cancer (Peled et al., 2012). Elevated levels of specific VOCs have been observed in breath samples from patients with lung cancer, pulmonary diseases, and asthma (Rudnicka et al., 2014). In laboratory settings, breath samples from mice have successfully identified *Pseudomonas aeruginosa* (Zhu et al., 2013). In humans, *Clostridioides difficile* can be detected by analysing VOC profiles of stool samples (See Sethi et al., 2013 for review). Similarly, urine VOC profiles have been used to detect urinary tract infections (Sethi et al., 2013). VOC analysis has also been effective in identifying respiratory infections in humans such as *Mycobacterium tuberculosis*, *P aeruginosa*, and *Aspergillus fumigatus* (Sethi et al., 2013). During the SARS CoV-2 pandemic, the abundance of VOCs in breath samples from infected patients were used to discriminate between SARS CoV-2 infection, healthy individuals, and other respiratory infections (Sethi et al., 2013).

VOC signatures from honey bees could offer a potential biomarker for disease surveillance because a large portion of honey bee communication is done using VOCs, such as the 10 methyl and ethyl fatty acid esters that make up the brood ester pheromone (Le Conte et al., 1990; Asiri et al., 2024). Indeed, evidence for unique VOC profiles has been found in honey bees in response to chalkbrood

(Finstrom et al., 2023), AFB (Lee et al., 2020), and parasitism by *V. destructor* (Wagoner et al., 2021). Several studies have found that the removal of brood infested with *V. destructor* is associated with a shift in VOCs, such as brood ester pheromone (Nazzi et al., 2004; Schoning et al., 2012; Liendo et al., 2021; Wagoner et al., 2021). Identifying specific VOC biomarkers associated with the presence or absence of disease could therefore allow for the development of non-invasive VOC sensors capable of early detection of infection based on a VOC profile, providing a novel and rapid non-invasive method of monitoring infection in apiculture (Szczurek et al., 2020a; Asiri et al., 2024).

Here, we carried out a meta-analysis of the existing literature to assess if we can identify VOCs associated with common honey bee infections (AFB, Varroa, Sacbrood virus, Chalkbrood) and identify compounds that are uniquely associated with infection. Using a novel approach of network analysis, we assess whether we can detect a 'smell of infection' by quantifying shared VOCs across infections and across studies.

3.3 Methods

Data acquisition

We searched the existing literature using Scopus, Web of science Core Collection, CAB abstracts and BIOSIS, searched from 1928-2022 using the following search string:("honey bee*" OR honeybee* OR "apis mellifera") AND (voc OR vocs OR semiochemical* OR "volatile organic compound*" OR pheromone* OR odo*r OR odo*rs OR odo*rant OR odo*rants OR smell OR scent OR "chemical cue*" OR olfact* OR cue* OR "chemical signal*" OR "chemical detection" OR "cuticular hydrocarbon*" OR chc*) AND (disease* OR infection* OR parasit* OR pest OR pests OR illness* OR varroa OR "melissococcus plutonius" OR nosema OR "paenibacillus larvae" OR "Ascosphaera apis" OR aspergillus OR virus* OR viral OR foulbrood OR chalkbrood OR stonebrood OR "hygienic behavio*r" OR hygienic*). This resulted in a total of 1190 articles before screening.

Data inclusion criteria

The screening of publications was carried out according to PRISMA guidelines (Page et al., 2021) (Figure 3.1). The initial database search returned 1190 articles, which was reduced to 1164 after removing duplicates. Titles and abstracts were screened according to the following inclusion criteria: (1) Primary studies involving any semiochemicals associated with healthy, parasitised, and/or immune stimulated *Apis mellifera* at any life stage. (2) Primary studies investigating the semiochemicals associated with *in vitro* parasites of *Apis mellifera*. The exclusion criteria were the following: (1) Studies related to compounds associated with pesticides or chemical treatments; (2) Studies reporting behaviour in response to synthetic compounds (3) Pheromone lure studies; (4) Gene expression; (5) Studies on any other species of insect; (6) Study does not provide compound identity; (7) Study does not exclusively sample headspace volatiles (e.g. hexane extraction). This initial screening retained 266 articles for full-text assessment. Applying the same inclusion and exclusion criteria to the full texts yielded a final set of 23 articles that were included in the analysis (Figure 3.1).

In some cases, a single paper conducted multiple experiments on multiple treatments. For example, Bikaun et al. (2022) quantified headspace volatiles from five different parasites, alongside an uninfected control. We refer to these individual experiments as ‘study’, while referring to the entire paper as the ‘article. A full account of each reference, which parasites they studied, the sampling methods used, and how many studies were conducted is provided in Table S3.1. Where possible, raw data were extracted. If unavailable, the summarised and reported compounds in the study text were used.

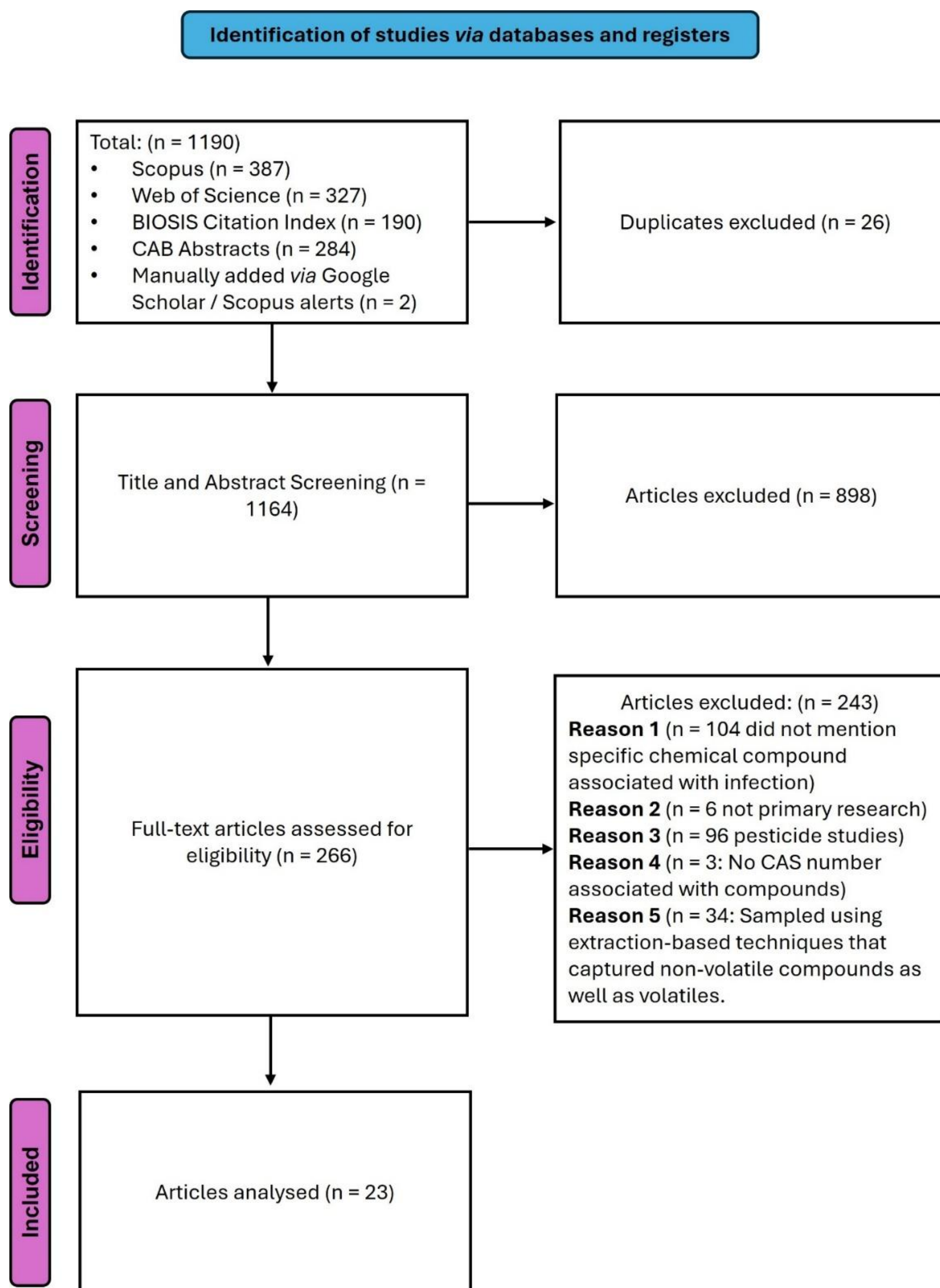

Figure 3.1: Article selection process.PRISMA flow diagram (Page et al., 2021) depicting the process of article identification, screening, and eligibility assessment which led to article inclusion.

Infection status

The VOC samples collated across the literature spanned four different parasites, including Varroa, AFB, chalkbrood and sacbrood, some of which were sampled *in vitro* and some *in vivo* (See Table 3.1 for full breakdown). We refer to these samples collectively as ‘infected’ treatments. Control treatments consisted of both live bees that were uninfected and ‘lab-killed’ uninfected bees, referred to collectively as uninfected treatments.

Table 3.1: Summary of studies on volatile organic compounds (VOCs) associated with infection in honey bees. The table details the infection status, parasite type, and the number of studies for each treatment across different honey bee life stages (studies = 49 from 23 articles).

Treatment	Infection status	Parasite type	Larvae	*In vitro*	Mixed	Adult	Capped larvae	Drone egg and	Drone larvae	Drone pupae	Egg	Pupae	Total studies
Uninfected	Uninfected	N/A	12	0	1	4	2	1	1	1	1	5	28
Lab-killed bees			4	0	0	0	0	0	0	1	0	2	7
American foulbrood	Infected	Bacteria	3	0	0	0	0	0	0	0	0	0	3
American foulbrood *in vitro*			0	1	0	0	0	0	0	0	0	0	1
Sacbrood virus		Virus	1	0	0	0	0	0	0	0	0	0	1
Chalkbrood		Fungi	3	0	0	0	0	0	0	0	0	0	3
Chalkbrood *In vitro*			0	1	0	0	0	0	0	0	0	0	1
Varroa mite		Mite	0	0	0	0	2	0	0	0	0	2	4
Varroa *in vitro*			0	1	0	0	0	0	0	0	0	0	1

Network analysis and community detection

We used network analysis to quantify how VOCs associated with given infections and uninfected bees cluster. The aim is to assess if network communities occur and whether they consist of VOCs that are associated with infection, allowing us to discern a 'smell of infection'. First, we constructed an undirected bipartite network which is characterised by its division into two disjoint and independent groups of nodes, where edges can only connect between nodes of different groups. In our case, one group of nodes comprises individual VOCs and the other represents treatments, edges represent co-occurrence in specific treatments (infected and uninfected; Table 3.1). From this bipartite network, we then created a unipartite projection, resulting in a network where VOCs were connected (or not) if they were emitted by the same treatment, which is presented here.

Community detection was used to identify clusters or communities (or 'smells') of VOCs based on their similarities. To determine the most suitable clustering algorithm, we applied five different clustering algorithms and evaluated their performance using exponential random graph modelling (ERGM). We compared the observed network to a randomly generated network with the same number of nodes and edges as the observed network to assess if clustering was stronger than expected by chance. We also examined whether nodes within the same detected community were more likely to be connected than expected in a random network by modelling nodematch (community) from the ERGM package (Hunter et al., 2008), which measures the tendency for nodes to form edges within rather than between communities. A positive and significant nodematch estimate indicates that the clustering method successfully captured meaningful structure in the network.

We tested five community detection algorithms: Fast Greedy modularity optimization (Clauset et al., 2004), Walktrap, (Pons and Latapy, 2005), Louvain multi-level modularity optimization (Blondel et al., 2008), Label propagation (Raghavan et al., 2007), and Spinglass (Reichardt and Bornholdt, 2006). Walktrap allows flexibility in the length of random walks used to form communities. Shorter walks form more tight-knit, local communities (Smith et al., 2020). Given that we are focused on identifying local clusters of VOCs associated with infection, we used the Walktrap algorithm with a walk length of 2 to best capture these local relationships.

To determine which algorithm provided the best representation of the network, we used each algorithm's output to define community memberships, which were then incorporated into ERGMs as nodematch terms. We fit an ERGM for each set of community assignments and compared Akaike Information Criterion (AIC) values and the estimated nodematch coefficients across models. Lower AIC values and higher nodematch coefficients were interpreted as indicating better model fit and stronger clustering by community assignment.

Network metrics

The weighted degree (K_w) metric was used to assess the relative importance of each VOC within its detected community. The weighted degree of a node is the sum of its edge weights, representing the total strength of its connections within the network. VOCs with high weighted degrees are those most frequently co-emitted across treatments, making them key contributors to the structure of their respective communities; here a proxy for 'smell'.

All statistical analyses were done in R version (4.2.2; R Core Team, 2023) using the "igraph" (Csárdi et al., 2025), "tidygraph" (Pedersen, 2025a), "ggplot2" (Wickham, 2011), "ERGM" (Hunter et al., 2008; Handcock et al., 2025), "ggraph" (Pedersen, 2025b), and "circlize" (Gu et al., 2014) packages. Network visualisation was conducted in Gephi (Bastian et al., 2009).

3.4 Results

Community detection of the smell of infection (VOCs) via network analysis

All tested community detection algorithms, except for 'Label Propagation', produced community clusters that differed significantly from random in our ERGMs (Table 3.2). The Walktrap algorithm performed best, yielding the highest nodematch estimate (3.6), indicating the strongest tendency for within-community ties, and the lowest AIC (39304), reflecting the best model fit. In contrast, Label Propagation performed worst (AIC: 50213), detecting only a single community. Based on these comparisons, we

selected the community clusters identified by the Walktrap algorithm for further analysis. Model coefficients and fit statistics for all algorithms are shown in Table 3.2.

Table 3.2: Coefficients from Exponential Random Graph Modelling (ERGM). Nodematch estimates, statistical significance, and Akaike Information Criterion (AIC) values for each community detection algorithm are shown. No significance values could be calculated for Label propagation as the algorithm only detected one community.

Algorithm	Nodematch estimate	Nodematch significance	AIC
Walktrap	3.6	*P* = <0.0001	39304
Spinglass	2.5	*P* = <0.0001	40874
FastGreedy	1.9	*P* = <0.0001	43568
Louvain	1.5	*P* = <0.0001	46339
Label propagation	N/A	N/A	50213

The network was comprised of 279 nodes (representing VOCs) with 25,063 edges (representing VOCs shared between treatments) (Figure 3.2). A total of 212 VOCs were associated with uninfected treatments and 177 with infected. Of these, 67 VOCs were uniquely associated with infection, 102 VOCs with uninfected, and 110 VOCs were shared between the two.

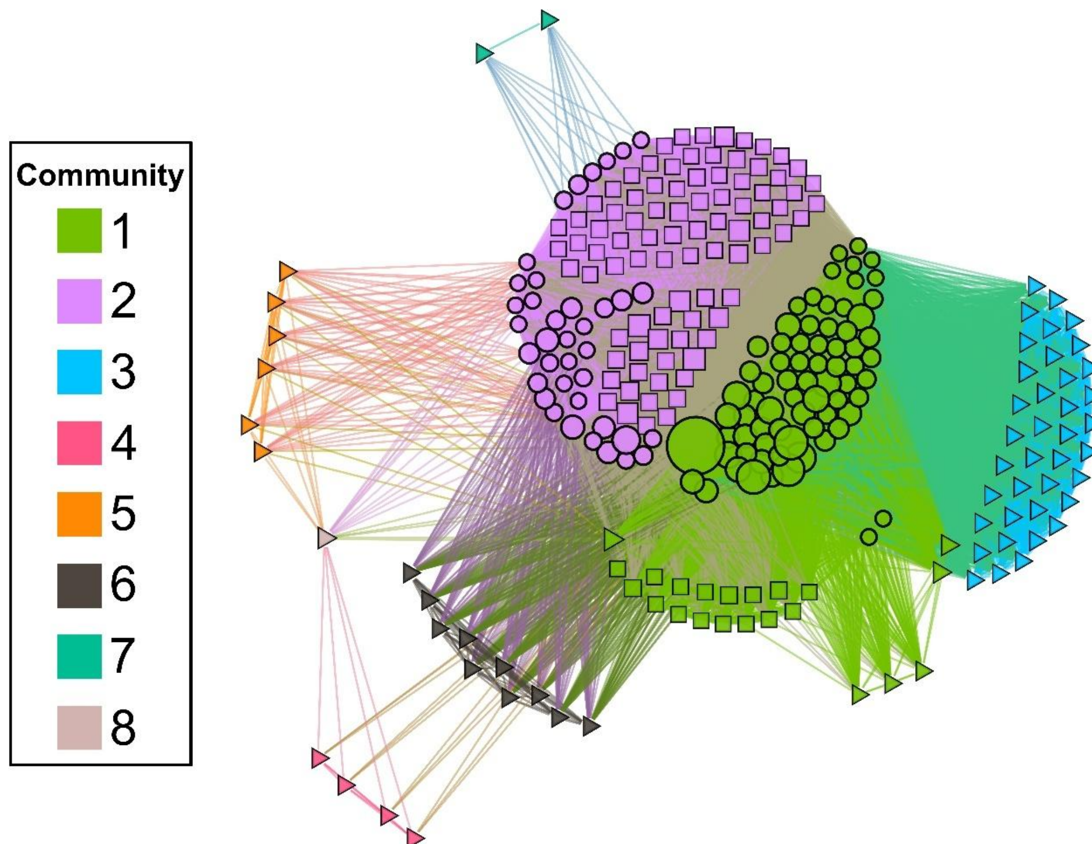

Figure 3.2: Network visualising VOCs detected across all life stages of *Apis mellifera* in studies using headspace sampling. Each node represents a unique VOC, and edges connect VOCs co-emitted by the same treatment. Edge colours blend the colours of the communities they connect, illustrating inter-community co-occurrence. Circular nodes indicate VOCs found in both infected and uninfected treatments, square nodes are unique to uninfected treatments, while triangular nodes represent VOCs uniquely associated with infected treatments. Node size corresponds to the number of studies reporting each VOC. Communities, detected using the Walktrap algorithm, are colour-coded and vary in study representation: Community 1 (studies = 41), Community 2 (studies = 40), Community 3 (studies = 2), Community 4 (studies = 1), Community 5 (studies = 2), Community 6 (studies = 2), Community 7 (studies = 1), Community 8 (studies = 2).

Using the Walktrap algorithm, we identified eight distinct communities within the network, which here is equivalent to distinct 'smells' (Figure 3.2). The largest community (Community 2) comprised 124 VOCs the majority of which (80.4%) were emitted by bees that were not infected (60.8% uninfected live bees and 19.6% uninfected lab-killed bees (Figure 3.2, Figure 3.3). The remaining 19.6% of VOCs

were associated with Varroa mite (8.3%), chalkbrood (5.4%), *in vitro* Varroa mite (4.4%), and sacbrood virus (1.5%) (Figure 3.2, Figure 3.3).There were no VOCs unique to infection in this cluster (Figure 3.2; Table S3.2).

The second largest community cluster of VOCs (Community 1, n = 94) was almost 50-50 uninfected and infected bee smell Figure 3.3. A total of 47.3% of VOCs were associated with uninfected bees; lab-killed uninfected bees (24.8%) and uninfected live bees (22.5%), with the remaining proportion associated with mixed infections: AFB (23.8%), sacbrood virus (18.3%), Varroa mites (8.4%), *in vitro* AFB (0.6%), *in vitro* chalkbrood (0.9%) and chalkbrood (0.6%) (Figure 3.3). This community was the most chemically diverse, encompassing all 17 chemical classes but also had five VOCs uniquely associated with sacbrood virus and AFB: 3-methylbutyrolactone, 2-methylcaproic acid, and 3-hydroxyisovaleric acid were associated with sacbrood virus, while alpha-isophorone and 1-decene were associated with both sacbrood virus and AFB (Figure 3.2; Table S3.2).

The remaining community clusters of 'smell' (community 3-8) were almost exclusively associated with VOCs that originated from parasites and a single treatment Figure 3.3. The only minor exception was community 8, which consisted of a single cyclic hydrocarbon (3,3-dimethyl-6- methylene-1- cyclohexene) emitted by chalkbrood and *in vitro* chalkbrood. Communities 3-7 all contained VOCs that were uniquely associated with a single parasite (Figure 3.2; Table S3.2). Community 3 contained 35 VOCs emitted by AFB (Figure 3.2; Figure 3.3. Community 4 contained VOCs that were all emitted by *in vitro* chalkbrood (Figure 3.2; Figure 3.3). Community 5 consisted of 6 VOCs emitted from chalkbrood infected larvae. Community 6 was associated with Varroa mite parasitism (Figure 3.2; Figure 3.3), whilst Community 7 was made up of two VOCs associated with *in vitro* Varroa mite (Figure 3.2; Figure 3.3).

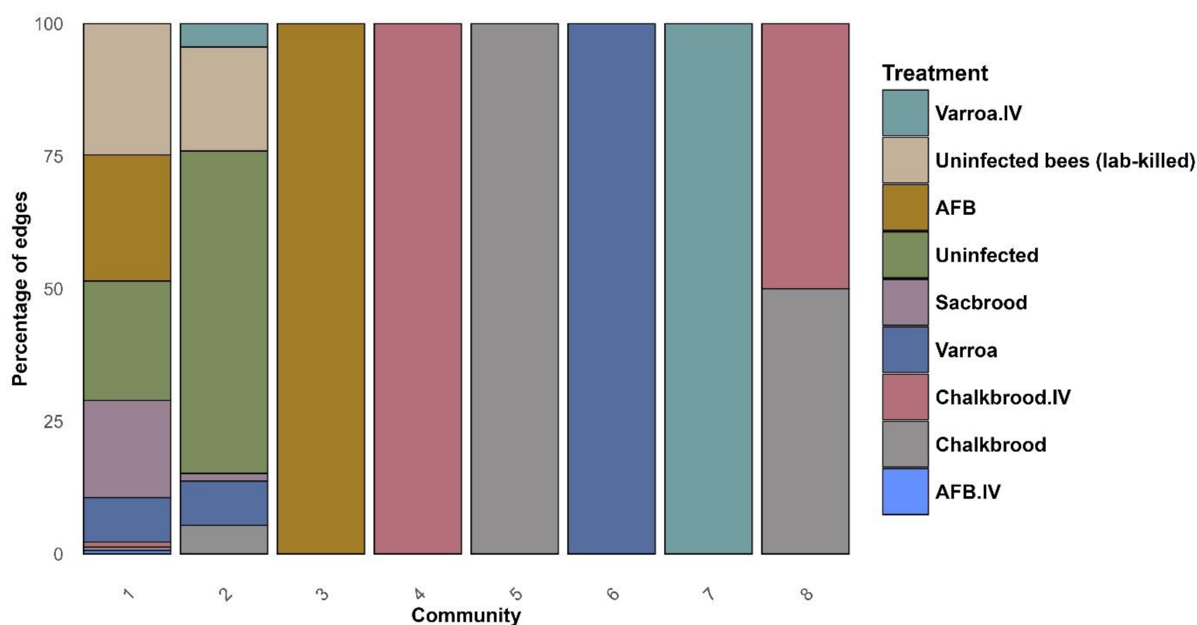

Figure 3.3: Percentage composition of treatments (edges) linking VOCs within each community in the network shown in Figure 3.2. Communities vary in the number of studies they contain: Community 1 (studies = 41), Community 2 (studies = 40), Community 3 (studies = 2), Community 4 (studies = 1), Community 5 (studies = 2), Community 6 (studies = 2), Community 7 (studies = 1), Community 8 (studies = 2).

The smell of infection within communities

To identify the most influential VOCs contributing to 'smell' clusters we examined the weighted degree (sum of the edges connected to a specific node) of each VOC within a community and focused on those in the top 5%. In community 1, the most connected VOCs included (E)-beta-ocimene ($k_w = 549$), acetoin ($k_w = 537$), and propionic acid ($k_w = 531$), which were also emitted by six of the eight treatments in the community (Figure 3.4; Table S3.2). Fourteen other highly connected VOCs ($k_w = 530$), primarily carboxylic acids and terpenoids (including (Z)-beta-ocimene), two alcohols (2,3-butanediol and isoamyl alcohol), and a single ester (methyl benzoate) were observed (Figure 3.4; Table S3.2). Community 2 was predominantly associated with uninfected bees and was characterised by phenethyl alcohol ($k_w = 386$) and methionol ($k_w = 367$). Other key VOCs included a series of alkanes, an aldehyde (nonanal), and a terpenoid (3-carene), with a weighted degree of 360 (Figure 3.4; Table S3.2).

In contrast to communities 1 and 2, communities 3-7 consisted exclusively of VOCs emitted by infected treatments. Within these communities, the weighted degree of each VOC remained constant, as each community was composed of a single treatment with evenly distributed edges (Table S3.2). Community 3, associated with American foulbrood, exhibited the greatest chemical diversity of the parasite clusters, encompassing 38 VOCs from a broad range of chemical classes. The most prevalent chemical classes (>10%) included aromatic hydrocarbons (18.4%), esters (15.8%), amides (13.2%) and alcohols (13.2%) (Figure 3.4; Table S3.2). Community 4 was associated with *in vitro* chalkbrood and contained four VOCs, primarily esters: phenethyl isobutyrate, phenethyl butyrate, phenethyl propionate, and rose oxide (Figure 3.4; Table S3.2). The chalkbrood-associated cluster in community 5 comprised six VOCs, primarily lactones (50%), including $_{\gamma}$-nonalactone, $_{\gamma}$ - heptalactone, $_{\gamma}$-dodecalactone) (Figure 3.4; Table S3.2). Community 6 was associated with Varroa mite and comprised mainly of esters (36%) and aromatic hydrocarbons (27%) Figure 3.4. Community 7, linked with *in vitro* Varroa mite, was characterised by two VOCs: oleic acid and hentriacontane (Figure 3.4; Table S3.2). Finally in community 8, consisting of VOCs from both chalkbrood and *in vitro* chalkbrood treatments, was dominated by a single cyclic hydrocarbon, 3,3-dimethyl-6- methylene-1- cyclohexene (Figure 3.4; Table S3.2).

When grouping VOCs by chemical class, there was substantial overlap among communities. No ‘smell’ community had a completely distinct chemical class profile. For example, Community 8 shared cyclic hydrocarbons with Communities 1-3, and amides and heteroaromatics appeared in both Community 1 and 3. All chemical classes found in infection-dominated communities were also present in at least one mixed community (Figure 3.4).

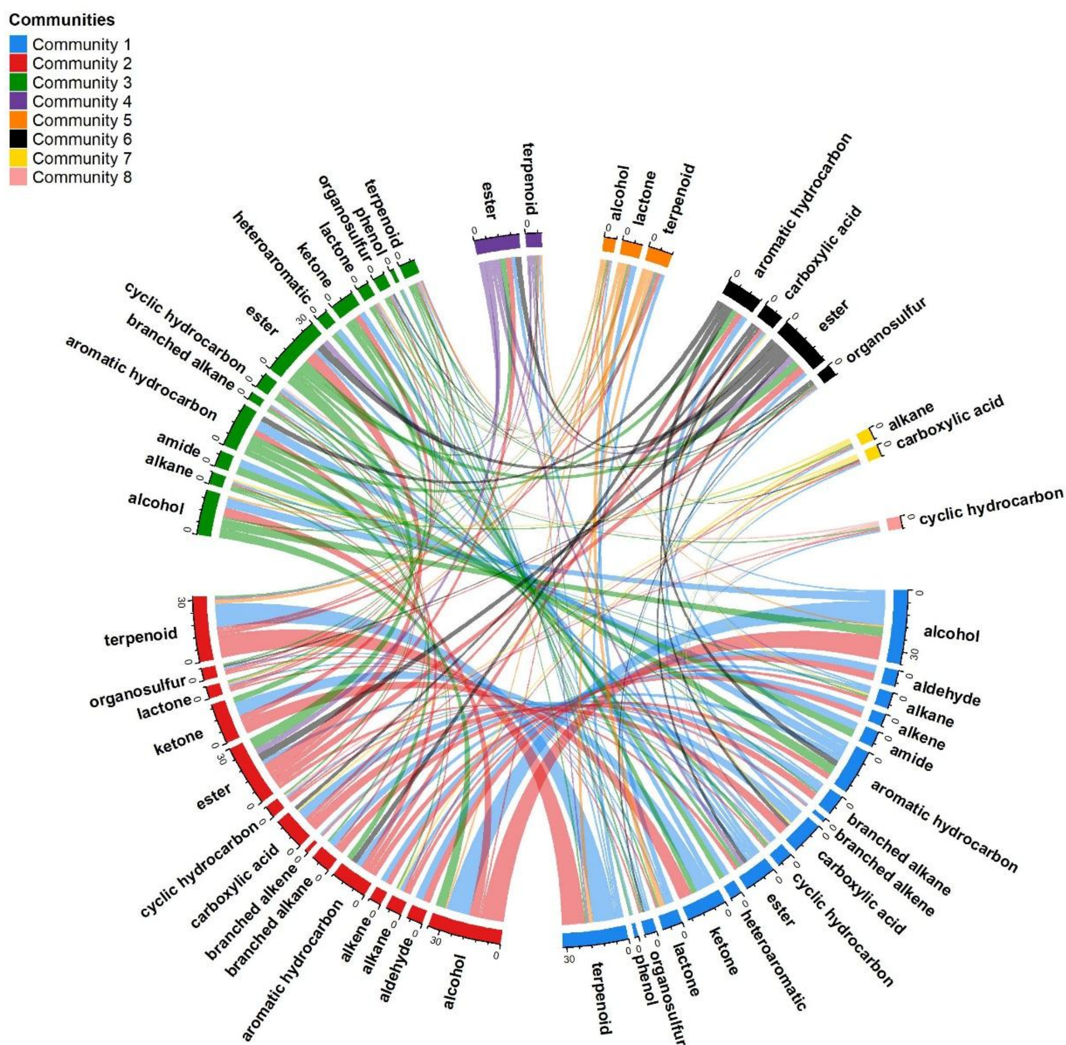

Figure 3.4: Composition of volatile organic compound (VOC) classes in each community. Width of each link represents the minimum number of shared VOC classes between each community. Communities vary in the number of studies they contain: Community 1 (studies = 41), Community 2 (studies = 40), Community 3 (studies = 2), Community 4 (studies = 1), Community 5 (studies = 2), Community 6 (studies = 2), Community 7 (studies = 1), Community 8 (studies = 2).

3.5 Discussion

Is there a smell of infection?

Our analysis provides strong evidence that certain honey bee pathogens produce distinguishable VOC profiles — a “smell of infection”, but that a distinct profile is not clear when VOCs are categorised at the broader class level. Using network analysis to synthesise VOC data across 49 studies and 23 articles, we identified eight VOC communities, six of which were composed exclusively of VOCs associated with infected treatments. These infection-specific communities corresponded to American foulbrood (AFB, *Paenibacillus larvae*), chalkbrood (*Ascosphaera apis*), and Varroa mite (*Varroa destructor*), and were composed of distinct, tightly co-occurring VOCs. This pattern supports the hypothesis that infection can generate unique chemical signatures in honey bees, which could underpin both colony-level behavioural responses and provide practical biosensing strategies for early disease detection.

We identified clusters of common volatiles that likely reflect the fact that, even during infection, the primary source of volatiles is still the bee and its environment. Two large communities (Communities 1 and 2) were composed of compounds detected in both infected and uninfected treatments. These included hive product-derived compounds, and pheromones associated with normal colony function. For instance, highly connected compounds such as beta-myrcene, alpha-pinene, and 2,3-butanediol (Community 1), and 3-carene and methionol (Community 2), are known to occur in floral nectar, pollen, and bee bread (Seisonen et al., 2015; Iglesias et al., 2020; Dekebo et al., 2022). Their consistent detection across treatments indicates that environmental and dietary sources dominate much of the honey bee volatilome. As a result, the background “smell” of a colony includes a wide array of constitutive VOCs that are not informative for diagnosis of disease.

Many volatiles commonly reported in association with infection were also consistently present in uninfected treatments, making it difficult to use a single VOC as a biomarker of infection. These broadly occurring compounds are part of the colony’s typical volatile profile, and may originate from bees, floral resources, hive materials, or microbial activity unrelated to infection. For example, (E)-β-ocimene, a brood pheromone and floral volatile (Maisonnasse et al., 2010; Zhang et al., 2019; Zhang et al., 2021; Dekebo et al., 2022), was among the most connected nodes in

the network and occurred across nearly all treatments, including AFB and chalkbrood infections (Table S3.2). Phenethyl alcohol and its ester phenethyl acetate, both of which have been linked to hygienic behaviour in chalkbrood-infected colonies (Swanson et al., 2009), were also associated with uninfected treatments. Similarly, compounds like acetoin are commonly synthesised by many microbes (Romano and Suzzi, 1996; Xiao and Xu, 2007; Lee et al., 2015) and were associated with infected, uninfected, and *in vitro* treatments, further illustrating that such volatiles can arise independently of infection status. Many of these compounds are commonly found in healthy colonies, although their concentrations may vary during infection (Lee et al., 2020). Therefore, while these VOCs may still be biologically relevant, their presence in isolation is not sufficient to diagnose infection.

Our observation that single VOCs are not suitable as biomarkers highlights the need to identify the “smell of infection” as a combination of compounds. Parasite-specific VOCs do not appear to occur in isolation. Instead, their diagnostic value lies in the way they co-occur with each other and in how they alter the typical composition of an insect’s odour. Our network approach suggests that these co-occurrence patterns — or “VOC bouquets” — may serve as more reliable indicators of infection than any single compound alone.

Composition of the smell of infection

Understanding the composition of VOCs associated with infection is important as both artificial sensors and bees rely on pattern recognition to detect these signals. In electronic nose (E-nose) or gas sensor arrays, the selection of sensors is determined by the chemical diversity of the target volatiles. Each sensor type varies in its sensitivity and selectivity for specific compounds, and the set of sensors included shapes the array’s ability to recognise an odour profile (Li et al., 2023). The same principle applies to behavioural sensing in honey bees: odour detection depends on the expression of specific odorant binding proteins (OBPs), which determine an individual’s sensitivity to particular compounds and can vary with caste and behavioural role (Iovinella et al., 2018). Bees that exhibit enhanced hygienic behaviour often exhibit differential expression of OBPs, which may contribute to their ability to detect infection-related odours (Mondet et al., 2015). In contrast, some

pathogens may suppress these responses: viral infection, for example, can inhibit OBP expression and reduce olfactory sensitivity, potentially helping parasites evade detection (Silva et al., 2021). By characterising the composition of infection-related VOCs, we can begin to identify which compounds are likely to be sensed, and thus, which OBPs or sensor types may be relevant. This has practical implications for both non-invasive gas sensor surveillance and breeding disease-resistant bees, where OBP expression profiles could be used as selection markers. It also provides a framework for understanding how other stressors, such as pesticides or co-infections, may impact a bee's ability to perceive and respond to infection cues *via* interactions with OBP expression (Li et al., 2015a).

Here, we found distinct VOC compositions for three major honey bee infections. The unique VOCs associated with infection revealed a mix of pathogen-derived compounds and host stress signals. In the case of AFB, caused by *Paenibacillus larvae,* a diverse cluster of 38 compounds was identified belonging to 13 chemical classes. The 38 VOCs were almost as diverse in terms of chemical class as the common background VOCs found in Communities 1 and 2, which both contained almost twice as many VOCs. This included a variety of microbial metabolites (e.g. esters such as ethyl butanoate and pyrazines like 2,5-dimethyl pyrazine), host-derived stress signals (e.g. (S)-2-heptanol, 1-methylpyrrole), and decomposition products (e.g. dimethyl disulfide, dimethyl trisulfide), supporting the view that multiple sources, both host and pathogen-derived, contribute to the smell of AFB (Gochnauer and Margetts, 1981; Gochnauer and Shearer, 1981; McAfee et al., 2018; Lee et al., 2020; Bikaun et al., 2022).

Chalkbrood was far less diverse in VOC composition compared to AFB. We identified three distinct communities linked to chalkbrood, comprising VOCs from only six chemical classes. These included phenethyl alcohol esters (Community 4) from *in vitro* growth, gamma-lactones and benzyl alcohol (Community 5) from *in vivo* infections, and a single cyclic hydrocarbon (3,3-dimethyl-6-methylene-1-cyclohexene), detected in both *in vitro* and *in vivo* chalkbrood samples (Community 8). Gamma-lactones such as gamma-nonalactone and gamma-heptalactone are associated with fermentation and lipid metabolism and were absent in uninfected treatments, suggesting they may be pathogen-derived metabolites specific to active infection (Ferron et al., 2020; Zeng et al., 2009). Benzyl alcohol is a known honey

bee alarm pheromone that may play a role in triggering brood removal (Swanson et al., 2009; Wen et al., 2017). Its presence in chalkbrood-infected larvae, but not *in vitro* chalkbrood, suggests it may be host-derived, produced as a semiochemical signalling for removal. 3,3-dimethyl-6-methylene-1-cyclohexene may be a promising biomarker as it was detected in both *in vitro* and *in vivo* chalkbrood, suggesting it could be a core metabolite. However, it has no known function in honey bee or *A. apis* (the causative agent of chalkbrood) metabolism, and it remains unclear whether this reflects an artefact of low study replication or a highly specific biomarker for chalkbrood infection. More data is needed on the VOCs associated with chalkbrood infection to clarify this.

Varroa-associated VOCs were identified in two VOC communities, Community 6, associated with Varroa *in vivo*, and Community 7, linked to Varroa *in vitro*. As with chalkbrood, the Varroa-associated VOCs were less chemically diverse than those linked to AFB, comprising only six chemical classes. These VOCs included a mix of mite-derived emissions and host physiological responses to parasitism. Ethyl hexanoate, for example, was present only in parasitised pupae and is a known trigger of hygienic behaviour, suggesting it is a host-derived stress signal induced by Varroa parasitism (Liendo et al., 2021). Several other compounds (heptanoic acid, methyl nonanoate, and nonahexacontanoic acid) are fatty acid derivatives with no known function in bees, but which appear to be unique to Varroa-infected brood and may serve as indicators of parasitism. Other notable VOCs included methylsulfonylmethane, linked to oxidative stress, which can be induced by Varroa parasitism (Badotra et al., 2013). In addition, *in vitro* Varroa-derived volatiles like ethyl octanoate – recently confirmed in Varroa headspace samples (Zhao et al., 2025) – may provide more specific indicators of mite presence.

VOCs as biomarkers of infection

Our identification of infection-specific VOCs presents promising opportunities for their use as biomarkers for non-invasive disease surveillance. The technological feasibility of VOC-based biosensing is already being demonstrated in other biological systems. E-nose technologies, for example, have been used to monitor food spoilage using VOCs (Ma et al., 2018; Xing et al., 2023), and AFB and Varroa in

honey bees using gas sensor arrays (Bak et al., 2020; Bak et al., 2022). Biosensors should ideally detect VOCs associated with the earliest stages of infection, since late-stage infections – particularly with AFB – are often too advanced to treat effectively (Matovic et al., 2023). Accordingly, VOCs associated with late-stage infection and brood decay (e.g. dimethyl disulfide and dimethyl trisulfide) are poor candidates for early biomonitoring. More promising are VOCs produced during the early stages of infection, such as methylated esters and pyrazines in AFB (e.g. ethyl 2-methylbutanoate, trimethylpyrazine) that are known antimicrobial volatiles produced by *Bacillus* species (Li et al., 2015b; Seo et al., 2018; Toral et al., 2021; Sun et al., 2023). Similarly, the lactones identified in chalkbrood (e.g. gamma-nonalactone, gamma-heptalactone) are associated with fermentation, lipid metabolism (Zeng et al., 2009; Ferron et al., 2020) and quorum-sensing regulation (Williams et al., 2012), suggesting roles in microbial competition or infection dynamics.

In the case of Varroa, which is nearly ubiquitous in managed colonies (USDA-APHIS National Honey Bee Survey, 2024), biomonitoring efforts would benefit from quantifying Varroa load rather than simply detecting presence/absence. Sensors should target VOCs specifically associated with Varroa, rather than generalised stress signals from the host that may also vary in association with non-Varroa related factors, such as oxidative stress (e.g. methylsulfonylmethane, Badotra et al., 2013). While no single VOC may accurately reflect mite load, sensors trained on mixtures of Varroa-specific VOCs could provide cumulative signals, and early trials have already shown some success in using E-noses to estimate Varroa load (Szczurek et al., 2020b). In our analysis, ethyl octanoate was specific to Varroa-infested brood and has recently been identified in *in vitro* Varroa headspace (Zhao et al., 2025). Additionally, heptanoic acid, methyl nonanoate, and nonahexacontanoic acid were not reported in association with any other treatments, suggesting they may reflect Varroa-specific metabolism rather than bee-derived stress signals. These features make them promising targets for tracking mite burden independent of other colony conditions. Further identification of parasite/pathogen VOCs will help guide gas sensor design and improve the specificity of E-noses by informing which sensor channels to include and which background VOCs to exclude.

Importantly, our network analysis highlights the potential of using VOC combinations rather than single markers to monitor infection. The clustering of infection-associated VOCs into distinct communities supports the development of detection systems based on pattern recognition, rather than threshold detection of individual compounds. Future efforts should aim to validate these compounds under field conditions and determine the concentration thresholds at which VOC bouquets become reliably detectable by gas sensors.

What are the limitations?

Although our network analysis revealed strong clustering of infection-specific VOCs, several limitations affect how these patterns should be interpreted. The first and most significant is the relatively small number of studies representing each infection type. For example, only three studies reported on AFB and chalkbrood infections, and only four examined Varroa. With such low replication, some VOCs may appear unique to infection simply because they were not reported in other contexts. This introduces a risk of overestimating their specificity and underestimating natural variation within infections.

A further limitation stems from the nature of the data: our analysis relied on presence/absence rather than quantitative VOC data. While this approach helped navigate inconsistencies in sampling techniques and reporting formats, it does not capture changes in compound abundance. As mentioned above, many compounds, including semiochemicals like (E)-β-ocimene, are constitutively produced by honey bees and a deviation from normal emissions, such as a decrease in concentration, can signal infection (Lee et al., 2020). However, presence/absence data cannot distinguish whether the VOCs identified reflect meaningful biological concentration shifts or background variability. This limits our ability to evaluate compounds that, whilst produced by uninfected and/or infected bees, may fluctuate significantly during infection and thus be biologically relevant for behavioural cues or infection monitoring.

Methodological variability across studies also introduces uncertainty. VOCs were sampled using different techniques, including solid-phase microextraction, open-loop

stripping, and volatile traps, each of which captures different subsets of the volatilome. Variability in the sampling method, therefore, limits the number of true replicates for each treatment. For example, oleic acid – identified here as Varroa-specific – is a well-known death pheromone commonly present in the cuticular extracts of dead uninfected bees (McAfee et al., 2018), and we would expect it to be found in association with lab-killed uninfected bees. Oleic acid is typically a low volatility compound and the fact that it was only detected in the headspace of one Varroa study likely reflects artefacts of either unusually high concentrations, or detection using exceptionally sensitive techniques. The biological origin of many VOCs is also difficult to resolve, especially for compounds with multiple potential sources. Citronellol and citral, detected in chalkbrood-infected larvae, could plausibly originate from the host's defensive response, the fungus itself, or environmental contamination from hive materials or floral residues (Shearer and Boch, 1966; Butler and Calam, 1969; Gochnauer et al., 1979). This ambiguity makes it challenging to assign ecological or diagnostic significance without controlled comparative studies.

Some VOCs in the dataset were reported only in single studies, often in low concentrations, and may represent environmental contaminants or laboratory artefacts. For example, 1,4-dimethylnaphthalene and 2,7-dimethylnaphthalene were detected only once in association with Varroa parasitism and lack known biological relevance to bees or pathogens. Such compounds may have skewed community assignments in the network while providing limited diagnostic utility. Together, these limitations highlight the need for future empirical studies that investigate VOCs associated with these infections to include quantitative measurements and validate candidate compounds across multiple colonies and environmental contexts.

All studies included in this study investigated honey bee larvae, none focussed on the adults. This was due to a lack of literature investigating volatile cues in adult honey bees. Adults are key drivers of colony-level infection dynamics and may also represent overlooked sources of diagnostic VOCs. CHC profiles, for example, are known to shift in adults when infected with *Vairimorpha* spp. and Israeli Acute Paralysis Virus (Murray et al., 2015; Geffre et al., 2020). Other key parasites were also missing from the literature, including headspace VOCs for European foulbrood, which has been associated with unique CHC profiles, but has not had its headspace volatiles sampled (Kathe et al., 2021). These gaps point to important opportunities

for expanding the chemical understanding of infection across life stages and pathogen types.

Ecological implications of a ‘smell of infection’

Despite limitations, the presence of distinct infection-associated VOC profiles in honey bees has important implications for our understanding of social immunity, the collective behavioural defence by social insects against infection (Cremer et al., 2007), as well as for colony-level disease dynamics and host-pathogen coevolution. A key component of social immunity is the need to identify and communicate the presence of infection in the colony to enable workers to remove infected individuals before transmission (Cremer et al., 2018). Many of the compounds identified here, including ethyl hexanoate, phenethyl acetate, benzyl alcohol, and various lactones, are known to elicit hygienic behaviour – the targeted detection and removal of infected brood by nestmates (Swanson et al., 2009; Liendo et al., 2021; Wen et al., 2017). This behaviour is a central mechanism in honey bee disease defence, and its effectiveness hinges on reliable chemical cues of infection.

Studies have previously used semiochemicals associated with infection to identify honey bee colonies with Varroa mite resistance behaviours (Wagoner et al., 2021). Targeting and selecting for colonies with heightened sensitivity to infection-associated VOCs is a promising solution for infection control that avoids the need to apply chemical treatments, such as miticides, to colonies. In our study, several VOCs, such as ethyl hexanoate and gamma-decalactone, were uniquely associated with parasitised brood and have been linked to hygienic responses in prior work (Liendo et al., 2021; Finstrom et al., 2023). These, along with other infection-specific VOCs identified in our dataset, represent potential candidates for behavioural assays designed to evaluate colony chemosensitivity towards infection.

While VOCs can serve as cues for social immunity, parasite and host are in a constant evolutionary arms race, and parasites may actively manipulate VOC signals to avoid or exploit host detection. For example, Varroa mite is known to mimic the cuticular hydrocarbon profile of honey bees (Kather et al., 2015) and may also express this chemical camouflage through VOC production. Several compounds

found in Community 1 (a series of alkanes, an aldehyde and a terpenoid, including hexadecane, heptadecane, undecane, dodecane) were emitted by both uninfected bees and Varroa mite *in vitro*. These compounds are common volatile hydrocarbons found in the mandibular glands and hive products of honey bees (Engels et al., 1997; Starowicz et al., 2021) and their presence in both treatments suggests potential chemical mimicry. In addition to mimicry, some parasites may alter host chemistry to enhance their own transmission. Viral infections are known to shift honey bee CHC profiles in ways that increase their inter-colony acceptance (Geffre et al., 2020). The same could be true of VOCs, especially those detected in infected treatments but without clear links to hygienic behaviour. These could be parasite-derived odours that suppress detection or promote vectoring.

Overall, the smell of infection in honey bees is not just a byproduct of disease but an ecologically meaningful signal with functional roles in disease recognition, social behaviour, and host-pathogen coevolution. These findings highlight the importance of integrating chemical ecology with apicultural practice and the evolutionary biology of social insects.

Conclusion

This meta-analysis provides the first synthesis of volatile organic compounds associated with honey bee infections across multiple studies, revealing that distinct “smells of infection” exist for key pathogens including *P. larvae*, *A. apis*, and *V. destructor*. Using a network approach, we show that infection alters the VOC profile of honey bees, producing chemical signatures composed of both pathogen-derived volatiles and host metabolic responses. These signatures were composed of diverse chemical classes, including esters, lactones, pyrazines, alcohols, and aromatic hydrocarbons, many of which have known behavioural or microbial activity. These VOC communities have potential as early biomarkers of infection that can be used to monitor infection using gas sensor arrays or behavioural assays for breeding disease-resistant bees, particularly if used in combination, as their co-occurrence patterns may more diagnostic than individual compound presence alone. Promising candidates include early-stage infection markers such as ethyl hexanoate, benzyl alcohol, lactones, and microbial pyrazines. However, several limitations remain. The

small number of studies for each infection type, the reliance on presence/absence data, and methodological heterogeneity constrain our ability to determine how generalisable or robust these VOC profiles are under field conditions. Further research is needed to validate these findings with quantitative data, assess concentration thresholds for behavioural responses and gas sensors, and test whether candidate compounds can reliably distinguish infection from other colony stressors.

Ultimately, this work demonstrates that infection in social insects produces biologically meaningful changes to the volatilome that can be detected and potentially exploited as infection biomarkers. This does not only apply to honey bees, but any animal system that is difficult to manually survey for infection and has relevance for other social animals that may be impacted by infection, while also communicating chemically. By combining chemical ecology with biosensing technology and selective breeding strategies, there is clear potential to develop non-invasive tools that improve insect disease surveillance and support more sustainable insect farming practices.

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3.7 Supplementary Material

Table S3.1: A complete list of 23 articles included in a meta-analysis of VOCs to assess the 'smell' of infection. The table reports the number of studies within each paper, parasites and host life-stage sampled and the method used.

Article	Treatment	No. studies	Sampling method	Life stages
Bikaun, J.M., Bates, T., Bollen, M., Flematti, G.R., Melonek, J., Praveen, P. et al. 2022: Volatile biomarkers for non-invasive detection of american foulbrood, a threat to honey bee pollination services. — Science of The Total Environment, 845: p. 157123.	Uninfected bees (lab-killed), Uninfected, AFB, Sacbrood	5	SPME	Larvae
Carroll, M.J. & Duehl, A.J. 2012: Collection of volatiles from honeybee larvae and adults enclosed on brood frames. — Apidologie, 43: pp. 715-730.	Uninfected	1	Volatile trap	Mixed
Finstrom, M.S., Angove, M., Brooks, P. & Gerdts, J. 2023: Identification and discrimination of volatiles associated with chalkbrood infection in european honey bees (apis mellifera), eastern australia. —.	Chalkbrood, Chalkbrood.IV, Uninfected bees (lab-killed), Uninfected	4	SPME	Larvae, In vitro
Haber, M., Mishyna, M., Martinez, J.I. & Benjamin, O. 2019: Edible larvae and pupae of honey bee (apis mellifera): Odor and nutritional characterization as a function of diet. — Food chemistry, 292: pp. 197-203.	Uninfected bees (lab-killed)	2	SPME	Larvae, pupae
He, X.J., Zhang, X.C., Jiang, W.J., Barron, A.B., Zhang, J.H. & Zeng, Z.J. 2016: Starving honey bee (apis mellifera) larvae signal pheromonally to	Uninfected	1	Volatile trap	Larvae

Article	Treatment	No. studies	Sampling method	Life stages
worker bees. — Scientific reports, 6 (1): p. 22359.				
LIOLIOS, V., KANELIS, D., TANANAKI, C. & RODOPOULOU, M.-A. 2022. A Comparative Study of healthy and American Foulbrood-Infected Bee Brood (Apis mellifera L.) through the Investigation of Volatile Compounds. Agriculture, 12, 812.	AFB, Uninfected	2	Volatile trap	Larvae
Lee, S., Lim, S., Choi, Y., Lee, M. & Kwon, H. 2020: Volatile Disease Markers of American Foulbrood-Infected Larvae in Apis Mellifera. — Journal of Insect Physiology. 122: 122.	AFB, AFB.IV, Uninfected	3	SPME	Larvae, In vitro
Liendo, M.C., Muntaabski, I., Russo, R.M., Lanzavecchia, S.B., Segura, D.F., Palacio, M.A., Cladera, J.L., Fernández, P.C. & Scannapieco, A.C. 2021: Temporal Changes in Volatile Profiles of Varroa Destructor-Infested Brood May Trigger Hygienic Behavior in Apis Mellifera. — Entomologia Experimentalis et Applicata. 169: 563-574.	Varroa, Uninfected	2	Volatile trap	Pupae
Light M, Shutler D, Cutler GC, Hillier NK. Varroa destructor mite electrophysiological responses to honey bee (Apis mellifera) colony volatiles. Exp Appl Acarol. 2020;	Uninfected bees (lab-killed), Uninfected	4	Volatile trap	Drone pupae, larvae, drone egg and larvae
Liu, J., Zhang, R., Tang, R., Zhang, Y., Guo, R., Xu, G. et al. 2022: The role of honey bee derived aliphatic esters in the	Uninfected	2	SPME	Larvae, drone larvae

Article	Treatment	No. studies	Sampling method	Life stages
host-finding behavior of varroa destructor. — Insects, 14 (1): p. 24.				
Maisonnasse, A., Lenoir, J.-C., Beslay, D., Crauser, D. & Le Conte, Y. 2010: E-β-ocimene, a volatile brood pheromone involved in social regulation in the honey bee colony (apis mellifera). — PLOS one, 5 (10): p. e13531.	Uninfected	2	SPME	Larvae, pupae
Martin, C., et al. (2002). "Potential mechanism for detection by Apis mellifera of the parasitic mite Varroa destructor inside sealed brood cells." Physiological Entomology 27(3): 175-188.	Varroa.IV	1	SPME	In vitro
McAfee, A., Collins, T.F., Madilao, L.L. & Foster, L.J. 2017: Odorant Cues Linked to Social Immunity Induce Lateralized Antenna Stimulation in Honey Bees (Apis Mellifera L.). — Scientific Reports. 7.	Uninfected bees (lab-killed), Uninfected	2	SPME	Pupae
Nazzi, F., et al. (2002). "(Z)-8-heptadecene from infested cells reduces the reproduction of Varroa destructor under laboratory conditions." Journal of Chemical Ecology 28(11): 2181-2190.	Varroa, Uninfected	2	SPME	Capped larvae
Nazzi, F., et al. (2004). "A semiochemical from brood cells infested by Varroa destructor triggers hygienic behaviour in Apis mellifera." Apidologie 35(1): 65-70.	Varroa, Uninfected	2	SPME	Capped larvae

Article	Treatment	No. studies	Sampling method	Life stages
Noël, A., Dumas, C., Rottier, E., Beslay, D., Costagliola, G., Ginies, C. et al. 2023: Detailed chemical analysis of honey bee (apis mellifera) worker brood volatile profile from egg to emergence. — Plos one, 18 (2): p. e0282120.	Uninfected	4	SPME	Egg, larvae, pupae, adult
Piechowicz, B., Kwiatek, A., Sadło, S., Zaręba, L., Koziorowska, A., Kloc, D. et al. 2023: Use of gas chromatography and spme extraction for the differentiation between healthy and paenibacillus larvae infected colonies of bee brood—preliminary research. — Agriculture, 13 (2): p. 487.	Uninfected bees (lab-killed)	1	SPME	Larvae
Schmitt, T., Herzner, G., Weckerle, B., Schreier, P. & Strohm, E. 2007: Volatiles of foraging honeybees apis mellifera (hymenoptera : Apidae) and their potential role as semiochemicals. — Apidologie, 38 (2): pp. 164-170.	Uninfected	1	SPME	Adult
Schoening, C., Gisder, S., Geiselhardt, S., Kretschmann, I., Bienefeld, K., Hilker, M. & Genersch, E. 2012: Evidence for Damage-Dependent Hygienic Behaviour Towards Varroa Destructor-Parasitised Brood in the Western Honey Bee, Apis Mellifera. — Journal of Experimental Biology. 215: 264-271.	Uninfected, Varroa	2	Open-loop stripping	Pupae
Swanson, J.A.I., Torto, B., Kells, S.A., Mesce, K.A., Tumlinson, J.H. & Spivak, M. 2009: Odorants That Induce Hygienic	Chalkbrood, Uninfected	2	Volatile trap	Larvae

Article	Treatment	No. studies	Sampling method	Life stages
Behavior in Honeybees: Identification of Volatile Compounds in Chalkbrood-Infected Honeybee Larvae. — Journal of Chemical Ecology. 35: 1108-1116.				
Torto, B., et al. (2005). "Response of the small hive beetle (Aethina tumida) to a blend of chemicals identified from honeybee (Apis mellifera) volatiles." Apidologie 36(4): 523-532.	Uninfected	1	Volatile trap	Adult
Wu, F., Ma, C., Han, B., Meng, L., Hu, H., Fang, Y. et al. 2019: Behavioural, physiological and molecular changes in alloparental caregivers may be responsible for selection response for female reproductive investment in honey bees. — Molecular Ecology, 28 (18): pp. 4212-4227.	Uninfected	1	SPME	Larvae
Zhao, H.X., Liang, Q., Lee, J.H., Zhang, X.F., Huang, W.Z., Chen, H.S. & Luo, Y.X. 2015: Behavioral Responses of Apis Mellifera Adult Workers to Odors from healthy Brood and Diseased Brood. — Sociobiology. 62: 564-570.	Chalkbrood, Uninfected	2	SPME	Larvae

Table S3.2: Complete table of all volatile organic compounds found in the analysis. The table reports chemical class, Walktrap community clustering, weighted degree within the network, and associated treatments.

CAS No.	Class	Community	Degree	Compound	Emitted by
3779-61-1	Terpenoid	1	550	(E)-beta-ocimene	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa, Chalkbrood
513-86-0	Ketone	1	538	acetoin	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa, Chalkbrood.IV
79-09-4	Carboxylic acid	1	532	propionic acid	AFB, AFB.IV, Uninfected, Varroa, Uninfected bees (lab-killed), Sacbrood
107-92-6	Carboxylic acid	1	531	butyric acid	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
112-05-0	Carboxylic acid	1	531	nonanoic acid	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa

CAS No.	Class	Community	Degree	Compound	Emitted by
116-53-0	Carboxylic acid	1	531	anteisovaleric acid	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
123-35-3	Terpenoid	1	531	beta-myrcene	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
123-51-3	Alcohol	1	531	isoamyl alcohol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
124-07-2	Carboxylic acid	1	531	octanoic acid	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
127-91-3	Terpenoid	1	531	beta-pinene	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
142-62-1	Carboxylic acid	1	531	caproic acid	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa

CAS No.	Class	Community	Degree	Compound	Emitted by
3338-55-4	Terpenoid	1	531	(Z)-beta-ocimene	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
503-74-2	Carboxylic acid	1	531	isovaleric acid	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
513-85-9	Alcohol	1	531	2,3-butanediol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
79-31-2	Carboxylic acid	1	531	isobutyric acid	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
80-56-8	Terpenoid	1	531	alpha-pinene	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa
93-58-3	Ester	1	531	methyl benzoate	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood, Varroa

CAS No.	Class	Community	Degree	Compound	Emitted by
103-81-1	Amide	1	479	benzeneacetamide	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
10482-56-1	Terpenoid	1	479	alpha-terpineol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
106-44-5	Phenol	1	479	4-methylphenol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
108-29-2	Lactone	1	479	gamma-valerolactone	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
108-82-7	Alcohol	1	479	diisobutylcarbinol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
108-88-3	Aromatic hydrocarbon	1	479	toluene	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
108-95-2	Phenol	1	479	phenol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
109-97-7	Heteroaromatic	1	479	pyrrole	AFB, Uninfected bees (lab-killed),

CAS No.	Class	Community	Degree	Compound	Emitted by
					Uninfected, Sacbrood
110-43-0	Ketone	1	479	2-heptanone	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
112-12-9	Ketone	1	479	2-undecanone	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
1569-60-4	Alcohol	1	479	sulcatol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
19132-06-0	Alcohol	1	479	2,3-butanediol, (threo)	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
431-03-8	Ketone	1	479	2,3-butanedione	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
460-01-6	Alkene	1	479	dihydromyrcene	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
541-35-5	Amide	1	479	butyramide	AFB, Uninfected, Uninfected bees

CAS No.	Class	Community	Degree	Compound	Emitted by
					(lab-killed), Sacbrood
541-46-8	Amide	1	479	isovaleramide	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
590-86-3	Aldehyde	1	479	isovaleraldehyde	AFB, Uninfected, Sacbrood, Uninfected bees (lab-killed)
6032-29-7	Alcohol	1	479	2-pentanol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
628-99-9	Alcohol	1	479	2-nonanol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
64-19-7	Carboxylic acid	1	479	acetic acid	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
67-63-0	Alcohol	1	479	isopropyl alcohol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
695-06-7	Lactone	1	479	gamma-caprolactone	AFB, Uninfected bees (lab-killed),

CAS No.	Class	Community	Degree	Compound	Emitted by
					Uninfected, Sacbrood
71-41-0	Alcohol	1	479	1-pentanol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
78-70-6	Terpenoid	1	479	linalool	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
78-83-1	Alcohol	1	479	2-methyl-1-propanol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
816-66-0	Carboxylic acid	1	479	isopropylpyruvic acid	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
821-55-6	Ketone	1	479	2-nonanone	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
87414-49-1	Lactone	1	479	butyrolactone	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
90-05-1	Phenol	1	479	2-methoxyphenol	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood

CAS No.	Class	Community	Degree	Compound	Emitted by
91-20-3	Aromatic hydrocarbon	1	479	naphthalene	Varroa, Uninfected bees (lab-killed), Uninfected, AFB, Chalkbrood.IV
96-04-8	Ketone	1	479	2,3-heptanedione	AFB, Uninfected bees (lab-killed), Uninfected, Sacbrood
96-17-3	Aldehyde	1	479	2-methylbutyraldehyde	AFB, Uninfected, Sacbrood, Uninfected bees (lab-killed)
100-41-4	Aromatic hydrocarbon	1	472	ethylbenzene	Varroa, Uninfected bees (lab-killed), Uninfected, AFB
110-93-0	Ketone	1	472	sulcatone	AFB, Uninfected bees (lab-killed), Uninfected, Varroa
111-84-2	Alkane	1	472	nonane	Uninfected bees (lab-killed), Varroa, Uninfected, AFB
123-92-2	Ester	1	472	isoamyl acetate	Uninfected, Varroa, AFB, Uninfected bees (lab-killed)

CAS No.	Class	Community	Degree	Compound	Emitted by
138-86-3	Terpenoid	1	472	limonene	Uninfected, Varroa, Uninfected bees (lab-killed), AFB
107-87-9	Ketone	1	420	2-pentanone	Uninfected bees (lab-killed), Uninfected, AFB
111-65-9	Alkane	1	420	octane	Uninfected bees (lab-killed), AFB, Uninfected
553-86-6	Cyclic hydrocarbon	1	420	2-coumaranone	AFB, Uninfected bees (lab-killed), Uninfected
628-02-4	Amide	1	420	caproamide	AFB, Uninfected bees (lab-killed), Uninfected
930-27-8	Heteroaromatic	1	420	3-methylfuran	AFB, Uninfected bees (lab-killed), Uninfected
5989-27-5	Terpenoid	1	415	d-limonene	AFB, Uninfected, Sacbrood, Varroa
763-32-6	Alcohol	1	415	isobutenylcarbinol	AFB, Uninfected, Sacbrood, Varroa
470-82-6	Terpenoid	1	363	eucalyptol	AFB, Uninfected, Sacbrood

CAS No.	Class	Community	Degree	Compound	Emitted by
109-52-4	Carboxylic acid	1	357	valeric acid	AFB, AFB.IV, Uninfected, Varroa
108-38-3	Aromatic hydrocarbon	1	304	1,3-xylene	AFB, Uninfected
124-13-0	Aldehyde	1	304	octanal	AFB, Uninfected
2345-28-0	Ketone	1	304	2-pentadecanone	AFB, Uninfected
593-08-8	Ketone	1	304	2-tridecanone	AFB, Uninfected
600-14-6	Ketone	1	304	2,3-pentanedione	AFB, Uninfected
95-47-6	Aromatic hydrocarbon	1	304	1,2-xylene	AFB, Uninfected
98-01-1	Aldehyde	1	304	furan-2-carbaldehyde	AFB, Uninfected
120-72-9	Heteroaromatic	1	227	indole	AFB, Uninfected bees (lab-killed)
4613-38-1	Carboxylic acid	1	227	neric acid	AFB, Uninfected bees (lab-killed)
78-59-1	Ketone	1	170	alpha-isophorone	AFB, Sacbrood
872-05-9	Alkene	1	170	1-decene	AFB, Sacbrood
10574-36-4	Branched alkene	1	116	(Z)-3-methyl-2-hexene	Uninfected bees (lab-killed)
13877-91-3	Terpenoid	1	116	ocimene	Uninfected bees (lab-killed)

CAS No.	Class	Community	Degree	Compound	Emitted by
15869-80-4	Branched alkane	1	116	3-ethylheptane	Uninfected bees (lab-killed)
15869-86-0	Branched alkane	1	116	4-ethyloctane	Uninfected bees (lab-killed)
15869-93-9	Branched alkane	1	116	3,5-dimethyloctane	Uninfected bees (lab-killed)
22025-20-3	Alcohol	1	116	3,3-dimethyl-2-hexanol	Uninfected bees (lab-killed)
3741-00-2	Cyclic hydrocarbon	1	116	pentylcyclopentane	Uninfected bees (lab-killed)
3899-36-3	Branched alkene	1	116	3-methyl-3-hexene	Uninfected bees (lab-killed)
4984-01-4	Branched alkene	1	116	1-octene, 3,7-dimethyl	Uninfected bees (lab-killed)
515-00-4	Terpenoid	1	116	2-pinen-10-ol	Uninfected bees (lab-killed)
51655-64-2	Alkene	1	116	nonane, 3-methylene	Uninfected bees (lab-killed)
589-81-1	Branched alkene	1	116	heptane 3-methylene	Uninfected bees (lab-killed)
62108-25-2	Branched alkane	1	116	decane, 2,6,7 trimethyl	Uninfected bees (lab-killed)
74630-08-3	Alkene	1	116	3-Ethyl-1-octene	Uninfected bees (lab-killed)
74630-65-2	Branched alkene	1	116	(Z)-9-methyl-5-undecene	Uninfected bees (lab-killed)
75-18-3	Organosulfur	1	116	dimethyl sulfide	Uninfected bees (lab-killed)

CAS No.	Class	Community	Degree	Compound	Emitted by
706-14-9	Lactone	1	85	gamma-decalactone	Sacbrood, Chalkbrood, Chalkbrood.IV
1679-49-8	Lactone	1	59	3-methylbutyrolactone	Sacbrood
4536-23-6	Carboxylic acid	1	59	2-methylcaproic acid	Sacbrood
625-08-1	Carboxylic acid	1	59	3-hydroxyisovaleric acid	Sacbrood
60-12-8	Alcohol	2	387	phenethyl alcohol	Uninfected bees (lab-killed), Uninfected, Sacbrood, Chalkbrood
505-10-2	Organosulfur	2	368	methionol	Uninfected bees (lab-killed), Uninfected, Sacbrood
112-40-3	Alkane	2	361	dodecane	Uninfected bees (lab-killed), Uninfected, Varroa
1120-21-4	Alkane	2	361	undecane	Uninfected bees (lab-killed), Uninfected, Varroa
124-19-6	Aldehyde	2	361	nonanal	Uninfected bees (lab-killed), Varroa, Uninfected

CAS No.	Class	Community	Degree	Compound	Emitted by
13466-78-9	Terpenoid	2	361	3-carene	Uninfected bees (lab-killed), Uninfected, Varroa
544-76-3	Alkane	2	361	hexadecane	Uninfected bees (lab-killed), Varroa, Uninfected
629-78-7	Alkane	2	361	heptadecane	Varroa, Uninfected bees (lab-killed), Uninfected
103-45-7	Ester	2	328	2-phenethyl acetate	Uninfected bees (lab-killed), Uninfected, Chalkbrood
112-31-2	Aldehyde	2	328	decanal	Uninfected bees (lab-killed), Uninfected, Chalkbrood
3796-70-1	Terpenoid	2	328	geranyl acetone	Uninfected, Chalkbrood, Uninfected bees (lab-killed)
112-95-8	Alkane	2	319	eicosane	Uninfected bees (lab-killed), Varroa.IV, Uninfected
593-49-7	Alkane	2	319	heptacosane	Varroa.IV, Uninfected bees

CAS No.	Class	Community	Degree	Compound	Emitted by
					(lab-killed), Uninfected
629-94-7	Alkane	2	319	heneicosane	Uninfected, Varroa.IV, Uninfected bees (lab-killed)
108-93-0	Alcohol	2	309	cyclohexanol	Uninfected bees (lab-killed), Uninfected
112-30-1	Alcohol	2	309	1-decanol	Uninfected bees (lab-killed), Uninfected
112-39-0	Ester	2	309	methyl palmitate	Uninfected, Uninfected bees (lab-killed)
1120-72-5	Ketone	2	309	2-methyl cyclopentanone	Uninfected bees (lab-killed), Uninfected
119-36-8	Ester	2	309	methyl salicylate	Uninfected bees (lab-killed), Uninfected
124-18-5	Alkane	2	309	decane	Uninfected bees (lab-killed), Uninfected
13151-34-3	Branched alkane	2	309	3-methyldecane	Uninfected bees (lab-killed), Uninfected

CAS No.	Class	Community	Degree	Compound	Emitted by
141-78-6	Ester	2	309	ethyl acetate	Uninfected bees (lab-killed), Uninfected
143-13-5	Ester	2	309	nonyl acetate	Uninfected bees (lab-killed), Uninfected
1462-03-9	Alcohol	2	309	1-methyl cyclopentanol	Uninfected bees (lab-killed), Uninfected
1757-42-2	Ketone	2	309	3-methyl cyclopentanone	Uninfected bees (lab-killed), Uninfected
18729-48-1	Alcohol	2	309	3-methyl cyclopentanol	Uninfected bees (lab-killed), Uninfected
2216-34-4	Branched alkane	2	309	4-methyloctane	Uninfected bees (lab-killed), Uninfected
24070-77-7	Alcohol	2	309	2-methyl cyclopentanol	Uninfected bees (lab-killed), Uninfected
2579-04-6	Alkene	2	309	8-heptadecene	Uninfected bees (lab-killed), Uninfected
4340-76-5	Alcohol	2	309	2-eicosanol	Uninfected bees (lab-killed), Uninfected

CAS No.	Class	Community	Degree	Compound	Emitted by
4588-18-5	Branched alkene	2	309	2-methyl-1-octene	Uninfected bees (lab-killed), Uninfected
5009-32-5	Ketone	2	309	8-nonen-2-one	Uninfected bees (lab-killed), Uninfected
565-67-3	Alcohol	2	309	2-methyl 3-pentanol	Uninfected bees (lab-killed), Uninfected
623-37-0	Alcohol	2	309	3-hexanol	Uninfected bees (lab-killed), Uninfected
623-55-2	Alcohol	2	309	5-methyl-3-hexanol	Uninfected bees (lab-killed), Uninfected
626-93-7	Alcohol	2	309	2-hexanol	Uninfected bees (lab-killed), Uninfected
629-50-5	Alkane	2	309	tridecane	Uninfected bees (lab-killed), Uninfected
77-74-7	Alcohol	2	309	3-methyl 3-pentanol	Uninfected bees (lab-killed), Uninfected
97-64-3	Ester	2	309	2-hydroxyethyl propionate	Uninfected bees (lab-killed), Uninfected

CAS No.	Class	Community	Degree	Compound	Emitted by
98-86-2	Ketone	2	309	acetophenone	Uninfected bees (lab-killed), Uninfected
104-50-7	Lactone	2	271	gamma-octalactone	Uninfected, Sacbrood, Chalkbrood
100-52-7	Aldehyde	2	245	benzaldehyde	Uninfected, Varroa
106-30-9	Ester	2	245	ethyl heptanoate	Uninfected, Varroa
1195-32-0	Terpenoid	2	245	p-cymenene	Uninfected, Varroa
16369-12-3	Alkene	2	245	(z)-8-heptadecene	Varroa, Uninfected
18435-45-5	Alkene	2	245	nonadecene	Uninfected, Varroa
2591-86-8	Aldehyde	2	245	piperidine-1-carbaldehyde	Uninfected, Varroa
593-45-3	Alkane	2	245	octadecane	Uninfected, Varroa
629-59-4	Alkane	2	245	tetradecane	Uninfected, Varroa
629-92-5	Alkane	2	245	nonadecane	Uninfected, Varroa
99-85-4	Terpenoid	2	245	1-methyl-4-propan-2-ylcyclohexa-1,4-diene	Uninfected, Varroa
99-87-6	Terpenoid	2	245	p-cymene	Uninfected, Varroa

CAS No.	Class	Community	Degree	Compound	Emitted by
106-24-1	Terpenoid	2	212	geraniol	Uninfected, Chalkbrood
460-01-5	Terpenoid	2	212	cosmene	Chalkbrood, Uninfected
4724-89-4	Cyclic hydrocarbon	2	212	1,3,5,5-tetramethyl-3-cyclohexadiene	Chalkbrood, Uninfected
5256-65-5	Terpenoid	2	212	p-menth-2-ene	Chalkbrood, Uninfected
7399-49-7	Aromatic hydrocarbon	2	212	2-isopropenyltoluene	Chalkbrood, Uninfected
98-06-6	Aromatic hydrocarbon	2	212	tert-butylbenzene	Chalkbrood, Uninfected
57-10-3	Carboxylic acid	2	203	palmitic acid	Uninfected, Varroa.IV
57-11-4	Carboxylic acid	2	203	stearic acid	Uninfected, Varroa.IV
629-97-0	Alkane	2	203	docosane	Varroa.IV, Uninfected
629-99-2	Alkane	2	203	pentacosane	Varroa.IV, Uninfected
630-03-5	Alkane	2	203	nonacosane	Varroa.IV, Uninfected
638-67-5	Alkane	2	203	tricosane	Uninfected, Varroa.IV
1002-84-2	Carboxylic acid	2	193	pentadecanoic acid	Uninfected
104-76-7	Alcohol	2	193	2-Ethyl-1-hexanol	Uninfected

CAS No.	Class	Community	Degree	Compound	Emitted by
10486-19-8	Aldehyde	2	193	tridecanal	Uninfected
105-46-4	Ester	2	193	sec-butyl acetate	Uninfected
105-68-0	Ester	2	193	3-methylbutan-1-yl propionate	Uninfected
106-25-2	Terpenoid	2	193	nerol	Uninfected
106-26-3	Terpenoid	2	193	z-citral	Uninfected
109-21-7	Ester	2	193	butyl butyrate	Uninfected
110-19-0	Ester	2	193	isobutyl acetate	Uninfected
111-27-3	Alcohol	2	193	1-hexanol	Uninfected
111-61-5	Ester	2	193	octadecanoic acid, ethyl ester	Uninfected
111-62-6	Ester	2	193	ethyl oleate	Uninfected
111-70-6	Alcohol	2	193	1-heptanol	Uninfected
111-71-7	Aldehyde	2	193	heptanal	Uninfected
111-87-5	Alcohol	2	193	1-octanol	Uninfected
112-44-7	Aldehyde	2	193	undecanal	Uninfected

CAS No.	Class	Community	Degree	Compound	Emitted by
112-54-9	Aldehyde	2	193	dodecanal	Uninfected
1191-16-8	Ester	2	193	3-methyl-2-buten-1-yl acetate	Uninfected
122-78-1	Aldehyde	2	193	phenylacetaldehyde	Uninfected
123-86-4	Ester	2	193	butyl acetate	Uninfected
124-06-1	Ester	2	193	ethyl myristate	Uninfected
13150-81-7	Branched alkane	2	193	2,6-dimethyldecane	Uninfected
134-20-3	Ester	2	193	methyl anthranilate	Uninfected
140-11-4	Ester	2	193	benzyl acetate	Uninfected
141-27-5	Terpenoid	2	193	e-citral	Uninfected
142-92-7	Ester	2	193	hexyl acetate	Uninfected
17302-28-2	Branched alkane	2	193	2,6-dimethylnonane	Uninfected
18172-67-3	Terpenoid	2	193	(1S,5S)-beta-Pinene	Uninfected
18368-95-1	Terpenoid	2	193	1,3,8-p-menthatriene	Uninfected
1937-62-8	Ester	2	193	9-octadecanoic acid , methyl ester,(e)-	Uninfected

CAS No.	Class	Community	Degree	Compound	Emitted by
2213-23-2	Branched alkane	2	193	2,4-dimethylheptane	Uninfected
2553-96-0	Ester	2	193	methyl 4,6-dimethyloctanoate	Uninfected
2765-11-9	Aldehyde	2	193	pentadecanal	Uninfected
2980-69-0	Branched alkane	2	193	4-methylundecane	Uninfected
3016-19-1	Terpenoid	2	193	allo-ocimene	Uninfected
3221-61-2	Branched alkane	2	193	2-methyloctane	Uninfected
34995-77-2	Terpenoid	2	193	(E)-linalool furanoxide	Uninfected
373-49-9	Carboxylic acid	2	193	palmitoleic acid	Uninfected
39028-58-5	Alcohol	2	193	(E)-linalool pyranoxide	Uninfected
469-61-4	Terpenoid	2	193	alpha-cedrene	Uninfected
502-99-8	Terpenoid	2	193	alpha-ocimene	Uninfected
50396-87-7	Ketone	2	193	4-hexen-3-one	Uninfected
543-49-7	Alcohol	2	193	2-heptanol	Uninfected
544-63-8	Carboxylic acid	2	193	myristic acid	Uninfected

CAS No.	Class	Community	Degree	Compound	Emitted by
54546-22-4	Ester	2	193	ethyl 9-hexadecenoate	Uninfected
556-82-1	Alcohol	2	193	3-methyl-2-buten-1-ol	Uninfected
591-78-6	Ketone	2	193	2-hexanone	Uninfected
617-94-7	Alcohol	2	193	alpha-cumyl alcohol	Uninfected
62442-62-0	Alcohol	2	193	11-eicosen-1-ol	Uninfected
628-97-7	Ester	2	193	hexadecanoic acid, ethyl ester	Uninfected
629-62-9	Alkane	2	193	pentadecane	Uninfected
629-80-1	Aldehyde	2	193	hexadecanal	Uninfected
629-90-3	Aldehyde	2	193	heptadecanal	Uninfected
646-31-1	Alkane	2	193	tetracosane	Uninfected
693-54-9	Ketone	2	193	2-decanone	Uninfected
7146-60-3	Branched alkane	2	193	2,3-dimethyloctane	Uninfected
7216-56-0	Terpenoid	2	193	(E,Z)-alloocimene	Uninfected
89-78-1	Alcohol	2	193	menthol	Uninfected

CAS No.	Class	Community	Degree	Compound	Emitted by
93-89-0	Ester	2	193	ethyl benzoate	Uninfected
95-93-2	Aromatic hydrocarbon	2	193	durene	Uninfected
105-54-4	Ester	3	111	ethyl butanoate	AFB
108-64-5	Ester	3	111	ethyl 3-methylbutanoate	AFB
1119-29-5	Amide	3	111	4-methylvaleramide	AFB
1124-11-4	Heteroaromatic	3	111	2,3,5,6-tetramethylpyrazine	AFB
120-92-3	Ketone	3	111	cyclopentanone	AFB
123-32-0	Heteroaromatic	3	111	2,5-dimethylpyrazine	AFB
128-37-0	Phenol	3	111	butylated hydroxytoluene	AFB
13434-12-3	Amide	3	111	isoamyl acetamide	AFB
13475-82-6	Branched alkane	3	111	2,2,4,6,6-pentamethylheptane	AFB
137-32-6	Alcohol	3	111	2-methylbutan-1-ol	AFB
142-82-5	Alkane	3	111	heptane	AFB
14667-55-1	Heteroaromatic	3	111	trimethylpyrazine	AFB

CAS No.	Class	Community	Degree	Compound	Emitted by
17057-82-8	Cyclic hydrocarbon	3	111	1,2-dimethyl-2,3-dihydro-1h-indene	AFB
2345-27-9	Ketone	3	111	2-tetradecanone	AFB
264-09-5	Cyclic hydrocarbon	3	111	Benzocycloheptatriene	AFB
2870-04-4	Aromatic hydrocarbon	3	111	2-ethyl-1,3-dimethylbenzene	AFB
3658-80-8	Organosulfur	3	111	dimethyl trisulfide	AFB
3682-42-6	Ester	3	111	methyl 3-methyl-2-oxopentanoate	AFB
4706-81-4	Alcohol	3	111	2-tetradecanol	AFB
471-84-1	Terpenoid	3	111	alpha-fenchene	AFB
4798-58-7	Alcohol	3	111	4-hexen-3-ol	AFB
52126-90-6	Lactone	3	111	dl-pantolactone	AFB
556-24-1	Ester	3	111	methyl 3-methylbutanoate	AFB
563-83-7	Amide	3	111	isobutyramide	AFB
60-35-5	Amide	3	111	acetamide	AFB
6033-23-4	Alcohol	3	111	(S)-2-heptanol	AFB

CAS No.	Class	Community	Degree	Compound	Emitted by
611-14-3	Aromatic hydrocarbon	3	111	1-ethyl-2-methylbenzene	AFB
6175-49-1	Ketone	3	111	2-dodecanone	AFB
620-14-4	Aromatic hydrocarbon	3	111	1-ethyl-3-methylbenzene	AFB
622-96-8	Aromatic hydrocarbon	3	111	1-ethyl-4-methylbenzene	AFB
624-92-0	Organosulfur	3	111	dimethyl disulfide	AFB
64-17-5	Alcohol	3	111	ethanol	AFB
675-20-7	Amide	3	111	delta-valerolactam	AFB
700-12-9	Aromatic hydrocarbon	3	111	1,2,3,4,5-pentamethylbenzene	AFB
7452-79-1	Ester	3	111	ethyl 2-methylbutanoate	AFB
934-80-5	Aromatic hydrocarbon	3	111	4-ethyl-1,2-dimethylbenzene	AFB
96-54-8	Aromatic hydrocarbon	3	111	1-methylpyrrole	AFB
97-62-1	Ester	3	111	ethyl 2-methylpropanoate	AFB
103-48-0	Ester	4	7	phenethyl isobutyrate	Chalkbrood.IV
103-52-6	Ester	4	7	phenethyl butyrate	Chalkbrood.IV

CAS No.	Class	Community	Degree	Compound	Emitted by
122-70-3	Ester	4	7	phenethyl propionate	Chalkbrood.IV
16409-43-1	Terpenoid	4	7	rose oxide	Chalkbrood.IV
100-51-6	Alcohol	5	19	benzyl alcohol	Chalkbrood
104-61-0	Lactone	5	19	gamma-nonalactone	Chalkbrood
105-21-5	Lactone	5	19	gamma-heptalactone	Chalkbrood
106-22-9	Terpenoid	5	19	citronellol	Chalkbrood
2305-05-7	Lactone	5	19	y-dodecalactone	Chalkbrood
5392-40-5	Terpenoid	5	19	citral	Chalkbrood
103-65-1	Aromatic hydrocarbon	6	52	propylbenzene	Varroa
106-32-1	Ester	6	52	ethyl octanoate	Varroa
111-14-8	Carboxylic acid	6	52	heptanoic acid	Varroa
123-66-0	Ester	6	52	ethyl hexanoate	Varroa
1731-84-6	Ester	6	52	methyl nonanoate	Varroa
2639-63-6	Ester	6	52	hexyl butanoate	Varroa

CAS No.	Class	Community	Degree	Compound	Emitted by
40710-32-5	Carboxylic acid	6	52	nonahexacontanoic acid	Varroa
571-58-4	Aromatic hydrocarbon	6	52	1,4-dimethylnaphthalene	Varroa
582-16-1	Aromatic hydrocarbon	6	52	2,7-dimethylnaphthalene	Varroa
67-71-0	Organosulfur	6	52	methylsulfonylmethane	Varroa
112-80-1	Carboxylic acid	7	10	oleic acid	Varroa.IV
630-04-6	Alkane	7	10	hentriacontane	Varroa.IV
20185-16-4	Cyclic hydrocarbon	8	26	3,3-dimethyl-6-methylene-1-cyclohexene	Chalkbrood, Chalkbrood.IV

Chapter 4: Wake Up and Smell the Infected Bees: Volatile cues of *Vairimorpha* infection in honey bees

4.1 Abstract

Chemical communication underpins colony organisation and defence in social insects. Infection is well known to alter volatile organic compounds (VOCs) across taxa, including humans, cattle, and honey bee brood diseases, yet no studies have tested whether adult honey bee infections alter VOCs, and none have examined VOCs associated with *Vairimorpha* spp., globally important gut parasites linked to colony declines. This represents a key gap, as adult workers contribute heavily to the colony odour environment and are central to both pathogen transmission and social immunity. We used dynamic headspace sampling and GC×GC-MS to characterise VOC emissions from Vairimorpha-infected and uninfected worker bees over a 14-day infection time series. Across both treatments, 71 VOCs were detected. All were shared between control and infected bees, but their profiles differed in relative abundances and emission patterns. VOC profiles differed significantly between control and infected bees both when pooled across all time points, and at both 6 and 12 days post-infection. Random forest and redundancy analysis identified subsets of compounds that were more strongly associated with discrimination at certain days post-infection, including alkanes (e.g. tetradecane, dodecane) characteristic of early-stage infections, a benzaldehyde derivative (3,4-dimethylbenzaldehyde) that was relatively more abundant during late-stage infections, and one unidentified compound that increased earlier in infected bees than in controls. Several additional VOCs were elevated in infected bees during mid-stage infections, reinforcing that odour changes were dynamic, but still resulted in detectable differences in VOC emissions between infected and uninfected bees. Our findings provide the first evidence that *Vairimorpha* infection alters the volatile profile of adult honey bees. While no single biomarker was unique to infection, robust shifts in VOCs suggest that colonies may have access to semiochemical cues of disease, and that these

changes could be harnessed for applied surveillance. Gas sensors trained on infection-associated VOC bouquets could enable rapid, non-invasive monitoring under field conditions. More broadly, the dynamic nature of these shifts highlights a potential role for infection odours in regulating social immunity, offering new directions for understanding how parasites interact with host communication systems in social animals.

4.2 Introduction

Chemical sensing is one of the most ancient senses, shared across all forms of life from plants and bacteria to animals (Wyatt, 2014). Because of this deep evolutionary origin, chemical signalling is one of the most widespread and vital modes of communication in the animal kingdom. Semiochemicals (any chemical involved in interactions between organisms) can function as evolved signals between members of the same species or be exploited as cues by other species (Regnier, 1971). In all cases, they communicate information about the sender to a receiver. Semiochemicals vary widely in their form and function. Some are relatively large molecules that operate over short distances, such as the cuticular hydrocarbons (CHCs) of insects. These long-chain, typically low volatility compounds (LVCs) both prevent desiccation and serve diverse communicative roles, from nestmate recognition to acting as sex and primer pheromones, where they are detected *via* antennation (Wang et al., 2016; Blomquist et al., 2020). In contrast, volatile organic compounds (VOCs) are smaller molecules that typically act at longer range and often serve more specialised communicative roles (Wyatt, 2014). They are widespread across taxa, from insects to mammals (Liberles, 2014; Wyatt, 2014; Blomquist et al., 2020).

Crucially, this chemical sensing also plays a role in disease, where infection often alters the semiochemical profiles of animals. Such changes are often reflected in VOCs, with infection-linked shifts documented in humans (Shirasu and Touhara, 2011; Ahmed et al., 2017), mice (Ehman and Scott, 2001) and cattle (Peled et al., 2012). Because VOCs are closely tied to host and pathogen metabolism, they can serve as distinctive biomarkers of infection (Calcagnile et al., 2019; Gaude et al., 2019). For many social animals, infection-associated semiochemicals act as

inadvertent cues that healthy individuals can detect and use to avoid diseased conspecifics, thereby reducing transmission (Kiesecker et al., 1999). In eusocial insects, these semiochemical changes may not include only metabolic by-products of infection but also evolved pheromonal signals that promote altruistic behaviours such as the removal of infected nestmates, resulting in a collective behavioural response known as social immunity (Cremer et al., 2007; Cremer et al., 2018). Because chemical communication underpins nearly every aspect of insect life (Leonhardt et al., 2016), infection semiochemicals have the potential to directly influence disease dynamics within colonies. For example, in leaf-cutting ants, workers can detect infection before individuals become infectious (Walker and Hughes, 2009), underscoring the role of semiochemicals as powerful early-warning signals.

Because they provide reliable cues of infection and can communicate over long distances, VOCs hold promise as tools for disease surveillance and management (Chapter 2; Asiri et al., 2024). Just as animals use infection odours to detect and avoid diseased conspecifics, humans can also exploit these semiochemicals as cues when monitoring for disease. In managed animal systems, VOC profiling offers a rapid, non-invasive, and cost-effective alternative to molecular diagnostics such as PCR, which typically require destructive sampling (Maciel-Vergara and Ros, 2017). Gas sensors trained on infection-associated VOC profiles show potential for broad-scale, in-field disease surveillance (Bak et al., 2020; Asiri et al., 2024).

Honey bee health and pathogens

Infection surveillance is especially important in honey bees (*Apis mellifera*). They are intensively farmed pollinators, eusocial, and heavily reliant on chemical communication (Bortolotti and Costa, 2014). Pollinators, such as honey bees, play a critical role in global agriculture, providing pollination services to a wide range plants (Klein et al., 2007; Ollerton, 2017; Reilly et al., 2024). Yet honey bees consistently experience high colony mortality due to pesticide exposure and pathogens (Potts et al., 2010; Goulson et al., 2015). Colonies are vulnerable to a diverse array of pathogens, including bacterial infections such as American foulbrood (*Paenibacillus larvae*) (Genersch, 2010) and European foulbrood (*Melissococcus*

plutonius) (Forsgren, 2010), ectoparasites like *Varroa destructor* (Traynor et al., 2020), numerous viruses (Gisder and Genersch, 2017), and fungal diseases such as chalkbrood (*Ascosphaera apis*) (Aronstein and Murray, 2010), and *Vairimorpha* (formerly *Nosema*) spp. (Pasca et al., 2019).

Many of these infections are associated with changes in non-volatile CHCs. For example, CHCs change during infection by *Vairimorpha* spp. (McDonnell et al., 2013; Murray et al., 2015), European foulbrood (Kathe et al., 2021), *Varroa destructor* (Wagoner et al., 2019), and Israeli acute paralysis virus (Geffre et al., 2020). The same is true, however, of VOCs. Distinctive VOCs are associated with many of the common honey bee pathogens, such as American foulbrood (*Paenibacillus larvae*) (Gochnauer and Margetts, 1981; Gochnauer and Shearer, 1981; Lee et al., 2020; Bikaun et al., 2022), Varroa mite (*Varroa destructor*) (Nazzi et al., 2002; Nazzi et al., 2004; Schoning et al., 2012; Bikaun et al., 2022; Zhao et al., 2025), Chalkbrood (*Ascosphaera apis*) (Swanson et al., 2009; Finstrom et al., 2023), and sacbrood virus (Bikaun et al., 2022). We previously conducted a meta-analysis of VOCs associated with bee pathogens and identified distinctive infection-associated VOCs for Varroa mite, American foulbrood, and Chalkbrood infections (Chapter 3). However, we found that no studies have investigated VOCs associated with *Vairimorpha* infections or adult bee infections in general. This is a critical gap, as adult bees are central to disease transmission dynamics through their roles in nest maintenance and foraging, and are consequently frequently exposed to pathogens (Fefferman et al., 2007).

Vairimorpha infection in honey bees

Vairimorpha spp. are pathogens infecting adult bees, causing Nosemosis disease. Two species infect honey bees: *Vairimorpha ceranae* and *Vairimorpha apis*, both implicated in colony weakening and collapse (Higes et al., 2008; Higes et al., 2009; Martín-Hernández et al., 2018), though *V. ceranae* has become the dominant species in Europe (Higes et al., 2010). Whilst distinctive CHC profiles have been documented in *Vairimorpha*-infected bees (McDonnell et al., 2013; Murray et al., 2015), no studies have tested whether infections also alter VOCs.

Identifying VOCs associated with *Vairimorpha* spp. infection could shed light on how bees detect and respond to infected nestmates and support the development of new, non-invasive diagnostic tools (Asiri et al., 2024). Effective, scalable diagnostics are needed because beekeepers often lack laboratory access to PCR facilities and *Vairimorpha* infections may not present clear clinical symptoms (Holt and Grozinger, 2016). Current field methods, which rely on subsampling workers and identifying infections using microscopy, are labour-intensive, unreliable, and disruptive (Botías et al., 2012; Mulholland et al., 2012). Furthermore, if VOC changes precede visible symptoms or spore development, as suggested in leaf-cutting ants (Walker and Hughes, 2009), they could allow earlier and more effective detection and treatment.

In this study, we tested whether *Vairimorpha* spp. infection alters the VOC profile of adult honey bees. Using dynamic headspace sampling over a 14-day time series, we tracked VOC emissions from infected and uninfected honey bees to determine whether – and when – *Vairimorpha* spp. infection produces a detectable VOC signature.

4.3 Methods

Honey bee rearing and maintenance

Honey bee (*Apis mellifera*) colonies were maintained at the Cardiff University Research Apiary. Prior to collection, all source colonies were screened and confirmed to be free of *Vairimorpha* spp., as well as American foulbrood (*Paenibacillus larvae*) and European foulbrood (*Melissococcus plutonius*). All colonies exhibited natural levels of *Varroa destructor* infestation, consistent with typical UK field conditions.

Adult workers of mixed ages were collected directly from super frames in three unrelated, queenright colonies and housed in hoarding cages under controlled environmental conditions (33 °C, ~65% relative humidity). Each cage consisted of a ventilated plastic deli cup, provisioned *ad libitum* with 50% (w/v) sucrose solution supplied *via* a modified Eppendorf tube, filter paper to absorb waste, and a sealed VOC sampling port (Figure 4.1).

Vairimorpha spp. infections

To generate infectious material, adult forager bees were collected from colonies at Fonmon Apiaries (Cardiff, UK) with suspected *Vairimorpha* spp. infection. Infections were confirmed by phase-contrast microscopy and quantified using a haemocytometer, following standard protocols (Fries et al., 2013).

To prepare the spore suspension, the entire alimentary tract, including midgut, ileum, and rectum, was dissected from infected bees and pooled. Tissues were homogenised in sterile distilled water, and spore purification followed the method described by Fries et al. (2013). Briefly, the homogenate was first filtered through a 70 μm mesh to remove coarse tissue debris. The resulting filtrate underwent three sequential rounds of centrifugation (5000 × g for 5 min), with the pellet re-suspended in sterile water after each spin. The final spore pellet was re-suspended in 50% (w/v) sucrose solution to create the infectious inoculum.

Ten hoarding cages (35 bees per cage) were prepared in total. All bees were starved for 1 hour prior to feeding to encourage inoculum uptake. Bees in five treatment cages were bulk-fed with 50% sucrose solution containing 1.75×10^7 *Vairimorpha* spp. spores. This dosage equated to approximately 5×10^5 spores per bee, sufficient to establish high infection. Five control cages received sterile 50% (w/v) sucrose solution. Following inoculation, treatment and control cages were maintained in separate incubators under constant darkness at 33 °C and ~65% relative humidity.

Volatile collection across the infection time course

To determine whether *Vairimorpha* infection alters the volatile profile of honey bees, headspace volatiles (the air volume around bees in a sample bag) were sampled from infected and control hoarding cages at five time points post-infection: 5 hours (day 0), and 3, 6, 9, and 14 days post-infection (dpi). At each time point one sample of volatiles were collected from each of five replicate cages per treatment group (infected: n = 175 bees total; control: n = 175 bees total), using a custom static headspace sampling system (Figure 4.1). Each cage was enclosed within a sampling bag constructed from open-top nalophene bags and closed at the top with

clips. Bags were fitted with a sampling port consisting of a modified Eppendorf tube that connected directly to the interior of the hoarding cage (Figure 4.1). Bees were left undisturbed in the sealed bag for 30 minutes to allow VOCs to accumulate within the headspace. Following headspace accumulation, a thermal desorption (TD) tube (C2-EAXX-5314; inert-coated SafeLok stainless steel, Markes International) was uncapped and inserted into the sampling port. The opposite end of the TD tube was connected to an ACTI-VOC pump (Markes international), which extracted 3 L of air from the bag at a flow rate of 200 mL/min over 15 minutes. TD tubes were immediately sealed and stored at ambient temperature until analysis.

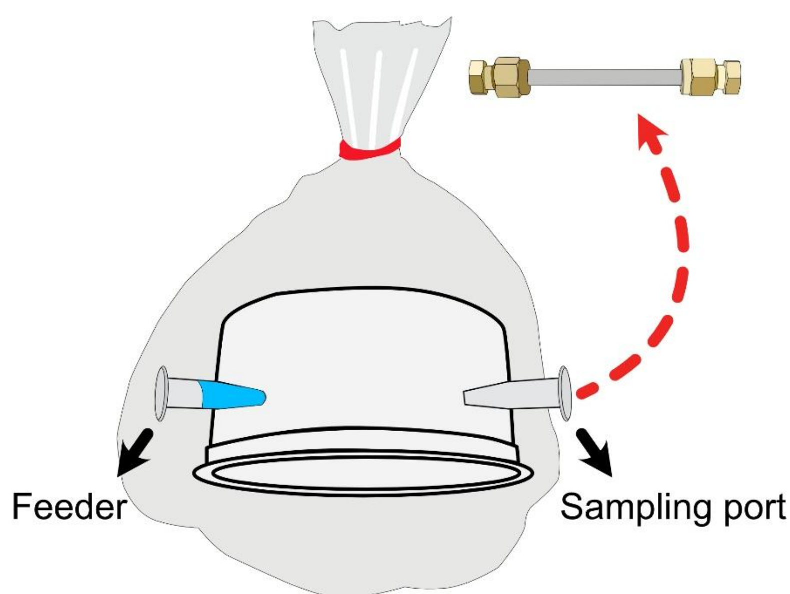

Figure 4.1: Custom headspace sampling bags consisting of a hoarding cage, 50% w/v sucrose feeder, and Eppendorf sampling port connected to the interior of the cage. 3 L of headspace was extracted onto thermal desorption tubes using an ACTI-VOC pump at 200 mL/min over 15 minutes.

At each sample time point a blank was also collected using an identical setup to the experimental cages, but without bees. These consisted of an empty hoarding cage enclosed within a sampling bag, containing filter paper and a 50% (w/v) sucrose feeder, and left to equilibrate before sampling to control for background emissions from the materials and ambient air.

Infection monitoring and validation

To monitor how infection load changes over time, we needed to destructively sample bees, therefore we matched experimental conditions using three additional cages of 35 bees (one per colony of origin) that we infected in parallel with the VOC-sampled bees using the same bulk-feeding inoculation protocol and maintained under identical laboratory conditions; we term these 'reference' bees.

At the same six time points used for VOC sampling (0, 3, 6, 9, 12, and 14 dpi), three reference bees were randomly sampled from each cage, pooled together and their *Vairimorpha* spp. spore loads were quantified microscopically using a haemocytometer (as described above). For the bees used in VOC sampling, after the final sampling point (14 dpi), all surviving infected bees were pooled within cages and their average spore loads measured ($n = 24 \pm 3$ bees per cage) and compared with reference bees to confirm successful and consistent infection levels across replicates. All surviving control bees ($n = 29 \pm 3$ per cage) were likewise pooled and screened at 14 dpi to ensure no infection had established.

Volatile sample analysis

Headspace volatile samples were analysed using a thermal desorption two-dimensional gas chromatography time-of-flight mass spectrometry system (TD-GC×GC-MS), comprising a CENTRI160 inlet system (SepSolve Analytical), an Agilent 8890 GC equipped with a flow modulator (INSIGHT, SepSolve Analytical), and a BenchTOF2-TI mass spectrometer (Markes International).

Thermal desorption was performed with the CENTRI inlet using the following settings: an initial 2-minute dry purge with nitrogen at 50 mL/min, followed by two-stage desorption at 120 °C and 280 °C for 5 minutes each, with nitrogen flows of 40 and 50 mL/min, respectively. VOCs were recollected on a trap at 25 °C, then desorbed at 300 °C for 3 minutes following a 1 minute dry purge with nitrogen (50 mL/min). Total flow during desorption was 2.5 mL/min helium, with 0.5 mL/min directed to the GC and 2.0 mL/min to re-collection (split ratio 4:1).

Samples were separated on a two-dimensional column set: a 20 m × 180 µm, 0.18 µm BPX5 column (SGE) as the primary column, followed by a 5 m × 250 µm,

0.1 μm BPX50 column (SGE) as the secondary, with modulation every 2 s using the INSIGHT flow modulator. Helium was used as the carrier gas, with flow rates of 0.5 mL/min in the first dimension and 20 mL/min in the second. The GC oven was held at 40 °C for 2 minutes, ramped at 3 °C/min to 240 °C, and held for a further 5 minutes.

Mass spectra were acquired using electron ionisation at 70 eV, scanning from m/z 35 to 600 at a data rate of 50 Hz. The ion source and transfer line were maintained at 230 °C and 240 °C, respectively.

Retention indices (RIs) were calculated using a standard alkane mixture (C8 to C20, 40 mg/L per component; Supelco). The standard was injected (1 μL) onto a TD tube and analysed under the same conditions as the experimental samples. RI standards were run in sequence before and after (using the re-collected standard) each set of samples.

Data processing

Raw chromatograms were processed using *ChromCompare*+ v2.2 (Markes International) for alignment, noise reduction, and peak integration. All chromatograms were first aligned to the sample containing the highest number of detected peaks. Noise reduction was then performed using dynamic baseline correction, with a specified peak width of 2 seconds.

Chromatograms from the alkane retention index (RI) standards were reviewed for consistency. A representative chromatogram was selected to define the RI pattern used for subsequent compound integration.

Peak integration was carried out using the deconvolution function in *ChromCompare*+, with a minimum peak area threshold of 2,000. Additional integration parameters included the use of the peak merge function and a higher minimum area threshold of 10,000 for library searching. Compound identification was performed by matching against a custom-built, retention-indexed mass spectral library, with a minimum match factor of 700 for both forward and reverse searches. The RI match window was set to ±3 RI units, with the RI penalty set to “strong.”

The custom compound library was created by searching selected chromatograms (processed under the same integration settings) against the NIST library (2020), using an RI window of ±10 and a medium RI penalty. Compounds that matched both spectra and RI were added as named compounds. Matches with spectral similarity but mismatched RI were classified by chemical class (e.g. “alkane#”), and recurrent, unidentifiable components with consistent RI but no plausible spectral match were categorised as “unknown#”.

Integrated peak lists for all samples were exported as csv files, combined, and reviewed in Microsoft Excel. Duplicate entries (based on RI) were identified using pivot tables and validated against the original chromatographic data. Where peak splitting by the integrator occurred, duplicate compound names were retained. However, genuinely distinct components (i.e. with similar RI but clearly different spectra) were either renamed to match an existing compound with a similar profile or added to the dataset as new components.

A final compound matrix was constructed using the pivot table sum function, resulting in a data table containing integrated peak areas for 71 unique compounds. Compounds occurring fewer than three times within the same set of replicates were excluded from further statistical analysis to reduce noise. Empty cells were imputed with one-tenth of the smallest non-zero area in the dataset, and peak areas were normalised within each sample to total peak area.

Statistical methods

Multivariate analyses of VOC profiles were conducted in R v4.3.2 (R Core Team, 2023). All plots were made using *ggplot2* (Wickham, 2011). Normalised VOC abundances were used as the response variable throughout.

To test whether infection altered VOC composition, we first performed canonical analysis of principal coordinates (CAP) using the CAPdiscrim function in the *BiodiversityR* package (Kindt and Coe, 2005). CAP constrains ordination by predefined groups and maximises separation while accounting for within-group variability. We fitted models using Euclidean distances with: (i) treatment (control vs. infected) as the grouping variable, to assess overall differences pooled across all

time points; and (ii) a combined factor of treatment and day post-infection (dpi), to assess how infected and control VOC profiles diverged over time.

We fitted a random forest model using the *randomForest* package (Liaw and Wiener, 2002) to evaluate the discriminatory power of VOC profiles and identify compounds most important for classifying treatment and dpi combinations. Variable importance was quantified as the mean decrease in classification accuracy when each compound was permuted. We defined the top ranked VOCs by applying a cut-off at the point where variable importance showed a clear drop in mean decrease accuracy (Figure S4.2). To test whether the most informative compounds improved temporal separation between treatments, we re-ran CAP with the combined factor of treatment and dpi as the predictor, and the subset of top-ranked VOCs identified in the random forest as the response matrix.

We formally tested differences in VOC profiles using permutational multivariate analysis of variance (PerMANOVA) with 999 permutations and Euclidean distances *via* the adonis function in 'vegan' (Oksanen et al., 2025). First, we fitted a simple model with treatment (control vs infected) as the only predictor to test for an overall effect of infection across all days pooled together. Second, we fitted a factorial model including treatment, dpi, and their interaction to test whether the effect of infection was dependent on days-post infection. This separation avoided interpreting main effects in the presence of an interaction, while allowing us to address both questions.

As a complementary approach, redundancy analysis (RDA) was performed using the 'vegan' package (Oksanen et al., 2025). We included treatment, dpi, and their interaction as predictor variables. RDA constrains the ordination of VOC abundances by the predictor, quantifying the proportion of variance explained by predictor variables. To assess the contribution of individual VOCs, we examined their loadings on the constrained axes and calculated magnitude as the Euclidean distance of each compound vector from the origin. We then ranked compounds by magnitude and identified the most influential VOCs by applying a cutoff at the point where the ranked values showed a clear drop (Figure S4.3).

Finally, to identify the most robust signals of infection, we compared the outputs of RDA and random forest analyses. VOCs consistently ranked as important across both approaches were considered key compounds, as they contributed both to group

discrimination and to variance explained by infection status and time. We assessed the significance of overall model fit, individual predictors, and constrained axes using permutation tests using the 'anova.cca' function from *vegan* with 999 permutations. We then compared the relative abundance of each key VOC by computing Z-scores, thereby allowing differences in abundance to be compared across VOCs with different baseline levels. To complement this, we also examined compounds identified as important by only one of the two methods. Because RDA and random forest capture different features of the data – linear variance structure versus non-linear classification power – method-specific compounds may highlight additional, biologically relevant shifts in VOC profiles that do not appear consistently across approaches. This allowed us to assess both the conservative set of consensus markers and the broader set of potentially informative VOCs associated with infection.

4.4 Results

Vairimorpha spp. infections

Vairimorpha spp. infections progressed rapidly across all replicates. Reference bees sampled destructively at 0–14 dpi showed typical infection trajectories, as observed in previously published work (Forsgren and Fries, 2010; Huang and Solter, 2013; Fan et al., 2024) with spore loads rising from $6.0 \pm 2.8 \times 10^4$ at 3 dpi to $2.0 \pm 0.27 \times 10^6$ at 6 dpi reaching an asymptote at $2.7 \pm 1.8 \times 10^7$ at 9 dpi, and exceeding 10^8 spores per bee by 12 dpi. At 14 dpi, both reference and experimental bees exhibited similarly high spore loads ($1.18 \pm 0.14 \times 10^8$), confirming that they followed a comparable infection trajectory (Figure S4.1). At 14 dpi, *Vairimorpha* spores were detected in one control cage which was therefore excluded from all analyses. No spores were found in the remaining control replicates.

Separation of infected and control VOC profiles pooled across time

After VOC data processing, only three infected replicates at 6 dpi were retained, as some samples did not meet the inclusion threshold of containing at least three consistently detected compounds across replicates. A total of 71 VOCs were found across both infected and uninfected treatments over the 14-day time series (Table S4.1). No VOCs were specific to either control or infected bees, but the relative abundance and pattern of VOCs significantly differed between control and infected bees when pooled across all time points (PerMANOVA: p = 0.002, R^2 = 0.038).

In the CAP analysis, infected bees were correctly classified 85.7% of the time, compared with only 50% for controls, giving an overall classification success rate of 69.2%. Ordination showed considerable overlap between treatments, though this was driven primarily by the high variability of control bees. Infected bees clustered more tightly, whereas control samples were more dispersed, leading to their overlap with the infected group (Figure 4.2).

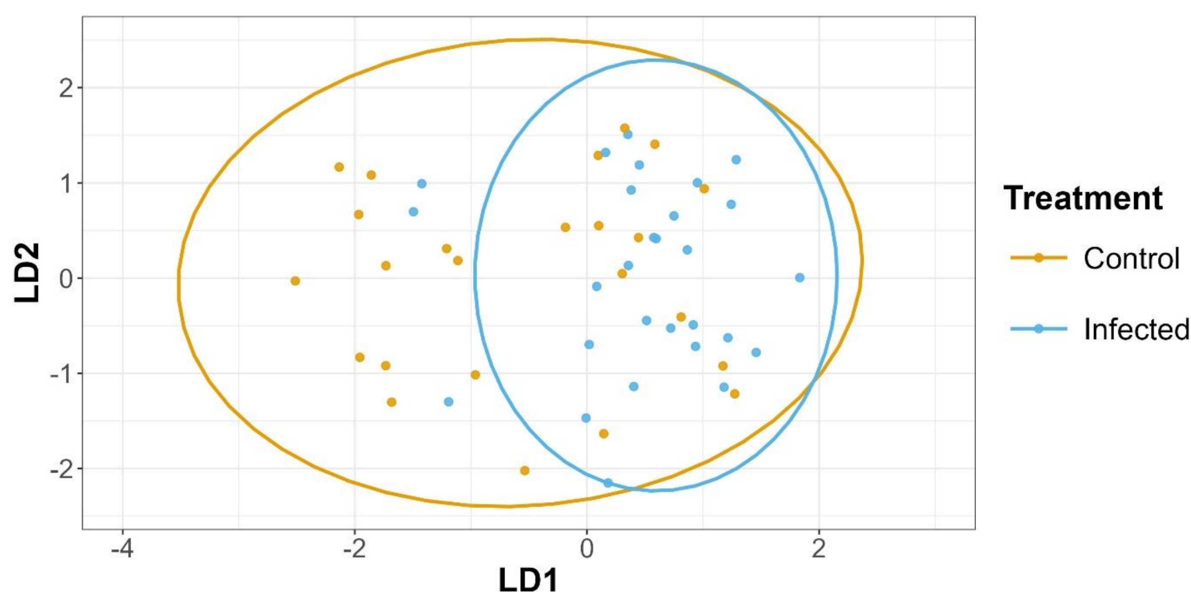

Figure 4.2: Canonical analysis of principal coordinates (CAP) based on Euclidean distances and normalised abundances of all 71 volatile organic compounds (VOCs) pooled across all days post-infection. Plot shows VOC profiles of adult worker honey bees of mixed ages either infected with *Vairimorpha* spp. or uninfected. Ellipses represent 95% confidence intervals around group centroids.

Temporal changes in VOC profiles during infection

Using the full set of 71 compounds, VOC profiles were significantly influenced by treatment (PerMANOVA: $p = 0.001$, $R^2 = 0.038$), dpi (PerMANOVA: $p = 0.001$, $R^2 = 0.31$) and their interaction (PerMANOVA: $p = 0.005$, $R^2 = 0.11$). CAP confirmed that the difference between the VOC profiles of infected and uninfected bees were time-dependent. Discrimination between infected and control bees was strongest at 12 dpi (CAP: 100% for controls, 80% for infected), moderate at 6-9 dpi (CAP: 33-60%), and absent at 0 and 14 dpi, where infected and control bees were indistinguishable (CAP: 0%; Table S4.2), yielding an overall classification success of 46%.

To identify which compounds drove these patterns, we used random forest analysis and identified a subset of 12 VOCs that most strongly discriminated between infected and uninfected bees across time (Figure S4.2). This reduced set of VOCs explained more of the overall variance (72% compared to 46% with all 71 VOCs) in PerMANOVA and improved CAP classification accuracy by 21% (overall classification success: 67%; Figure 4.3; Table S4.3). Using the subset of VOCs in CAP analysis revealed temporal dynamics of the ‘smell of infection’ more clearly (Figure 4.3). Infected and control bee VOC profiles were indistinguishable at 0-3 dpi but distinct at 6 dpi (CAP: 100% accuracy for infected, 75% control). At 9 dpi the VOC profiles of infected and uninfected bees converged, only to diverge again at 12 dpi (CAP: 100% accuracy for both; Table S4.3). By 14 dpi, however, treatments once more overlapped and could not be discriminated (Figure 4.3). This alternating pattern suggests that infection effects of VOCs are transient, with clear signals emerging only at certain time periods of the infection.

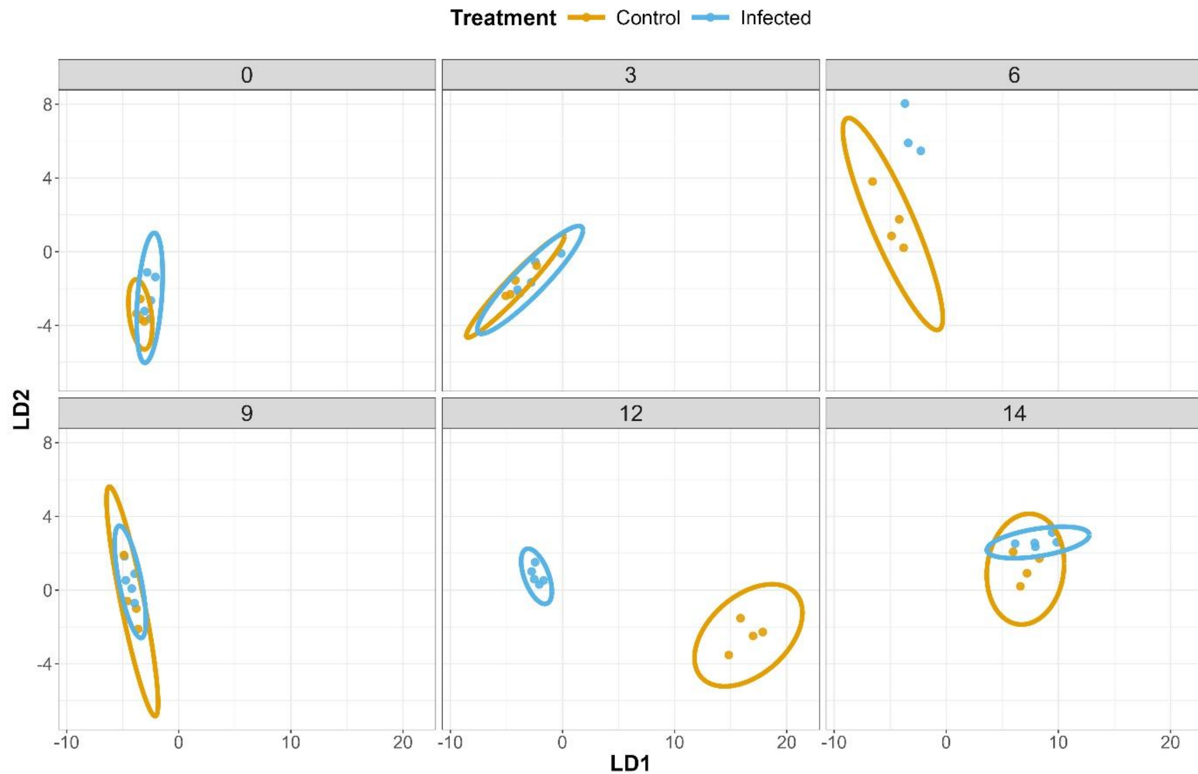

Figure 4.3: Canonical analysis of principal coordinates (CAP) based on Euclidean distances and normalised abundances of the 12 most important volatile organic compounds (VOCs) identified by the random forest model. Plots show adult worker honey bees of mixed ages, either infected with *Vairimorpha* spp. or uninfected, across days post-infection (dpi). Ellipses represent 95% confidence intervals. Only three samples for infected bees at 6 dpi were retained after VOC data were processed; therefore, confidence intervals could not be computed for infected bees at this time point.

Compound associations with infection stages

Redundancy analysis (RDA) confirmed that infection status, days post-infection (dpi), and their interaction all significantly shaped VOC profiles (treatment: F = 2.8, p = 0.001; dpi: F = 4.6, p = 0.001; treatment*dpi: F = 1.6, p = 0.004). The constrained ordination explained 46% of the total variance in VOC profiles, and the first three RDA axes (RDA1-RDA3) were significant ($p < 0.01$), indicating that each axis explained a distinct, biologically meaningful component of variation linked to infection status, dpi, or their interaction. Sample scores were strongly ordered by dpi along RDA1, whereas RDA2 separated infected and control bees, and RDA3 reflected

their interaction. Therefore, to highlight separation driven by treatment and the way this varied over time, we plotted RDA2 against RDA3 (Figure 4.4).

Because RDA places samples and compounds in the same ordination space, clustering shows which samples had similar VOC emissions and which compounds defined those regions, revealing the VOCs most characteristic of the samples that fall in that region. The compounds with the strongest loadings in the RDA model (Figure S4.3) were associated with regions of ordination space dominated by particular days post infection. Infected bees at 0 and 3 dpi clustered in the positive space of RDA2 and negative space of RDA3, overlapping with 0 dpi controls (Figure 4.4) and were linked with tetradecane (C063), dodecane (C048), and decane (C046). The positive space of RDA3 and negative space of RDA2 primarily contained both mid-late stage (6-12 dpi) infected and control bees that were associated with undecane (C066), Alkane07 (C015), and an unidentified compound (C071) (Figure 4.4). The negative space of both axes was almost exclusively occupied by infected samples from multiple stages of infection (3-14 dpi), apart from two controls at 14 dpi that overlapped with infected bees. This region was characterised by benzaldehyde, 3,4-dimethyl- (C036), 3-ethyl-3-methylheptane (C010), 1-octanol, 2,2-dimethyl- (C001), several long-chain alkanes (C030, C028, C027, C022), and toluene (C064).

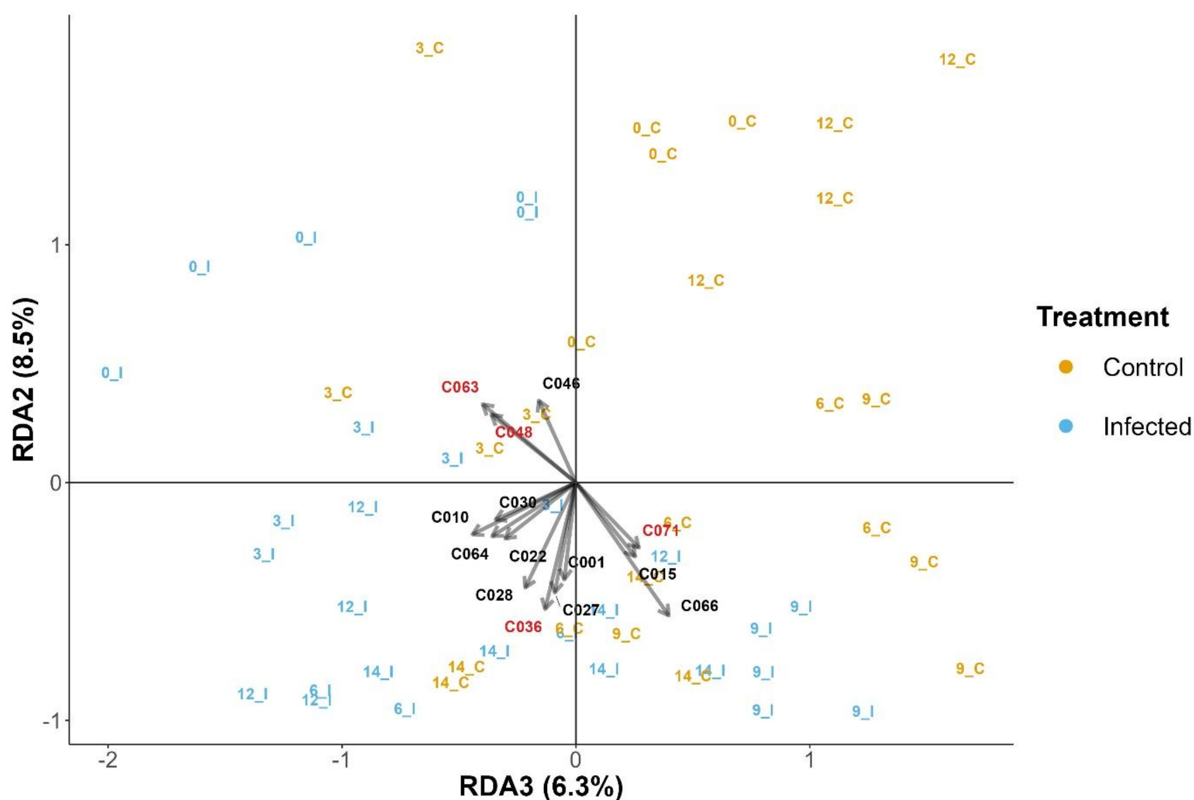

Figure 4.4: Redundancy analysis (RDA) biplot of VOC profiles, containing the 71 identified volatile organic compounds (VOCs) constrained by treatment, days post-infection (dpi), and their interaction. Sample IDs indicate an individual sample taken at a combined dpi and treatment (e.g., 3_I = dpi 3, infected treatment). The top VOCs contributing to constrained variance show on the plot with arrows indicating how strongly a compound is correlated with the constrained axes. VOCs that did not contribute strongly to constrained ordinance are not shown on the plot. Top compounds with consensus between random forest and RDA are shown in red.

Reducing complexity and identifying robust markers of infection

A consensus between RDA and random forest identified four compounds consistently associated with treatment separation: tetradecane (C063), dodecane (C048), benzaldehyde, 3,4-dimethyl- (C036), and one unknown compound (C071) (Figure 4.5). Tetradecane and dodecane were elevated at 0–3 dpi, with tetradecane higher in infected bees at 0 dpi and dodecane higher in controls at 3 dpi. From 6 dpi onwards, both compounds declined steadily in relative abundance, reaching their lowest levels at 14 dpi (Figure 4.5). In contrast, benzaldehyde, 3,4-dimethyl- was initially low in both treatments but increased markedly by 6 dpi, particularly in

infected bees. It remained more abundant in infected bees for the remainder of the experiment, despite some variation at 9 dpi (Figure 4.5). The unknown compound (C071) was scarce at 0 dpi but increased in abundance over time in both treatments. Infected bees showed an earlier rise from 3 dpi, whereas in controls abundance only increased from 9 dpi onwards, at which point levels were comparable between treatments (Figure 4.5).

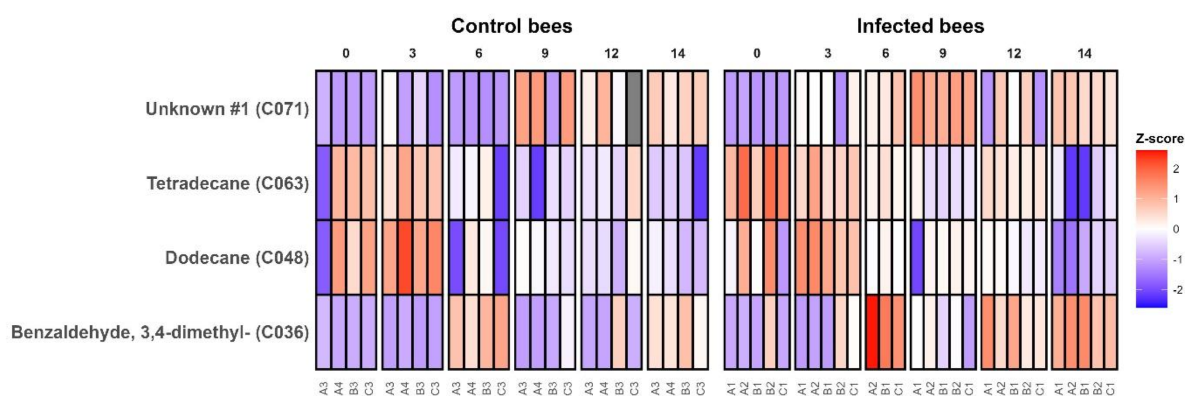

Figure 4.5: Heatmap showing standardised volatile organic compound (VOC) abundances in adult honey bee workers across a 14-day post-infection time series. Each tile represents the Z-scored abundance of a compound (row) in each sample (column) at a given day post-infection (dpi). Panels are faceted by treatment group (control vs. *Vairimorpha* spp.-infected bees), and sample labels indicate the original donor hive (A, B, or C) and cage ID. Z-scores were calculated separately for each compound across all samples to highlight relative increases (red) and decreases (blue) in abundance. The four compounds shown were identified by consensus across redundancy analysis and random forest models as those most strongly associated with differences between control and infected groups.

Additional VOCs contributing to treatment separation

We visualised the relative abundance of compounds ranked as important by either RDA or random forest (but not both). While not confirmed across methods, these compounds provide a complementary view of treatment differences. Infected bees often emitted a broader set of compounds at higher levels than controls, with the exception of a small group of aromatics at 12 dpi (Figure 4.6). Several alkanes (C030, C028, C027, C022, C015) were more abundant in infected bees from 3 dpi

onwards, with consistent elevation relative to controls at 6 dpi. At 12 dpi, aromatic hydrocarbons including benzene derivatives (C043, C039) and naphthalene (C053) were more abundant in controls, although alkanes and most other volatiles remained elevated in infected bees. By 9 and 14 dpi, abundances of most VOCs were similar between treatments.

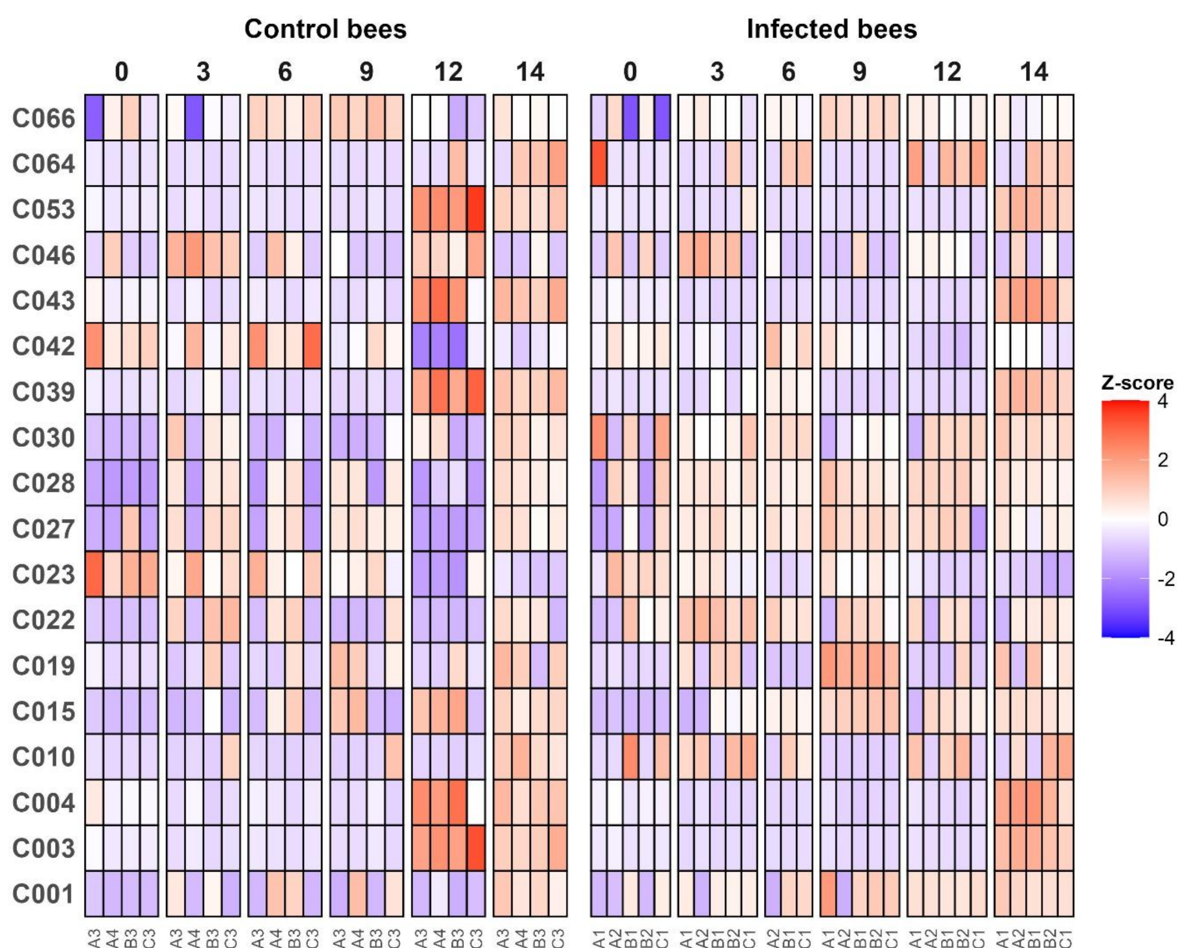

Figure 4.6: Heatmap showing standardised volatile organic compound (VOC) abundances in adult honey bee workers across a 14-day post-infection time series. Compound codes (e.g. C028) correspond to identities listed in Table S4.1. The compounds shown were identified as important by either the Random Forest or RDA models, but not by both (i.e. no consensus between methods). Each tile represents the Z-scored abundance of a compound (row) in an individual sample (column) at a given day post-infection (dpi). Panels are faceted by treatment group (control vs. *Vairimorpha*-infected bees), and sample labels indicate the original donor hive (A, B, or C) and cage ID. Z-scores were calculated separately for each compound across all samples to highlight relative increases (red) and decreases (blue) in abundance.

4.5 Discussion

Understanding the semiochemical cues associated with infection is vital for uncovering the mechanisms of disease transmission in animals and presents a unique opportunity to eavesdrop on animal chemical communication as a non-invasive technique for disease surveillance. Here, we show for the first time that infection of adult honey bees by *Vairimorpha* spp. alters the VOC profile in a detectable way. All VOCs identified were emitted by both control and infected bees, but the relative abundances and patterns of their emission varied between treatments. The strongest differences in VOC profiles between infected and control bees occurred at 6- and 12-days post-infection. At a broad level, multiple alkanes, an alcohol, and an aromatic hydrocarbon were more heavily associated with infected bees. Within this pattern, four compounds (tetradecane, dodecane, 3,4-dimethylbenzaldehyde and one unidentified compound) were consistently identified as important for both discriminating between infected and uninfected bees and explaining variation in their VOC profiles. Together, these findings highlight potential biomarkers of infection and show that VOC profiles of infected and control bees diverge most strongly at mid-infection stages, providing a foundation for understanding how volatile cues vary during infection with *Vairimorpha* spp.

Variation in VOC emissions overall and across time

When pooled across all time points, the VOC profiles of *Vairimorpha* infected and uninfected bees significantly differed, in line with previous studies reporting changes in the low volatility cuticular hydrocarbon (CHC) during *Vairimorpha* infection (Murray et al., 2016; McDonnell et al., 2013). However, we also found considerable variation in both control and infected bees VOC profiles over time. We found that that infection-associated VOC changes were not linear but instead discrimination peaked at 6 and 12 dpi, with convergence at 9 and 14 dpi.

Interestingly, McDonnell et al. (2013) reported that CHC profiles also followed a non-linear pathway between *Vairimorpha*-infected and uninfected bees but at different time points to our observations; they converged at 5 dpi and instead became distinct at 10 dpi. Given that our results show divergence in VOCs at 6 dpi, this raises two

possibilities: either VOC changes precede the onset of CHC divergence, or infection alters VOC and CHC signalling in a temporally staggered way. This offset suggests VOCs may act as earlier, colony-level cues of infection, whereas CHCs provide later, contact-based confirmation, together forming complementary channels of chemical communication. Because CHCs are non-volatile, they require direct contact and typically convey identity-related information such as nestmate recognition, division of labour, or fertility signals (Drijfhout et al., 2009). In contrast, VOCs are volatile, act over longer distances, and can serve both as communication signals and as cues tied to host or pathogen metabolism, meaning they could elicit different responses in nestmates than CHCs.

The infection trajectories reported in our study (Figure S4.1) followed that typical of previous studies (Forsgren and Fries, 2010; Huang and Solter, 2013; Fan et al., 2024). Because spore proliferation is minimal in the first few days post-infection, the absence of VOC divergence at 0–3 dpi is consistent with this slow early replication phase, whereas the peaks at 6 and 12 dpi coincide with rapid growth, suggesting that volatile cues are closely linked to the metabolic processes following periods of rapid parasite replication. Supporting this, transcriptomic analyses have shown that *Vairimorpha* spores, and the bees themselves, differentially express metabolic genes at distinct time points during infection (Badaoui et al., 2017; Fan et al., 2022; Li et al., 2022). By 14 dpi, however, we found that two control samples shifted into the same RDA space as infected bees. While this may reflect ongoing metabolic changes in the host-parasite interaction, it is also plausible that prolonged caging induced stress in some control bees, which is known to alter physiology and metabolism could have altered VOC emissions (Alburaki et al., 2019; Lattorff, 2022). Nevertheless, the strong link with *Vairimorpha* growth dynamics in the mid-stage infections indicate that VOC changes likely reflect dynamic host-parasite metabolic interactions, rather than static infection status, explaining why chemical divergence was strongest at certain days post-infection.

Robust markers and shifts in VOC profiles

Across Random Forest and RDA models, four compounds consistently drove separation in the VOC profiles of infected from uninfected bees. Two were alkanes

(tetradecane and dodecane), both common components of honey bee brood VOC profiles (Light et al., 2020; Liendo et al., 2021; Liu et al., 2022). The third, 3,4-dimethylbenzaldehyde, was a benzaldehyde derivative not previously reported from honey bees, though benzaldehyde itself occurs in brood volatiles (Schoning et al., 2012; Liolios et al., 2022), and related derivatives possess antimicrobial and antifungal properties (Kim et al., 2019). The fourth was an unidentified compound (C071), which showed higher abundance in infected bees from 3 dpi onwards. Together, these findings indicate that infection with *Vairimorpha* spp. induces distinct changes in the VOC profile of adult honey bees, with specific compounds showing stage-dependent variation.

Tetradecane and dodecane were relatively more abundant during early infections and were clustered with early-stage infected bees in the RDA, alongside control bees at 3 dpi. This pattern suggests that tetradecane was driving treatment separation at 0 dpi, and, given its relatively higher abundance in infected bees at this stage, that it may be emitted at elevated levels immediately following infection. Notably, tetradecane has been shown to strongly trigger hygienic behaviour in *Varroa*-infested brood (Noël et al., 2025), making its early association with infection a plausible signal for removal by the host colony before spores mature and become infectious. *Vairimorpha* spores can germinate within 30 minutes of ingestion (Goblirsch, 2018) suggesting that metabolic changes in the host may initiate this early signalling. Dodecane followed the same general abundance and clustering patterns as tetradecane. It is a common honey bee alkane, though it does not trigger aggression when tested in isolation (Breed and Stiller, 1992). However, it may have yet unreported effects on honey bee perception and detection of nestmates. The reduction of tetradecane and dodecane as infection progresses could indicate that bees initially signal for removal prior to spore maturation, or alternatively that the infection itself suppresses host signalling capacity. Indeed, *V. ceranae* is known to suppress host immune responses and down-regulate cuticle genes and odorant binding proteins (Badaoui et al., 2017). Suppression of signalling could, therefore, extend to VOCs.

In contrast, 3,4-dimethylbenzaldehyde was relatively more abundant in infected bees at later infection stages. Although not previously reported in honey bees, benzaldehyde is found in propolis and wax (Smith and Bromenshenk, 2002), and

closely related benzaldehyde derivatives have antimicrobial properties (Kim et al., 2019), raising the possibility that it reflects either a host defence compound or a microbial by-product emerging in late infection. The higher association of 3,4-dimethylbenzaldehyde with later stage infections, in parallel with a reduction in dodecane and tetradecane may signal a shift from investment in social immunity to personal immunity as the infection reaches its peak. Because social immunity can be costly, such a switch could represent a metabolic reallocation away from signalling for removal towards producing antimicrobial metabolites that act directly at the individual level (Cremer et al., 2007; Cotter and Kilner, 2010a; Cotter and Kilner, 2010b).

The unidentified compound (C071) appeared to increase in relative abundance earlier in infected bees than controls. Because its chemical identity could not be established, no functional interpretation can be made, but its consistent role in distinguishing between control and infected bees highlights the need for future structural identification.

Beyond the consensus markers, several additional compounds also contributed to differences between infected and control bees. Alkanes were generally more abundant in infected bees from 3 dpi onwards, whereas aromatic hydrocarbons such as benzene derivatives and naphthalene were more prominent in controls, particularly at 12 dpi. Although the specific compounds emphasised differed between analyses, together these patterns reinforce that *Vairimorpha* infection alters adult VOC emissions in multiple ways and provide further support for the overall distinction between infected and control VOC profiles.

Although our methods were robust for distinguishing between different VOCs, their identities were not verified against reference standards, and functional interpretations should therefore be made with caution. Nevertheless, diagnostic markers may not be necessary. Bees themselves may detect infection as a deviation from the normal colony odour template (Tibbetts and Dale, 2007; Gherardi et al., 2012), and gas-sensor technologies could likewise be trained to recognise whole-profile variation rather than specific compounds (Chapter 2; Asiri et al., 2024). In this context, the relative shifts in overall VOC profiles observed in our study are highly

relevant, as they provide a realistic basis for both biological detection and applied surveillance.

Can VOCs be used for disease surveillance?

At first glance, the temporal variation in VOC profiles might appear to limit their usefulness for surveillance: transient shifts could mean that infection is only detectable at specific stages, such as 6 or 12 dpi. However, colony infections are rarely synchronous. Individual bees differ widely in spore load and stage of infection (Mulholland et al., 2012), meaning that colony-level headspace reflects a composite of multiple infection stages. In this context, analysing whole-colony odour and differentiating it from that of a healthy colony is likely to provide a more consistent and reliable signal than attempting to track stage-specific markers, and could therefore offer a practical estimation of infection intensity at the colony level. Supporting this, we found that pooled VOC profiles across all time points remained significantly different between treatments.

For surveillance applications, our results show that infection can be detected through whole-profile changes in VOCs, with differences driven by characteristic subsets of compounds. This has direct relevance for the development of gas sensor technology. In practice, colony headspace is chemically complex, containing not only bee-derived volatiles but also odours from hive products such as honey, pollen, and wax, as well as VOCs from hive materials including wood and paint (Smith and Bromenshenk, 2002). Any infection signal must therefore be strong enough to stand out against this background noise. In this context, identifying bouquets of VOCs that consistently contribute to treatment discrimination is especially valuable. While unique biomarkers would be valuable in principle (Bikaun et al., 2022), they may not always exist if infection alters compounds already present in the baseline odour rather than producing unique metabolites (Chapter 2; Asiri et al., 2024). Profile-level shifts are therefore particularly important because they can still be exploited even when specific biomarkers are absent. In our study, no VOCs were found to be uniquely emitted by *Vairimorpha*-infected bees compared with uninfected bees, but relative abundances and overall patterns changed at a profile level. In such cases,

training gas sensors to recognise multivariate patterns of VOCs provides a realistic and effective approach.

Using overall VOC profiles to identify infection has already been demonstrated in the context of *Varroa destructor*. Gas sensors have successfully distinguished between healthy and infested colonies, and in some cases can even quantify infestation levels (Szczurek et al., 2019; Bak et al., 2020; Szczurek et al., 2020). Notably, these studies did not focus on specific biomarkers but instead used arrays of sensors with different sensitivities to detect overall differences in VOC profiles between infested and healthy colonies. Given that our study shows infection-associated changes in *Vairimorpha*-infected VOC profiles, similar approaches could be applied to *Vairimorpha* spp. surveillance.

Why are VOC profiles important for infection dynamics?

Our results show that infection-associated VOCs in honey bees are dynamic, shifting in both the abundance of key compounds and in whole-profile divergence over time. While changes in CHCs during infection have been reported across insect taxa, (Murray et al., 2015; Qiu et al., 2015; Geffre et al., 2020; Dawson et al., 2024), far fewer studies describe infection-association changes in VOCs. Yet VOCs offer a distinctive advantage: they act as long-range signals, whereas CHCs provide high-resolution contact cues (Wang et al., 2016). This distinction suggests complementary roles in colony defence: VOCs could attract guards or nurses towards the area of infection, while CHCs allow precise identification of the infected individuals (McAfee et al., 2018; Wagoner et al., 2019). More broadly, VOCs could facilitate rapid information flow throughout the colony, functioning as a long-distance channel of communication that complements direct contact (Cremer et al., 2018).

Such colony-wide signalling is not without precedent. Termites, for instance, use vibrational cues to coordinate group-level responses to pathogens (Rosengaus et al., 1999). Honey bees routinely rely on volatile communication: alarm pheromone, for example, is released during disturbance and elicits defensive behaviour from nestmates (Trhlin and Rajchard, 2011), while the Nasanov gland produces a volatile blend that coordinates swarming, nest orientation, and recruitment (Wells et al.,

1993; Trhlin and Rajchard, 2011). Crucially, these pheromones are not single compounds but complex mixtures. The alarm pheromone consists of around 15 components, each varying in potency but often acting synergistically to trigger stinging behaviour (Pankiw, 2004). By analogy, infection cues could similarly act at the profile level. In our study, infected bees were characterised by distinct VOC profiles at specific stages of infection, and overall, profiles differed significantly between treatments. While we did not test behavioural responses directly, these differences demonstrate that infection alters the odour landscape in ways that could, in principle, generate colony-level signals.

Evidence from other systems supports this broader perspective. In mice, exposure to odours from diseased conspecifics reduces innate immune responsiveness in uninfected individuals (Alves et al., 2010). In insects (*Drosophila*), exposure to parasitoid wasp odours primes immune responses against future challenges (Madhwal et al., 2020). Whether infection-associated VOCs in social insects have comparable effects remains unknown. Nonetheless, our findings highlight the potential for such cues to influence colony level social immune responses, a possibility that warrants direct behavioural and physiological testing.

Conclusions

In conclusion, our study demonstrates that Vairimorpha infection alters the volatile profile of adult honey bees in ways that are both detectable and dynamic. Although variation and overlap were present, distinctive differences emerged at specific infection stages, particularly 6 and 12 days post-inoculation, coinciding with periods of rapid parasite replication. Rather than a single biomarker, subsets of compounds consistently contributed to treatment discrimination, suggesting that infection odours operate as profile-level signals. These findings have two main implications. First, they support the development of gas sensor technologies for non-invasive disease surveillance, where recognising whole-profile shifts may be more realistic than relying solely on unique compounds. Second, they point to a role for infection odours in regulating social immunity, with temporal shifts in VOCs potentially shaping when and how nestmates respond to diseased individuals. By linking infection physiology to colony-level processes through odour, our results provide both a foundation for

applied surveillance tools and a framework for testing how chemical cues underpin social immunity – not only in bees but across all social animals.

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4.7 Supplementary material

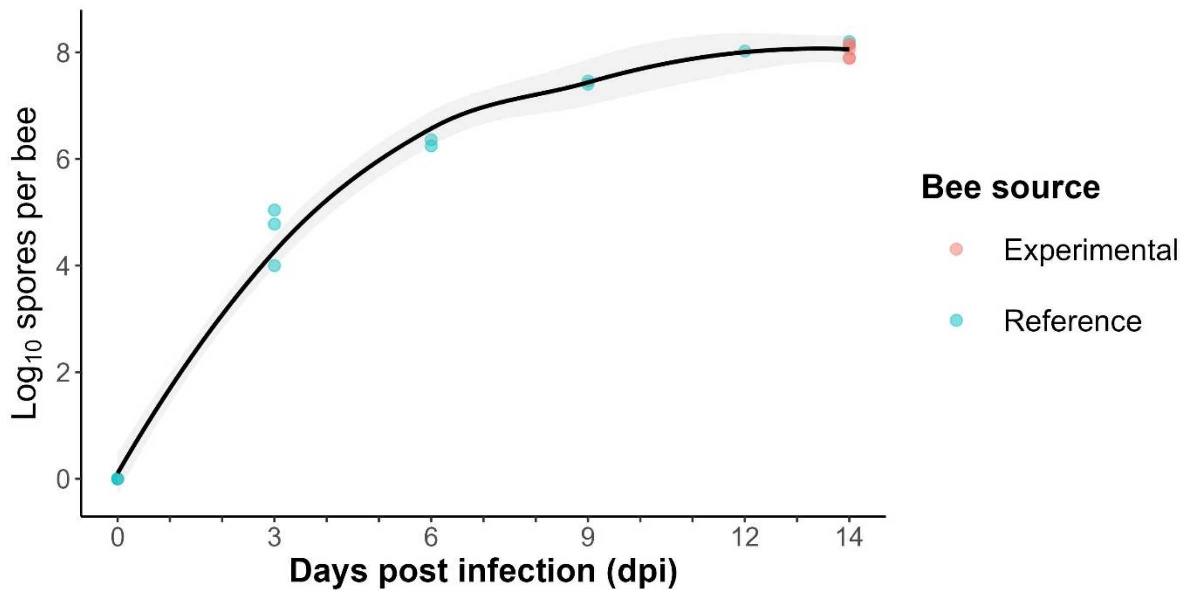

Figure S4.1: Infection dynamics of *Vairimorpha* spp. in reference and experimental bees. Reference bees were destructively sampled at 0, 3, 6, 9, 12, and 14 days post-infection (dpi) to confirm infection progression, while experimental bees used for volatile organic compound sampling were only sampled at 14 dpi. Each point represents a pooled sample of three bees. The black line and shaded ribbon show a loess smoother with ±95% confidence interval fitted across all data. Due to natural mortality, only one reference cage replicate was available at 12 and 14 dpi.

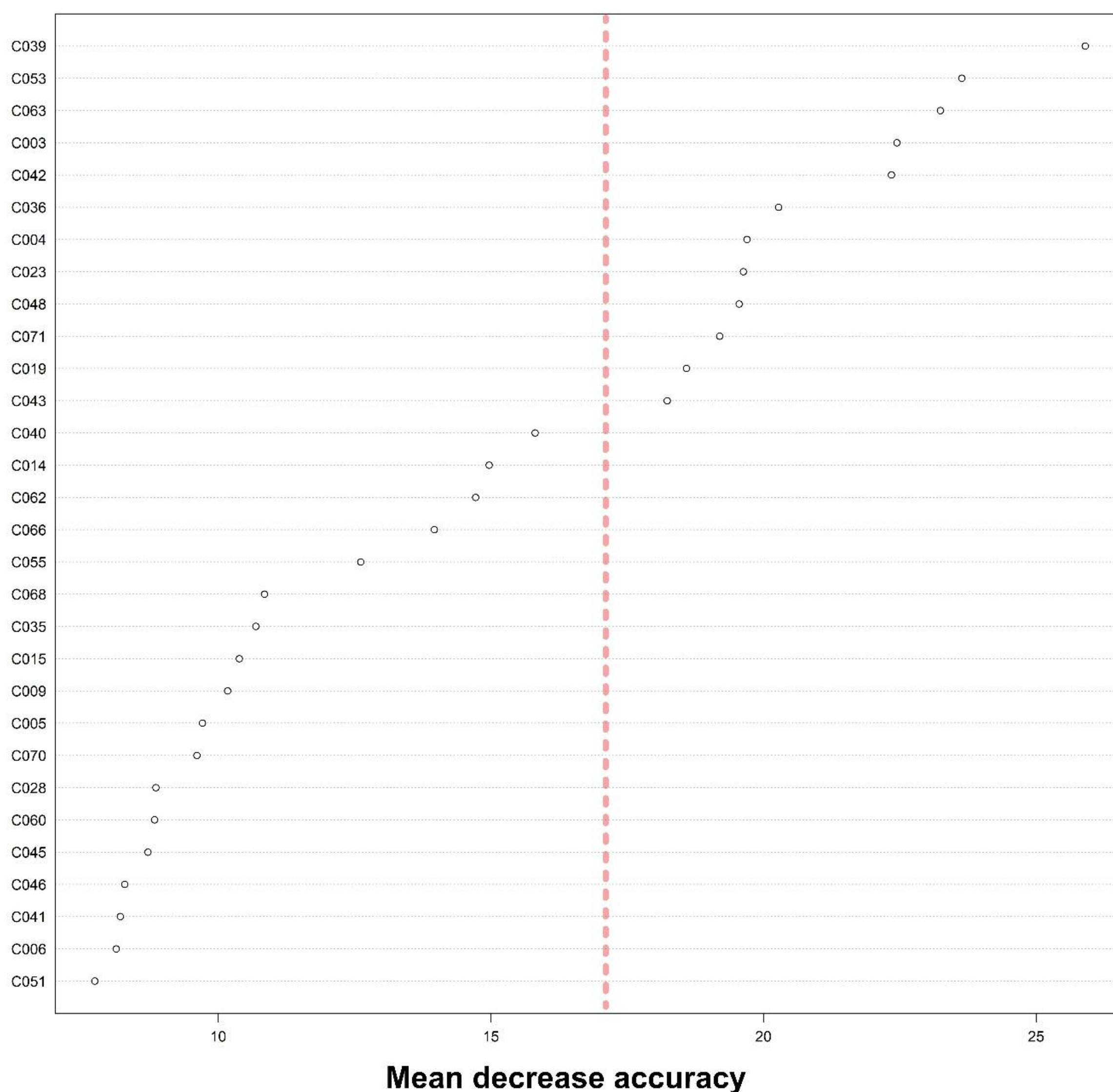

Figure S4.2: Top 30 volatile organic compounds (VOCs) ranked by their importance to classification accuracy in the random forest model based on all 71 VOCs. Higher values indicate a greater contribution to model performance. The dashed red line indicates the cut-off point used to select the most important compounds for discriminating between combined treatment and days post-infection (dpi) variables.

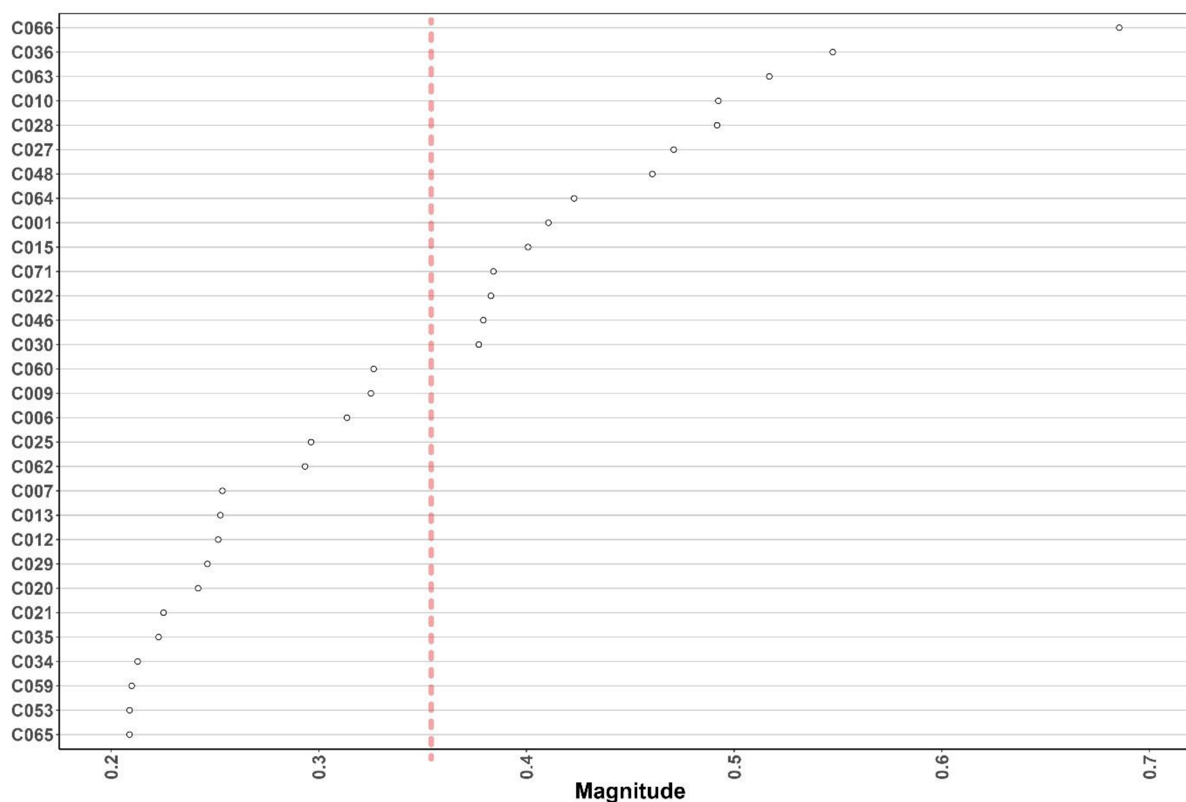

Figure S4.3: Top 30 volatile organic compounds (VOCs) ranked by their contribution to the redundancy analysis (RDA) model based on all 71 VOCs. Higher magnitudes indicate a stronger contribution to constrained variance. The dashed red line indicates the cut-off point used to select the most important compounds for discriminating between combined treatment and days post-infection (dpi) variables.

Table S4.1: The 71 volatile organic compounds (VOCs) identified in infected and uninfected bees across all timepoints.

Compound	Component	CAS number	Family
1-Octanol, 2,2-dimethyl-	C001	2370-14-1	alcohol
1-Octene, 2,6-dimethyl-	C002	6874-29-9	alkene
1,3-Cyclopentadiene, 1,2,3,4-tetramethyl-5-methylene-	C003	76089-59-3	cycloalkene
1H-Indene, 2,3-dihydro-4-methyl-	C004	824-22-6	aromatic hydrocarbon
1H-Indene, 2,3-dihydro-5-methyl-	C005	874-35-1	aromatic hydrocarbon
2,3-Dimethyl-2-heptene	C006	3074-64-4	alkene

2,4-Di-tert-butylphenol	C007	96-76-4	phenol
2,4-Dimethyl-1-heptene	C008	19549-87-2	alkene
2,6-Dimethyldecane	C009	13150-81-7	alkane
3-Ethyl-3-methylheptane	C010	17302-01-1	alkane
3,4-Dimethylcumene	C011	4132-77-8	alkylbenzene
Alkane02	C012	N.D.	alkane
Alkane03	C013	N.D.	alkane
Alkane04	C014	N.D.	alkane
Alkane07	C015	N.D.	alkane
Alkane13	C016	N.D.	alkane
Alkane14	C017	N.D.	alkane
Alkane15	C018	N.D.	alkane
Alkane17	C019	N.D.	alkane
Alkane18	C020	N.D.	alkane
Alkane19	C021	N.D.	alkane
Alkane21	C022	N.D.	alkane
Alkane22	C023	N.D.	alkane
Alkane23	C024	N.D.	alkane
Alkane24	C025	N.D.	alkane
Alkane25	C026	N.D.	alkane
Alkane26	C027	N.D.	alkane
Alkane27	C028	N.D.	alkane
Alkane28	C029	N.D.	alkane
Alkane30	C030	N.D.	alkane
Alkanol02	C031	N.D.	alkanol
Alkanol03	C032	N.D.	alkanol
Alkene09	C033	N.D.	alkene
Alkene18	C034	N.D.	alkene
Alkene19	C035	N.D.	alkene
Benzaldehyde, 3,4-dimethyl-	C036	5973-71-7	aldehyde
Benzene, 1-ethyl-3,5-dimethyl-	C037	934-74-7	aromatic hydrocarbon
Benzene, 1-methyl-3-(1-methylethyl)-	C038	535-77-3	aromatic hydrocarbon

Benzene, 1,2,3,4-tetramethyl-	C039	488-23-3	aromatic hydrocarbon
Benzene, 1,2,3,5-tetramethyl-	C040	527-53-7	aromatic hydrocarbon
Benzene, 1,2,4-trimethyl-	C041	95-63-6	aromatic hydrocarbon
Benzene, 1,3-bis(1,1-dimethylethyl)-	C042	1014-60-4	aromatic hydrocarbon
Benzene, 2-ethyl-1,3-dimethyl-	C043	2870-04-4	aromatic hydrocarbon
C3-Benzene01	C044	N.D.	aromatic hydrocarbon
Cyclohexane, 1,2,3-trimethyl-, (1α,2α,3β)-	C045	7667-55-2	cycloalkane
Decane	C046	124-18-5	alkane
Decane, 2-methyl-	C047	6975-98-0	alkane
Dodecane	C048	112-40-3	alkane
Ethylbenzene	C049	100-41-4	aromatic hydrocarbon
Heptadecane	C050	629-78-7	alkane
Heptane, 2,4-dimethyl-	C051	2213-23-2	alkane
Hexane, 2,3,3-trimethyl-	C052	16747-28-7	alkane
Naphthalene	C053	91-20-3	aromatic hydrocarbon
Naphthalene, 1-methyl-	C054	90-12-0	aromatic hydrocarbon
Nonanal	C055	124-19-6	aldehyde
Nonane, 2-methyl-	C056	871-83-0	alkane
Nonane, 2,5-dimethyl-	C057	17302-27-1	alkane
o-Xylene	C058	95-47-6	aromatic hydrocarbon
p-Xylene	C059	106-42-3	aromatic hydrocarbon
Pentadecane	C060	629-62-9	alkane
Styrene	C061	100-42-5	aromatic hydrocarbon
Terpene01	C062	N.D.	terpenoid

Tetradecane	C063	629-59-4	alkane
Toluene	C064	108-88-3	aromatic hydrocarbon
Tridecane	C065	629-50-5	alkane
Undecane	C066	1120-21-4	alkane
Undecane, 2-methyl-	C067	7045-71-8	alkane
Undecane, 2,6-dimethyl-	C068	17301-23-4	alkane
α-Pinene	C069	80-56-8	terpenoid
1-Octanol, Dimethyl-	C070	N.D.	alcohol
Unknown#1: RI1083	C071	N.D.	N.D.

Table S4.2: Classification success (%) from canonical analysis of principal coordinates (CAP) using all 71 VOCs across treatments and days post-infection. CAP achieved an overall classification success of 46%.

Days post-infection	Treatment	Classification success (%)
0	Control	75
0	Infected	0
3	Control	25
3	Infected	80
6	Control	50
6	Infected	33
9	Control	50
9	Infected	60
12	Control	100
12	Infected	80
14	Control	0
14	Infected	0

Table S4.3: Classification success (%) from canonical analysis of principal components (CAP) using the top 12 VOCs identified as most important in the random forest model across treatments and days post-infection. CAP achieved an overall classification success of 67%.

Days post-infection	Treatment	Classification success (%)
0	Control	75
0	Infected	60
3	Control	25
3	Infected	40
6	Control	75
6	Infected	100
9	Control	25
9	Infected	80
12	Control	100
12	Infected	100
14	Control	50
14	Infected	80

Chapter 5: Smell as a mechanism regulating the social response of honeybees to infection with *Vairimorpha ceranae*

5.1 Abstract

Social insects rely on chemical communication to detect and respond to disease threats within the colony. In honey bees (*Apis mellifera*), responses to infected individuals may be triggered by changes in scent, due to changes in cuticular hydrocarbons (CHCs) or volatile organic compounds (VOCs), but the causal role of olfactory cues in mediating behavioural interactions between individuals is unclear. We used a controlled dyadic assay to test whether olfactory signals can elicit behavioural avoidance responses toward nestmates infected with *Vairimorpha (Nosema) ceranae*, a common gut parasite. Bees (n = 416) were paired in arenas that either allowed physical touch (CHC + VOC exposure) or prevented it (VOC-only), and we quantified autogrooming, contact frequency, time spent in contact, and spatial proximity over a 2 hour observation period. Our results showed no consistent evidence that bees increased autogrooming or reduced contact in response to infected nestmates, regardless of whether they could physically contact or were only exposed to olfactory cues. Neither contact frequency nor grooming behaviour was significantly predicted by infection or infection intensity. While bees in touch-allowed arenas interacted more frequently and for longer durations, this effect was independent of infection status. These findings suggest that under laboratory conditions, olfactory cues from *V. ceranae*-infected bees are insufficient to trigger robust behavioural changes in nestmates. Our study provides mechanistic insight into the sensory basis of social immunity and highlights the need to explore context-dependence and the threshold dynamics of disease detection in honey bees.

5.2 Introduction

Living in social groups offers insects many benefits, from cooperative brood care to efficient foraging, but also introduces a critical vulnerability: the rapid spread of infectious disease. Eusocial insects, in particular, face intense pathogen pressure due to high-density living, frequent contact among individuals, and stable nest environments that favour pathogen persistence (Kappeler et al., 2015; Schmid-Hempel, 2021). In response, these species have evolved a complex set of collective defences known as social immunity, a term encompassing all group-level behaviours that reduce pathogen transmission within a colony (Cremer et al., 2007).

Social immunity in insect societies

Social immunity is recognised as a central pillar of disease resistance in eusocial insects, and it operates across multiple levels and stages of infection. For example, ants avoid contaminated food sources to prevent parasite uptake (Pereira and Detrain, 2020), termites use intensive grooming to remove fungal spores from nestmates before infection takes hold (Wen et al., 2017), and corpse removal is widespread in ants and bees, limiting exposure to infectious cadavers (Diez et al., 2014). Many of these behaviours reduce the burden on the individual immune system, allowing the colony to maintain health even under high pathogen loads (Cotter and Kilner, 2010).

A central requirement for collective behaviours is the detection and communication of infection. In social insects, communication of disease status is primarily mediated through chemical cues. These may be emitted directly by pathogens, such as fungal metabolites, or arise indirectly *via* physiological changes in the infected host (Dussaubat et al., 2010; Milutinović and Schmitt, 2022; Chapter 2; Asiri et al., 2024; Chapter 3; Chapter 4). For instance, viral infection in ant brood induces changes in specific cuticular hydrocarbons (CHCs) that prompt removal by workers (Dawson et al., 2024), and termites exhibit distinct responses to nestmates with altered CHC profiles following infection (Esparza-Mora et al., 2023). Chemical communication is especially effective in the crowded, low-light environments of insect nests, where volatile and contact-based signals can rapidly propagate social immune responses.

Despite strong evidence for colony-level coordination, it is increasingly clear that individual-level interactions are the foundation of social immunity. Collective outcomes arise from the accumulation of dyadic interactions; the moment-to-moment behavioural responses between two individuals, such as grooming, trophallaxis, or avoidance (Cremer et al., 2018). Studying these interactions provides critical mechanistic insight into how infection is detected and managed before large-scale responses occur. Ant studies, for example, have shown that just a few key interactions between infected and healthy individuals can trigger downstream colony-wide prophylactic behaviours (Hamilton et al., 2011). Focusing on dyads allows us to test the cues, thresholds, and decisions that initiate social immunity at its smallest scale, knowledge that is essential for linking physiology, communication, and collective behaviour.

Honey bees as a model for social immunity

The honey bee (*Apis mellifera*) represents an ideal system in which to explore these mechanisms (Chapter 1). As one of the most intensively studied eusocial insects, honey bees are known to engage in a broad range of social immune behaviours, from resin collection to prevent microbial growth (Simone-Finstrom et al., 2017), to exclusion of infected foragers by nest guards (Cappa et al., 2016), and the hygienic removal of diseased brood (Spivak and Gilliam, 1998; Spivak and Danka, 2021). Their social defences are particularly vital given their reduced innate immune gene repertoire compared to solitary insects (Evans et al., 2006).

Although most research has focused on social immunity behaviours towards brood, there is growing recognition that adult bees, too, are targets of social immune responses. Infected adults may experience reduced trophallaxis (Geffre et al., 2020), increased grooming, aggression, or even removal from the hive (Richard et al., 2008; Baracchi et al., 2012; Biganski et al., 2018; Pusceddu et al., 2021). These responses appear to be mediated by chemical cues – changes in CHCs and volatile organic compounds (VOCs) that mark individuals as infected (Masterman et al., 2000, Swanson et al., 2009; McAfee et al., 2017; Wagoner et al., 2019; Chapter 2; Asiri et al., 2024; Chapter 3; Chapter 4). However, while correlational studies suggest bees

respond to these cues, few studies have tested causality: does chemical information alone, without visual or tactile cues, drive changes in behaviour?

This question is especially relevant for pathogens like *Vairimorpha* (*Nosema*) *ceranae*, a widespread microsporidian parasite that infects the midgut of adult bees and is associated with physiological stress, impaired behaviour, and colony-level declines (Higes et al., 2008; Higes et al., 2009; Schüler, 2022). Transmission occurs primarily through ingestion of spores from contaminated nectar, pollen, or hive surfaces, and can also occur during trophallaxis or grooming (Graystock et al., 2015; Martín-Hernández et al., 2018). Spores may even become airborne within the hive (Sulborska et al., 2019) creating multiple potential routes for infection. Given that *V. ceranae* activates immune responses in bees (Li et al., 2017) and alters host CHC and VOC profiles (Chapter 4; McDonnell et al., 2013; Murray et al., 2015), it is plausible that nestmates can detect and respond to infected individuals using smell. However, evidence for behavioural changes is mixed, with some studies finding no social response (McDonnell et al., 2013; Murray et al., 2015) and others reporting variable outcomes, ranging from increased grooming to aggression, depending on infection severity (Biganski et al., 2018). Critically, the role of smell as a causal mechanism remains untested in a dyadic context.

Here, we use a controlled laboratory assay to investigate whether olfactory cues mediate social immunity in honey bee dyads involving *V. ceranae*-infected individuals. By pairing healthy bees with infected or uninfected nestmates and varying whether they can physically touch each other (CHC + VOC exposure) or only exchange airborne cues (VOC-only), we test how smell influences autogrooming, contact frequency, time spent in contact, and social distancing. This dyadic framework allows us to isolate the specific sensory modalities that regulate social immunity, linking individual perception with group-level disease defence.

5.3 Methods

Experimental overview

We tested whether honey bees alter their behaviour in response to infected nestmates, and whether these behaviours depend on the ability to physically touch the other individual or detect volatile cues. To do this, we used dyads of bees placed in arenas that either allowed or prevented physical contact. Each dyad consisted of either two uninfected bees (hereafter “control dyads”) or one bee infected with *Vairimorpha ceranae* and one uninfected bee (hereafter “infected dyads”). Each dyad was placed in one of two arena types: a touch-allowed condition permitting both contact and volatile cue exchange, and a touch-prevented condition that allowed only volatile cues to pass, creating a 2x2 factorial design.

Honey bee maintenance

Frames of capped brood were collected from four colonies maintained at the Cardiff University Research Apiary and Fonmon Apiary, Wales and incubated at 33 °C and 65% R.H. Newly emerging workers (0-24 h old) were collected and housed in hoarding cages (10-30 individuals) grouped by colony of origin and maintained at 33 °C and 65% relative humidity in darkness. The number of bees in each cage varied based on how many individuals emerged on the day of collection. All bees were fed 50% (w/v) sucrose solution *ad libitum*. Donor colonies were free of *Vairimorpha* spp., American foulbrood, and European foulbrood, but naturally infected with *Varroa destructor*.

Vairimorpha ceranae spore collection and inoculation

Bees were collected from colonies with suspected *V. ceranae* infection at Silwood Park apiary (Imperial College London). The alimentary tract, consisting of the midgut, small intestine, and rectum were dissected, pooled and homogenised in sterile distilled water to create spore suspensions. Infection was confirmed using phase-contrast microscopy (Fries et al., 2013). To achieve species-level identification, spore suspensions were analysed by the Graystock Lab, Imperial College London by

extracting the DNA using Qiagen DNeasy colum kits. Briefly, this involved lysis with ~100 µL of 0.10 mm glass beads and a 5 mm steel bead for 3 minutes at 30 Hz, then overnight digestion with protinase K before following the Qiagen Kit instructions for DNA isolation. DNA samples were stored at -20 °C ready for later molecular analysis. To identify the *Vairimorpha* species observed under microscope, a diagnostic PCR screen was performed that targeted the RPB1 gene to differentiate between the two common honeybee parasites and identified the species as being *V. ceranae* in accordance with standard PCR protocols (Gisder and Genersch, 2013).

To infect bees, spore suspensions were diluted using 50% (w/v) sucrose to $1.5\text{x}10^6$ – $9.1\text{x}10^6$ spores/ml. Hoarding cages of 3 day old bees were starved for 1 hour and fed the inoculum in bulk. Uninfected bees received sterile sucrose solution following the same protocol. Infected and uninfected bees were housed in separate incubators for 7 days to allow infections to establish and prevent cross-contamination.

Behavioural assays

Seven days post-inoculation, bees were cold-anaesthetised for 5 minutes by placing hoarding cages at -20 °C in accordance with standard methods (Human et al., 2013). Anaesthetised bees were then randomly assigned to one of four dyad types: (1) Control dyads in touch-allowed arena (2) Control dyads in touch-prevented area (3) Infected dyads in touch-allowed arena (4) Infected dyads in touch-prevented arena. To maintain comparable familiarity between individuals, both bees in dyads were selected from different cages, but always from the same donor colony.

Dyads were placed into a 90 x 14 mm Petri dish arena divided by a central partition, with the identity of the individual bee marked on either side of the partition. In touch-allowed arenas, the partition consisted of 2.5 mm gauge mesh, allowing physical touch (Figure 5.1A). In touch-prevented arenas, the partition was made of 0.04 mm gauge mesh, which permitted volatile exchange but prevented bees from physically touching each other (Figure 5.1B). Each side of the arena was equipped with a modified Eppendorf feeder containing 50% (w/v) sucrose, providing food *ad libitum*.

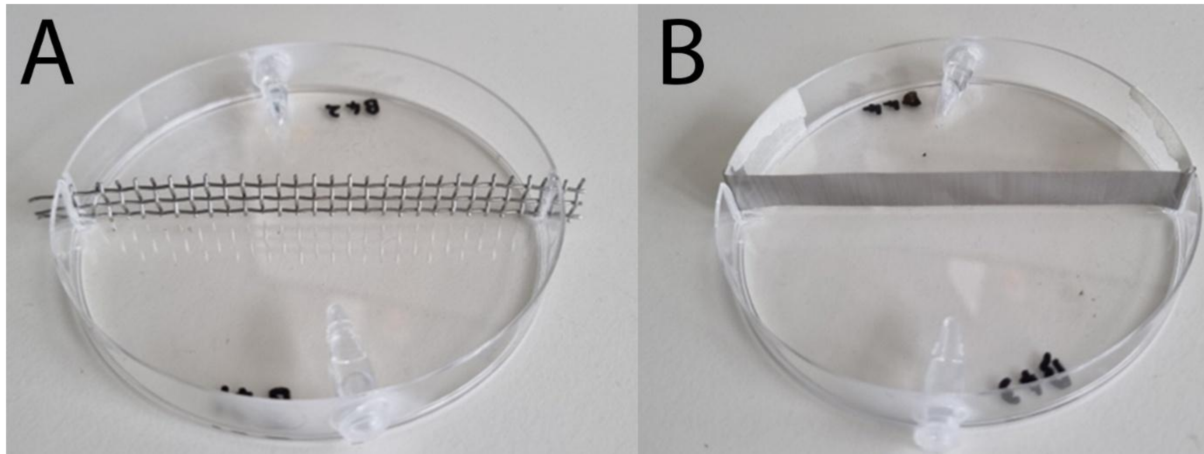

Figure 5.1: Experimental arenas used in behavioural assays. (A) Touch allowed (semi-partitioned) arena using 2.5 mm gauge mesh (B) Touch prevented (fully partitioned) arena using 0.04 mm gauge mesh. Both arenas were fitted with Eppendorf feeders on each side of the partition containing 50% w/v sucrose solution.

Arenas were placed in an incubator at 33 °C and 65% relative humidity and their behaviour recorded for 3 hours per dyad using a Logitech BRIO 4k webcam. Recording sessions were conducted between 09:45 and 20:00 over a five-week experimental period, resulting in 975 hours of video data across 325 dyads. The first recording hour was excluded from analysis to allow recovery from handling, as anaesthesia is known to affect odour-mediated behaviour (Pankiw and Page, 2003). If either bee in a dyad died during the observation period, the dyad was removed from analysis. Final sample size after natural in-experiment mortality was a total of 416 bees in 208 dyads: Infected dyads, touch-allowed: n = 69; Infected dyads, touch-prevented: n = 60; Control dyads, touch-allowed: n = 41; Control dyads, touch-prevented: n = 38. This yielded a total of 416 hours of video data for analysis.

Following behavioural assays, the gut of each bee was dissected and homogenised in sterile distilled water to assess infection load. Spore loads were quantified using phase-contrast microscopy at 400× magnification and expressed as total spores per bee in accordance with standard methods (Fries et al., 2013).

Behavioural quantification

Autogrooming was used as an indicator of whether bees increased self-grooming when detecting an infected partner, or whether infection itself induced elevated autogrooming. Frequency was scored manually using BORIS (Friard and Gamba, 2016) by scan sampling the 2 hour recordings for 30 seconds every 2 minutes. All manual observations were performed blind to dyad type.

Automated tracking was conducted using EthoVision XT 14 (Noldus) to quantify four additional behaviours across the 2 hour observation window. Total distance moved (cm) was used as a measure of activity, testing whether uninfected bees increased movement to avoid infected partners and whether infection itself reduced activity in line with sickness behaviour. Inter-individual Euclidean distance (cm), calculated from the calibrated X-Y coordinates of each bee, was used to assess spatial proximity, asking whether bees maintained greater separation from infected partners, a pattern that would indicate social distancing. Contact frequency quantified how often bees interacted, while time spent in contact measured the cumulative amount of time they remained together, allowing us to assess both the rate and extent of social interactions in response to infection. Analyses of time spent in contact included only dyads where at least one contact was recorded. Because direct physical touch could not be measured in touch-prevented arenas, we operationally defined a contact event as both bees being within a Euclidean distance of ≤1.6 cm for at least 0.05 s while moving at a velocity of ≤2 cm/s. This threshold was established using still images of bees in close contact (Figure S5.1).

Statistical methods

We fitted generalised linear mixed models (GLMMs) with appropriate error distributions using the *lme4* package (Bates et al., 2003) to test whether infection status and/or infection intensity were associated with variation in bee behaviour. All models included recording batch as a random intercept to account for variation between recording sessions because only a limited number of arenas could be recorded at once, and hive of origin to account for variation between colonies.

Analyses were conducted in R version 4.3.2 (R Core Team, 2023), and plots were generated using *ggplot2* (Wickham, 2011).

To test for behavioural differences between infected and uninfected dyads, we modelled each behavioural response individually (autogrooming frequency, contact frequency, time spent in contact, total distance moved, spatial proximity) as a function of dyad type (infected vs. control), arena type (touch-allowed vs. touch-prevented), and a two-way interaction between dyad type and arena type. Post hoc pairwise comparisons of estimated marginal means (*emmeans* package; Lenth, 2025) were used to test for differences between groups (treatment and arena type), with Tukey's adjustment for multiple testing.

To test whether infection intensity predicted behavioural changes, we fitted separate GLMMs in which the response variable was the behaviour of the uninfected bee in each dyad, and the key predictor was the log-transformed spore load of its infected partner. These models included arena type and its interaction with spore load as fixed effects. No post hoc comparisons were conducted for these models.

For all models, we tested the significance of the interaction between fixed effects using likelihood ratio tests, comparing the full model containing the interaction with a reduced model including only the main effects.

5.4 Results

Does infection, or exposure to infection, trigger autogrooming?

Autogrooming frequency varied somewhat between dyad and arena types, but there was no evidence that bees groomed themselves more often when paired with an infected partner compared to controls, regardless of whether they could physically touch or only detect their partner's VOCs.

In touch-allowed arenas, control bees tended to autogroom more frequently (4.60 ± 0.72 and 3.73 ± 0.59) than either the infected bees (3.42 ± 0.46) or their uninfected partners (2.87 ± 0.37; Figure 5.2), but not significantly so (both $p > 0.2$; Table S5.1). Within infected dyads, infected bees autogroomed slightly more often than their uninfected partners, though again this was not significant ($p = 0.61$; Table S5.1).

In touch-prevented arenas, the pattern shifted slightly: control bees autogroomed at very similar levels (2.68 ± 0.53 and 2.11 ± 0.27) to infected bees (2.15 ± 0.34; Figure 5.2; *p* = 0.78; Table S5.1) and somewhat less than the uninfected partners of infected bees (3.14 ± 0.57; Figure 5.2), but this difference was not significant (*p* = 0.53; Table S5.1). Infected bees autogroomed only marginally less than their uninfected partners (2.15 ± 0.34; Figure 5.2; *p* = 0.78; Table S5.1), though again this comparison was not significant (*p* = 0.20; Table S5.1; Figure 5.2).

The only consistent effect was between arena types: across all dyads, bees in touch-allowed arenas autogroomed more frequently than those in touch-prevented arenas (*p* = 0.006, z = -2.75, β = -0.31; Figure 5.2).

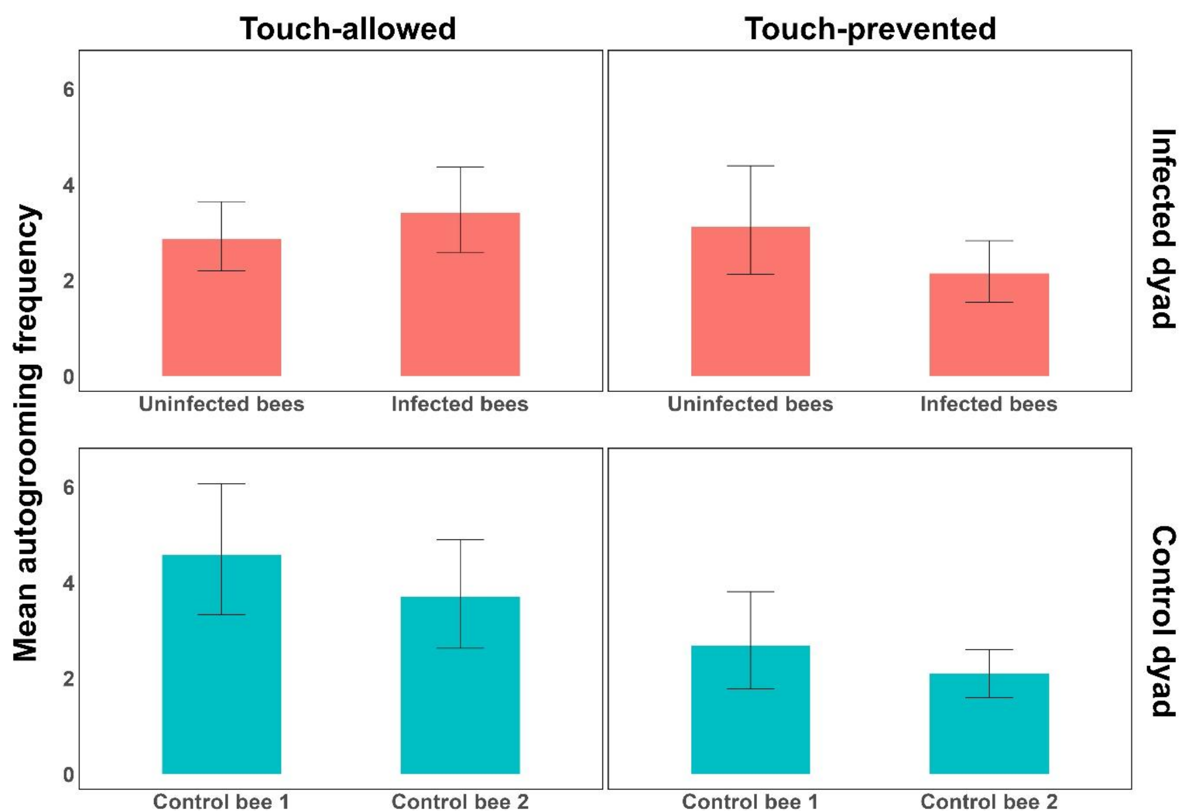

Figure 5.2: Mean autogrooming frequency of individual bees within infected and control dyads across arena types. Infected dyads contained one infected and one uninfected bee, while control dyads contained two uninfected bees. Behaviours were measured in both touch-allowed (partitioned with 2.5 mm gauge mesh) and touch-prevented (partitioned with 0.04 mm gauge mesh) arenas. Control bees are shown separately for completeness but were grouped in analyses, as they represent the same treatment. Error bars represent 95% bootstrapped confidence intervals.

We examined whether autogrooming frequency was related to infection severity, finding autogrooming of the uninfected bees (in infected dyad pairs) decreased with increasing spore load of their infected partner, but the effect was weak and non-significant (p = 0.13, z = -1.5, β = -0.25), and did not differ between touch-allowed and touch-prevented arenas (LRT: χ^2_1 = 0.69, p = 0.4; Figure 5.3). For infected bees, autogrooming frequency was also unrelated to their own spore load (p = 0.22, z = 1.2, β = 0.19; Figure 5.3). Although there was a slight tendency for bees in touch-allowed arenas to autogroom more when heavily infected, this relationship did not significantly differ between arenas (LRT: χ^2_1: = 0.93, p = 0.33; Figure 5.3).

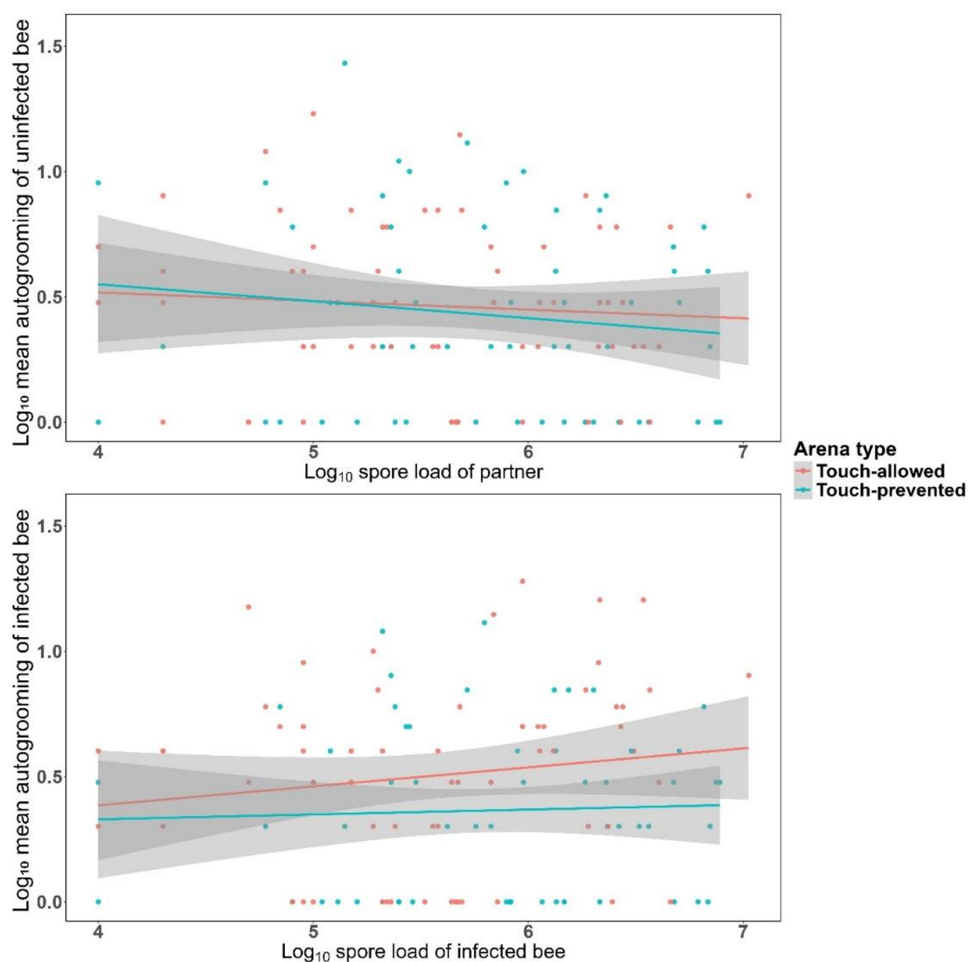

Figure 5.3: Mean autogrooming frequency of bees from infected dyads in relation to *Vairimorpha ceranae* spore load. The top panel shows the mean autogrooming frequency (log10) of uninfected bees in infected dyads (one infected and one uninfected bee) over the two-hour recording period in relation to the spore load (log10) of the infected partner. The bottom panel shows the mean autogrooming frequency of infected bees in relation to their own spore load. Behaviours were measured in both touch-allowed (partitioned with 2.5 mm gauge mesh) and touch-prevented (partitioned with 0.04 mm gauge mesh) arenas.

Does infection alter social interactions?

In touch-allowed arenas, there was no evidence that infected dyads interacted less than controls with respect to contact frequency. Although control dyads contacted one another more frequently (542 ± 69.8) than infected dyads (355 ± 52.8), this difference was not significant (p = 0.06, z = -1.9, β = 0.48). In touch-prevented arenas, contacts were significantly less frequent than in touch allowed (p < 0.01, z = -2.7, β = -0.76), but the difference between control (253 ± 50.1) and infected dyads (211 ± 37.7) was minimal and non-significant (p = 0.48, z = 0.7, β = 0.19; Fig. 5.4).

As another metric of social interactions we measured 'time spent in contact' specifically to examine whether contact frequencies might trade-off with interaction time. However, the total time each dyad spent in contact mirrored the pattern for contact frequency, suggesting that differences in contact frequency was not a function of time spent in contact. In touch-allowed arenas, control dyads spent more time in contact (2494 s ± 283) than infected dyads (2091 s ± 245), though this difference was not significant (p = 0.51, z = 0.65, β = 0.15). In touch-prevented arenas, both control (1039 s ± 200) and infected dyads (1102 s ± 168) spent similar amounts of time in contact (p = 0.94, z = –0.08, β = -0.19; Fig. 5.4). At the arena level, dyads in touch-allowed arenas spent significantly more time in contact (>2000 s) than those in touch-prevented arenas (~1000 s; p < 0.01, t = -2.98, β = -0.76).

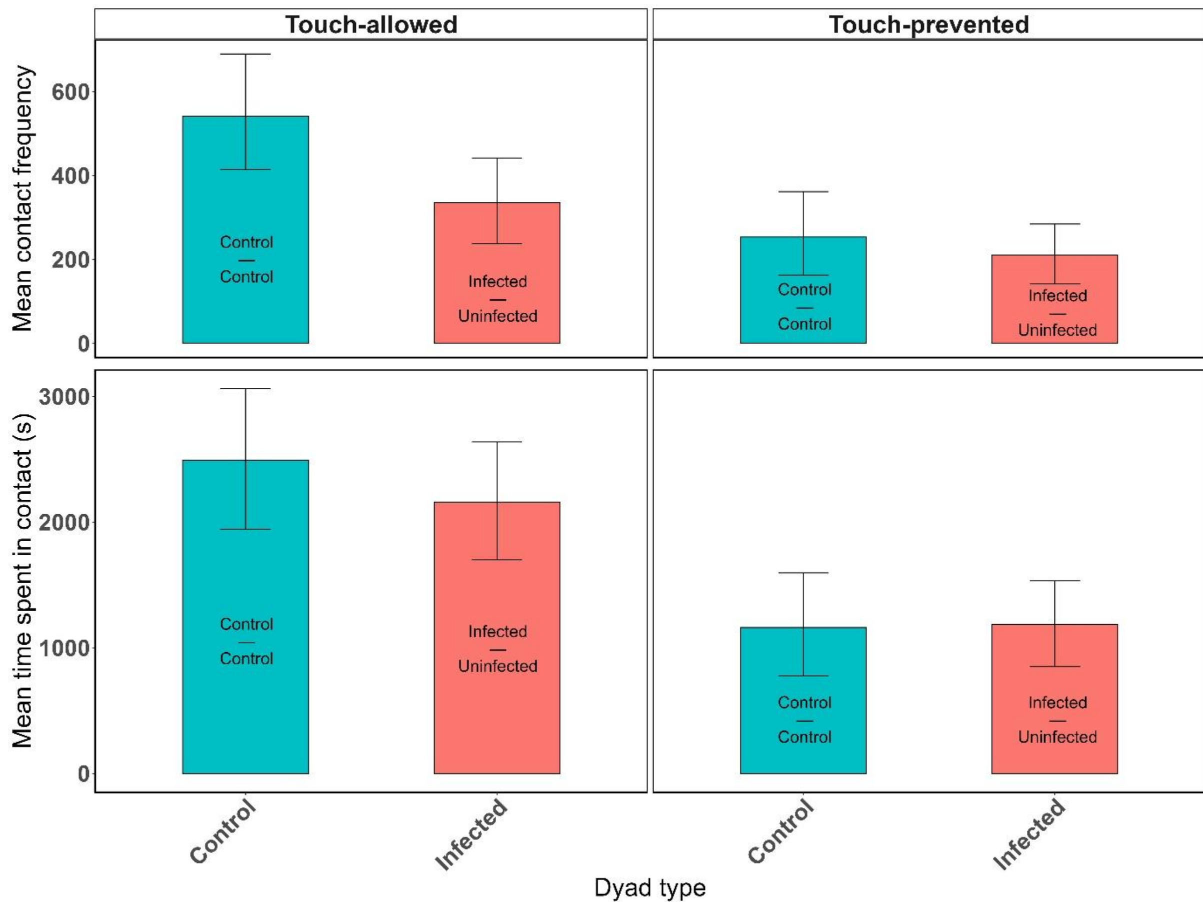

Figure 5.4: Mean contact frequency and time spent in contact of infected and control dyads across arena types. Infected dyads contained one infected and one uninfected bee, while control dyads contained two uninfected bees. Behaviours were measured in both touch-allowed (partitioned with 2.5 mm gauge mesh) and touch-prevented (partitioned with 0.04 mm gauge mesh) arenas. Error bars represent 95% bootstrapped confidence intervals.

We tested whether interactions between bees (contact frequency and time spent in contact) varied with infection intensity. In touch-allowed arenas, contact frequency showed a slight positive trend with spore load (Fig. 5.5), but the effect was weak and non-significant (p = 0.36, z = 0.90, β = 0.19). This trend was similar across arena types, with no evidence that the relationship between spore load and contact frequency differed between touch-allowed and touch-prevented arenas (LRT: χ^2_1 = 0.25, p = 0.62). Similarly, there was no relationship between spore load and time spent in contact (p = 0.17, t = 1.38, β = 0.28). Although bees in touch-allowed arenas tended to spend more time in contact when infection intensity was higher, while

those in touch-prevented arenas showed the opposite trend (Fig. 5.5), the interaction between spore load and arena type was not significant (LRT: $\chi^2_1 = 2.1$, $p = 0.15$).

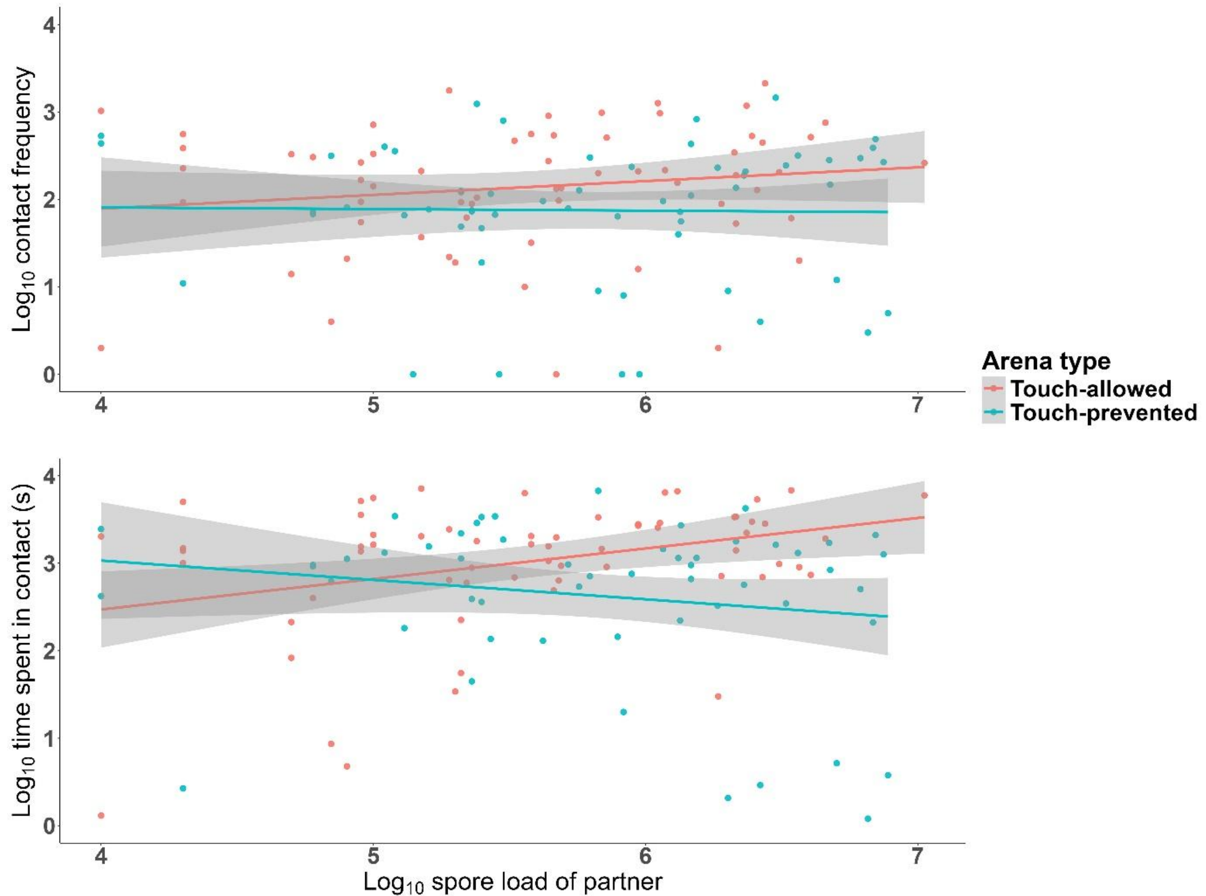

Figure 5.5: Contact frequency (log10) and time spent in contact (log10) between bees in infected dyads in relation to *Vairimorpha ceranae* spore load. Dyads contained one infected and one uninfected bee, and contact behaviours are shown in relation to the spore load (log10) of the infected partner. Measurements were made in both touch-allowed (partitioned with 2.5 mm gauge mesh) and touch-prevented (partitioned with 0.04 mm gauge mesh) arenas.

Does infection induce social distancing?

To test whether infection led to spatial avoidance, we examined the inter-individual Euclidean distances (cm) between dyads. If bees were avoiding each other, we would expect infected dyads to spend more time at the farthest distances and less

time close together when compared to controls. This pattern did not emerge: infected and control dyads showed very similar proximity patterns (p = 0.98, t = 0.03, β = 0.00; Figure 5.6). Across both arena types, dyads spent most of their time at intermediate distances of 3-6 cm (Figure 5.6). The one clear difference arose from arena structure: dyads in touch-allowed arenas remained significantly closer together on average than those in touch-prevented arenas (p < 0.001, t = -43.13, β = -0.85). This was driven by a distinct peak at 1-2 cm in the touch-allowed arenas (Figure 5.6), showing that bees stayed in close contact more often when physical interactions were possible. Beyond this peak, the overall distribution of distances was similar across arena types.

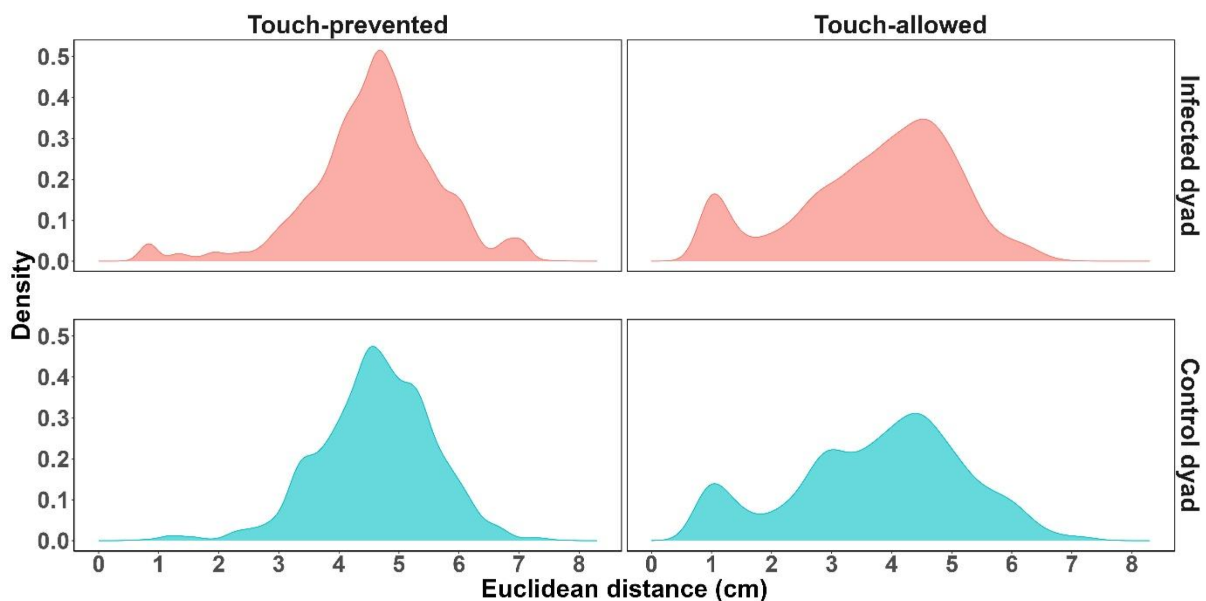

Figure 5.6: Kernel density distributions of inter-individual distances for infected and control dyads across arena types. Infected dyads contained one infected and one uninfected bee, while control dyads contained two uninfected bees. Distributions are shown for both touch-allowed arenas (partitioned with 2.5 mm mesh) and touch-prevented arenas (partitioned with 0.04 mm mesh). The y-axis shows relative density, illustrating how much time dyads spent at different distances from one another.

Do bees move more when exposed to infection?

We used total distance moved as a measure of activity to test whether uninfected bees altered their movement when paired with an infected partner, and whether infected bees themselves showed reduced activity when compared with controls.

In touch-allowed arenas, control bees on average moved farther (8427 ± 992 cm; 8537 ± 1090 cm) than either infected bees (7355 ± 729 cm) or their uninfected partners (6563 ± 685 cm), but none of these differences were statistically significant (all $p > 0.5$; Table S5.2). Within infected dyads, infected and uninfected bees moved similar distances to one another ($p = 0.54$; Table S5.2) A similar trend was seen in touch-prevented arenas, where controls again moved farther (10,508 ± 1180 cm; 9478 ± 1062 cm) than infected bees (8569 ± 700 cm) or their uninfected partners (8301 ± 782 cm), though here too the differences were not significant (both $p > 0.9$; Table S5.2). Within infected dyads, infected and uninfected bees moved similar distances to one another ($p = 0.95$; Table S5.2).

The only consistent effect was arena type: across all dyads, bees in touch-prevented arenas moved significantly more than those in touch-allowed arenas ($p = 0.02$, $z = 2.4$, $\beta = 0.24$; Figure 5.7).

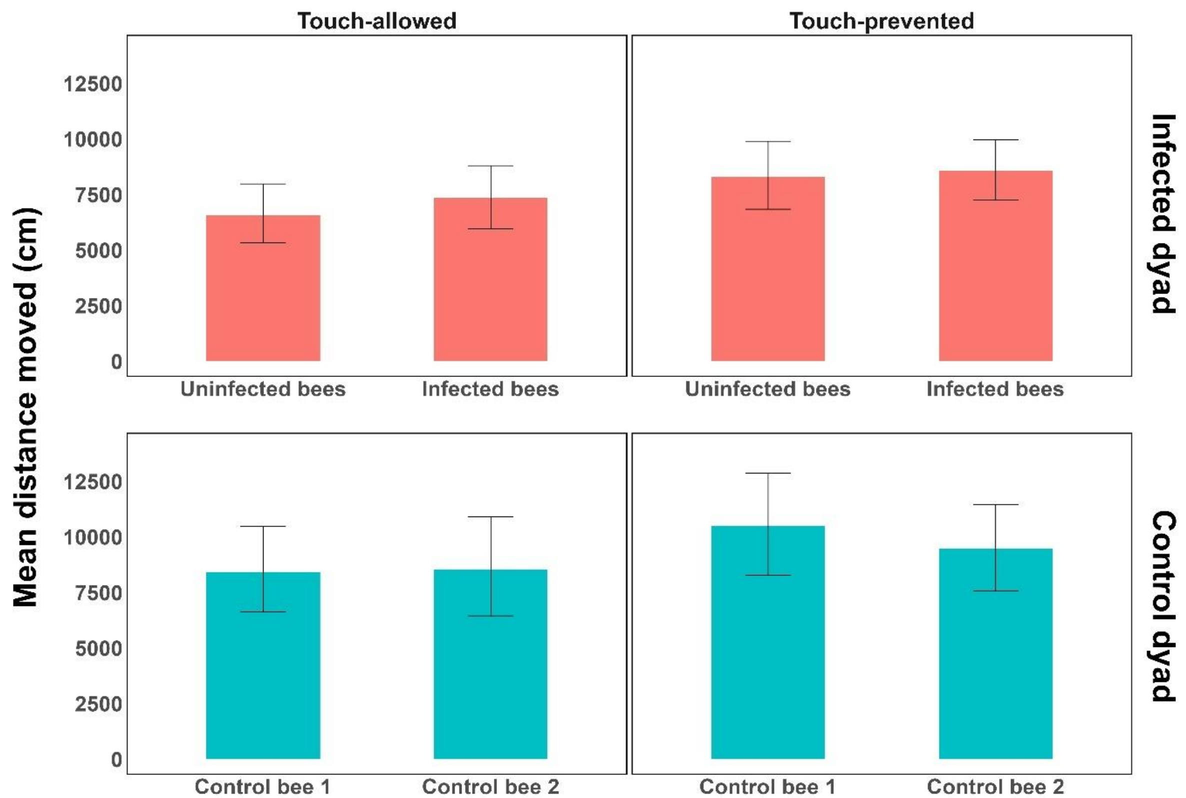

Figure 5.7: Mean distance moved (cm) by individual bees in infected and control dyads across arena types. Infected dyads contained one infected and one uninfected bee, while control dyads contained two uninfected bees. Behaviours were measured in both touch-allowed (partitioned with 2.5 mm gauge mesh) and touch-prevented (partitioned with 0.04 mm gauge mesh) arenas. Control bees are shown separately for completeness but were grouped in analyses, as they represent the same treatment. Error bars represent 95% bootstrapped confidence intervals.

5.5 Discussion

Social immunity relies on the ability of animals to detect and respond to infected conspecifics, often *via* chemical cues. Here we tested whether olfactory signals mediate behavioural changes towards *Vairimorpha ceranae*–infected honey bees in dyadic assays. Across all metrics, we found no consistent evidence that infection or infection intensity altered autogrooming, contact frequency, time spent in contact, or spatial proximity. Neither infected bees nor their uninfected partners interacted differently from controls. As expected, bees in touch-allowed arenas interacted more frequently and for longer than those separated by fine mesh. Crucially, however, infection did not alter behaviour in either arena type, indicating that under our laboratory design there is no evidence that olfactory cues alone are sufficient to elicit defensive responses towards infected nestmates, or that bees respond behaviourally to *Vairimorpha* infection at the dyad level.

Do Vairimorpha ceranae infections elicit behavioural responses?

Contrary to expectation, we found no significant differences in social interaction between control dyads and dyads containing an infected bee in either arena. This contrasts with studies reporting increased aggression or contact when *V. ceranae*–infected bees were introduced to uninfected nestmates in laboratory assays (Biganski et al., 2018). However, other work has similarly documented a lack of social immune responses, reporting no changes in agonistic behaviour or interaction frequency (McDonnell et al., 2013; Murray et al., 2015). Taken together, these findings suggest that responses to *V. ceranae* are highly variable, with some colonies detecting and responding to infection while others do not.

One possible explanation lies in the chemical cues themselves. While *V. ceranae* infection is consistently associated with changes in cuticular hydrocarbons (McDonnell et al., 2013; Murray et al., 2015), it remains unclear at what intensity volatiles, such as those identified in Chapter 4, are detectable to nestmates. This distinction may help explain the lack of response in touch-prevented arenas, where only volatiles could be exchanged: bees may simply have lacked access to sufficient

concentrations of cues, whereas in touch-allowed arenas they also had access to CHCs. If detection relies more heavily on contact-based hydrocarbons than airborne volatiles, our design would predictably yield weak or inconsistent behavioural responses in touch-prevented arenas. Yet even when contact was possible, we observed no significant changes in behaviour towards infected individuals, however, we did see trends consistent with infection dampening social contact: control dyads contacted one another more frequently (542 ± 69.8) than infected dyads (355 ± 52.8), a difference that approached significance (p = 0.06), and they also groomed more often. These subtle differences mirror the direction of effects reported by Biganski et al. (2018), that suggest that infection alters the behaviour of uninfected bees towards the infected individual.

Another explanation as to why we found no significant differences is high behavioural variability among workers. Honey bees are known to show strong inter-individual differences in interaction propensity and aggression (Walton and Toth, 2016) which may have obscured consistent effects at the dyadic scale. Although our overall sample size was large (n = 416 bees), the factorial design necessarily reduced replication within each treatment, which may have limited power to resolve such trends. Behavioural responses may also rely on a group environment to provide the social context for colony defence. Defensive behaviour in honey bees is a complex, multisensory process underpinned by division of labour (Nouvian et al., 2016). Most defensive tasks are performed by guard bees, typically 2–3 weeks old, which identify intruders and alert nestmates through alarm signals (Nouvian et al., 2016). Away from the nest, individual bees rarely show aggression (Nouvian et al., 2016), highlighting that the motivation to defend is tied to colony context. When directly confronted with a simulated threat, isolated bees can sting, but their responses are strongly modulated by group size and the behaviour of nestmates (Petrov et al., 2022). Defence is therefore both trigger-dependent and socially regulated. Furthermore, in ants, larvae only produce semiochemical cues when in the presence of adult workers (Dawson et al., 2024), raising the possibility that when bees were placed in dyads outside of a group context, no semiochemicals associated with infection were emitted. This perspective may explain why dyads in our study did not show robust responses, whereas Biganski et al. (2018) observed changes in groups of up to 50 bees. However, other group-based assays have also

reported muted responses to *V. ceranae* (McDonnell et al., 2013; Murray et al., 2015). These mixed findings emphasise that both scale (dyads vs. groups vs. colonies) and colony-specific variation must be considered.

Why is the response to Vairimorpha ceranae infection so variable?

If weak or inconsistent detection of infected adults is typical, this has important implications for how honey bee colonies manage disease. For *V. ceranae*, which often establishes chronic, sublethal infections, imperfect detection at the level of individual interactions could facilitate long-term persistence and contribute to its widespread distribution. Alternatively, detection may only become reliable once infection reaches a critical threshold in the colony (e.g. tens of infected individuals), meaning that small numbers of infected bees may go unnoticed. The variability observed in our study and across previous assays, reinforces the view that social immunity towards *V. ceranae* is highly context-dependent and our understanding is incomplete.

An additional consideration is parasite strain variation. Different *V. ceranae* strains show genetic polymorphisms that may have adaptive roles, and infections often consist of heterogeneous mixtures of spores rather than a single clonal strain (Sagastume et al., 2016). This means that the characteristics of infection, including any host chemical changes, can be highly variable. Such variability, combined with the well-documented behavioural diversity among honey bee workers (Petrov et al., 2022), may help explain why social responses to *V. ceranae* are inconsistent across studies. Previous behavioural assays have been conducted in different countries (New Zealand: Murray et al., 2015; France: McDonnell et al., 2013; Germany: Biganski et al., 2018), each likely involving genetically distinct parasite and host populations. To our knowledge, our study is the first to test social immunity towards *V. ceranae* in the United Kingdom, raising the possibility that either this strain of *V. ceranae* is adapted to avoid detection, or that local host populations are not adapted to respond to it.

It is intriguing that infection with *V. ceranae* is consistently associated with changes in semiochemical cues (McDonnell et al., 2013; Murray et al., 2015), yet bees did not respond behaviourally in our assays. Other honey bee pathogens can manipulate host scent to promote transmission. For example, Israeli acute paralysis virus alters host chemical cues in such a way that inter-colonial transmission becomes more likely (Geffre et al., 2020). *V. ceranae* strains may also differ in their ability to alter host chemistry, producing detectable changes that do not trigger strong defensive responses, and potentially conferring adaptive benefits such as enhanced inter-colonial transmission.

Conclusion

By isolating olfactory cues in a controlled dyadic assay, our study provides a causal test of whether volatile cues alone mediate behavioural responses to infection in honey bees. We found little evidence for strong behavioural changes towards *Vairimorpha ceranae*–infected nestmates, whether bees could physically touch or were only exposed to volatile cues. As expected, interactions were more frequent when physical touch was possible, though this was independent of infection status. The extent to which *V. ceranae* induces defensive behaviours in honey bees appears to be highly variable, shaped by context, parasite strain, and colony identity. This is the first evidence that *V. ceranae* infections in the United Kingdom do not elicit strong social immunity behaviours. However, given the variability in host and parasite responses, further investigation at the colony and field level, using different *V. ceranae* strains and more naturalistic environments is necessary. Extending our mechanistic approach for isolating olfaction as a pathway underlying social immunity to the colony scale offers a promising path forward for understanding how bees detect and respond to *V. ceranae*.

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5.7 Supplementary material

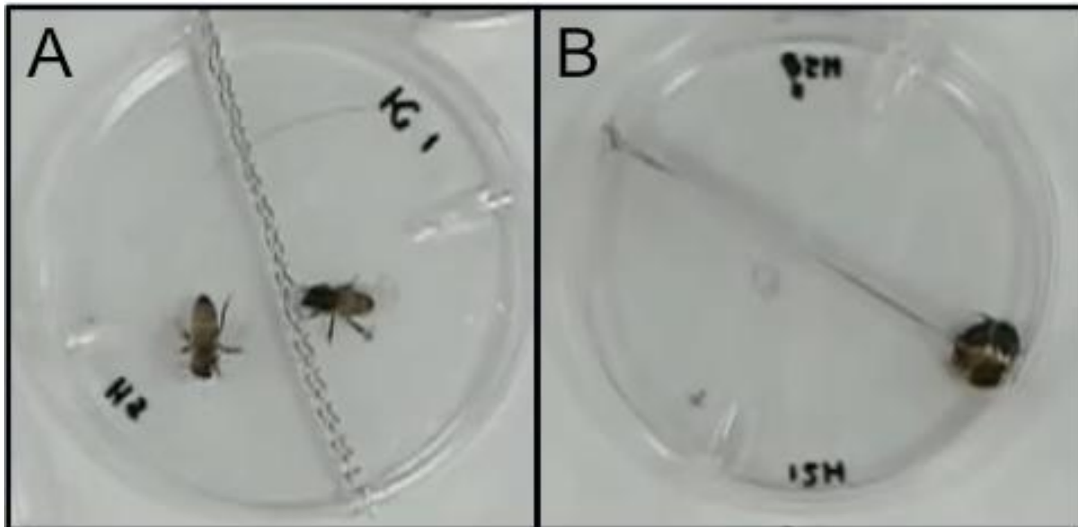

Figure S5.1 Still images of focal bees in arenas used to define contacts. (A) Bees not in contact (B) bees in contact.

Table S5.1: Tukey-adjusted post hoc comparisons of autogrooming frequency between dyads. Control = bees from control dyads (both uninfected). Infected = infected bee from infected dyads. Uninfected = uninfected partner from infected dyads. Control bees were grouped in the analysis since they represent the same treatment. Comparisons are shown separately for touch-allowed (2.5 mm mesh) and touch-prevented (0.04 mm mesh) arenas.

Dyad contrast	Arena type	Estimate	SE	Z ratio	P value
Control - Infected	Touch-allowed	0.142	0.187	0.759	0.728
Control - Uninfected	Touch-allowed	0.318	0.190	1.674	0.215
Infected - Uninfected	Touch-allowed	0.176	0.185	0.949	0.609
Control - Infected	Touch-prevented	0.138	0.205	0.672	0.780
Control - Uninfected	Touch-prevented	-0.215	0.201	-1.069	0.533
Infected - Uninfected	Touch-prevented	-0.353	0.205	-1.720	0.198

Table S5.2: Tukey-adjusted post hoc comparisons of total distance moved between dyads. Control = bees from control dyads (both uninfected). Infected = infected bee from infected dyads. Uninfected = uninfected partner from infected dyads. Control bees were grouped in the analysis since they represent the same treatment. Comparisons are shown separately for touch-allowed (2.5 mm mesh) and touch-prevented (0.04 mm mesh) arenas.

Dyad contrast	Arena type	Estimate	SE	Z ratio	P value
Control - Infected	Touch-allowed	0.010	0.145	0.070	0.997
Control - Uninfected	Touch-allowed	0.153	0.144	1.064	0.536
Infected - Uninfected	Touch-allowed	0.143	0.132	1.076	0.529
Control - Infected	Touch-prevented	0.022	0.146	0.150	0.988
Control - Uninfected	Touch-prevented	0.064	0.146	0.437	0.900
Infected - Uninfected	Touch-prevented	0.042	0.142	0.296	0.953

Chapter 6: The smell of infection as a mechanism underlying social immunity in social insects

6.1 Abstract

Colonies of social insects rely heavily on collective behavioural responses, known as social immunity, to limit pathogen transmission. Chemical cues have been implicated in social immunity, but empirical tests on infection-derived scent – “infection scent” – in social immune responses are lacking. Here, we test the role of scent in social immunity by individually introducing marked adult focal bees from four different treatments control (uninfected and unmanipulated), uninfected-smell (perfumed with uninfected bees), infected (infected with *Vairimorpha* spp.), and infected-smell (perfumed with *Vairimorpha* spp. infected bees) into observation colonies. Host responses toward, and behaviours performed by, focal bees were recorded over time, with 36 individual behaviours grouped into 7 categories (aggression, avoidance, antennation, grooming, maintenance, patrolling, social feeding). Multivariate analyses on all behaviours showed that the behavioural profiles of healthy bees diverged strongly from infected and infected-smell bees, which were similar to one another, with differences driven by aggression, avoidance, antennation, and intensive allogrooming. Aggression was strongly front-loaded: at introduction, infection-associated bees received significantly more aggression than healthy bees, then declined to low levels thereafter. In contrast, avoidance persisted, with infection-associated bees avoided more both at introduction and throughout later periods. Apparent survival across post-introduction intervals was lowest for infected bees, highest for uninfected-smell, and intermediate for controls and infected-smell. Together, these results show that infection scent can reproduce the colony response to infection to *Vairimorpha* infection. Olfactory recognition therefore underpins adult-focused social immunity in honey bees, with implications for transmission dynamics across social insects and behavioural immunity more broadly.

6.2 Introduction

Innate and adaptive immunity form the primary defences against parasites and pathogens in many animals, but behavioural strategies also play a critical role in reducing exposure to infection (Hart, 1990; Curtis, 2014). Empirical evidence demonstrates avoidance of diseased conspecifics across taxa: bullfrog tadpoles, *Rana catesbeiana* (Kiesecker et al., 1999), house finches, *Carpodacus mexicanus* (Zylberberg et al., 2013), and humans (Schaller and Park, 2011). Comparable behaviours are also widespread in invertebrates, where for example Caribbean spiny lobsters (*Panulirus argus*) avoid conspecifics carrying lethal viruses (Behringer et al., 2006), but this behavioural avoidance is especially common in social insects (Gliński and Buczek, 2003; Liu et al., 2019).

Insects lack the typical adaptive immune pathways found in vertebrates, though some host-parasite interactions reveal the capacity for immune specificity following prior exposure to pathogens (Sadd and Schmid-Hempel, 2006; Cooper and Eleftherianos, 2017; Gabriela and Adam, 2025). Beyond individual immunity, theory suggests insects have evolved additional strategies that reduce disease exposure and enhance colony fitness through collective behavioural defences, termed social immunity (Cremer et al., 2007; Pull and McMahon, 2020). Social immunity encompasses behaviours that limit infection risk for both diseased individuals and their nestmates, such as corpse disposal, nest sanitation, and the removal or exclusion of infected members (Cremer et al., 2007; Cotter and Kilner, 2010; Meunier, 2015). By acting collectively, colonies function as a defensive unit against parasites, a phenomenon extensively observed in eusocial insects (Gliński and Buczek, 2003; Stroeymeyt et al., 2014).

Social immunity can also exhibit adaptive features. Colonies sometimes mount stronger behavioural responses when re-exposed to parasites, in a way analogous to vertebrate immune memory. For example, ants previously exposed to fungal pathogens show elevated allogrooming of newly infected individuals (Walker and Hughes, 2009), which not only reduces pathogen load but can also facilitate low-level exposure that primes immune defences (Konrad et al., 2012). Collective mechanisms may be particularly important because social insects tend to express fewer genes linked to innate immune functions compared with solitary species

(Evans et al., 2006; Harpur and Zayed, 2013; Lopez-Uribe et al., 2016). Therefore, if social immunity is vital for both limiting parasite transmission and immune priming, a key question emerges: how do nestmates recognise when an infected individual has entered the colony and respond to it?

The mechanisms underlying social immunity

Effective social immunity first relies on animals detecting infection in conspecifics. The avoidance behaviour seen in bullfrog tadpoles, for example, can be triggered just by exposure to chemical cues from infected individuals (Kiesecker et al., 1999). This also applies to social insects: while some insect societies rely on visual or vibrational signals (Rosengaus et al., 1999, Davis et al., 2018), the use of chemicals is the oldest and most common mode of communication (Leonhardt et al., 2016). Chemical cues and signals in the context of nestmate recognition i.e. detecting nestmate from non-nestmate has been well studied in social insects, and the role of cuticular hydrocarbons (CHCs) is key to nestmate recognition functioning (Dani et al., 2005, Breed et al., 2015). The theoretical models of recognition systems developed in the nestmate recognition field help explain how scent-based social immunity responses arise. Recognition requires three components: cue production by the sender, cue perception by the receiver, and a behavioural response shaped by template matching (Sherman et al., 1997). In social insects, scent templates are dynamic and continuously updated (D'ettorre et al., 2006). Workers use these internal templates to classify individuals into meaningful categories such as “nestmate” versus “non-nestmate”, known as class-level recognition (Tibbetts and Dale, 2007; Gherardi et al., 2012). In the context of infection, class-level recognition could lead to infection being detected *via* changes in the chemical cue profile diverging away from the colony template (Tibbetts and Dale, 2007; Gherardi et al., 2012). According to the “undesirable-absent / desirable-present” model of recognition, nestmate acceptance depends on the balance of these cues: individuals are tolerated when undesirable scents are missing and desirable ones are sufficiently present (Guerrieri et al., 2009; Ratnieks et al., 2011). From this perspective, infection-associated scents may alter an individual’s chemical profile out of alignment with the current colony template, leading to increased aggression,

avoidance, or exclusion. Indeed, chemical cues and signals of infection arise through changes in CHCs or volatile organic compounds (VOCs) in many insect species (Milutinović and Schmitt, 2022; Asiri et al., 2024; Chapter 4), and some infection-associated scent changes have been associated with social immune responses in eusocial insects, such as ants (Dawson et al., 2024) and termites (Esparza-Mora et al., 2023), emphasising the central role of smell in coordinating collective defence.

Honey bees (*Apis mellifera*) provide a key model for testing the mechanisms underlying social immunity. Colonies face a wide range of parasites and pathogens, including Varroa mites (*Varroa destructor*), bacterial brood diseases, fungal pathogens such as chalkbrood, microsporidia such as *Vairimorpha* (formerly *Nosema*) spp., and numerous viruses (Aronstein and Murray, 2010; Grozinger and Flenniken, 2019; Emsen et al., 2020; Mejias, 2020; Warner et al., 2024; Chapter 1). Many of these infections are associated with shifts in host chemical profiles: including American and European foulbrood (Lee et al., 2020; Kathe et al., 2021; Chapter 3), chalkbrood (Finstrom et al., 2023; Chapter 3), *Vairimorpha* spp. (Murray et al., 2015; Chapter 4), Israeli Acute Paralysis Virus (Geffre et al., 2020), and Varroa mite (Wagoner et al., 2020; Zhao et al., 2025; Chapter 3). Colonies are thought to exploit such chemical information to mount social immune responses, for example, guards detect and block parasitised foragers at the hive entrance (Cappa et al., 2016), nurses remove infected brood (Wagoner et al., 2020; Spivak and Danka, 2021), and workers are more aggressive toward immune-stimulated nestmates (Richard et al., 2008), all of which are associated with altered CHC profiles. A well-documented form of honey bee social immunity is hygienic behaviour, in which nurse bees detect and remove infected brood before pathogens can spread (Spivak and Danka, 2021). Hygienic behaviour has been observed in response to multiple parasites, including American foulbrood (Spivak and Reuter, 2001), Varroa mites (Schoning et al., 2012), and chalkbrood (Spivak and Gilliam, 1998) and can convey resistance to these infections. These behaviours illustrate the importance of recognition templates and scent-based cues in understanding the mechanisms underlying colony-level social immunity.

While brood-targeted defences are well studied in honey bees, far less is known about the mechanisms underlying social immune responses towards infected adults. Yet adult workers are central to pathogen transmission as their roles in grooming,

nest sanitation, and foraging expose them to repeated infection risks (Fefferman et al., 2007). Colonies can adjust their behaviour towards infected workers: Israeli Acute Paralysis Virus-infected bees receive less trophallaxis (Geffre et al., 2020), and Deformed Wing Virus-infected bees are bitten and expelled (Baracchi et al., 2012). In some cases, however, pathogens appear to manipulate host recognition systems for their own benefit. Viral infection can alter the CHC profile of honey bees, making infected individuals more acceptable to foreign colonies and thereby enhancing inter-colonial transmission (Geffre et al., 2020).

For other adult infections the picture is less clear. Infections with *Vairimorpha* spp. are associated with changes in CHC and VOC profiles (McDonnell et al., 2013; Murray et al., 2015, Chapter 4), yet evidence for a consistent social immune response is mixed. Some studies report no behavioural changes toward infected bees (McDonnell et al., 2013; Murray et al., 2015; Chapter 5), whereas other describe increased interactions or even killing of infected conspecifics (Biganski et al., 2018). The variability in behavioural responses to *Vairimorpha* infection suggests that social immune defences may be contingent on how infection cues are perceived, making it important to clarify the mechanism underlying colony recognition of *Vairimorpha*-infected adults.

Vairimorpha infections of honey bees

Both *Vairimorpha apis* and *Vairimorpha ceranae* are linked to colony losses, with *V. ceranae* now widespread across Europe and North America (Higes et al., 2008). Transmission occurs when spores are ingested by foragers at flowers and spread orally within colonies (Higes et al., 2009; Forsgren and Fries, 2010; Graystock et al., 2015). Because *Vairimorpha* spp. primarily infect adult workers, host behaviour is particularly relevant for transmission dynamics. In our previous work, we introduced *Vairimorpha*-infected workers to healthy nestmates in laboratory assays and found no clear effect of infection or its associated odours on social interactions (Chapter 5). However, it remains untested whether olfactory cues alone – in the absence of true infection – are sufficient to trigger social immune responses, particularly at the colony level under natural conditions.

Here, we conducted a field-based behavioural experiment with *Vairimorpha*-infected honey bees to test whether both infection and the 'smell of infection' mediates social immunity behaviours in comparison with unifected bees. Our findings provide new insight into the role of chemical communication in disease transmission dynamics and highlight a key mechanism underlying the transmission of pathogens within social insect societies.

6.3 Methods

All *A. mellifera* honey bees were collected from the Cardiff University Research Apiary, herein referred to as Colony A and Colony B. Both colonies were free from existing American and European foulbrood, and *Vairimorpha* spp. infections, but were naturally infested with *V. destructor*. One day old bees (0-24 h old) were randomly assigned to one of four different treatments: Control (uninfected and unmanipulated), uninfected-smell (perfumed with uninfected bees), infected (infected with *Vairimorpha* spp.), and infected-smell (perfumed with *Vairimorpha* spp. infected bees) and placed into groups of 10 bees in hoarding cages made from ventilated plastic deli cups with feeders (Figure 6.1). A numbered Opalith tag (Zschopautaler Imker) was attached to the thorax to allow individual identification of focal bees and both dorsal and ventral sides of the abdomen were marked with queen marker paint (Posca) to allow for identification regardless of its orientation in the observation hive. To reduce the effect of observation colonies rejecting bees due to extended separation (as bees were maintained in the laboratory away from their original colonies prior to the experiment), during the incubation period, workers were housed with sections of comb from the original donor colony and only introduced into observation colonies consisting of bees from the colony they originated from. Cages were maintained at 33 °C and 65% relative humidity in full darkness and bees were fed 50% w/v sucrose solution *ad libitum*.

Vairimorpha infections

To create *Vairimorpha* inocula, the alimentary tract, consisting of the midgut, small intestine, and rectum from bees collected from apiaries, at Fonmon Apiary, Cardiff,

with suspected *Vairimorpha* spp. infection (confirmed by phase-contrast microscopy) were pooled and homogenised in sterile distilled water (Fries et al., 2013). The resulting spore suspensions were purified using the standard methods described by Fries et al. (2013). In brief, spore suspensions were filtered using mesh with a 70 μm pore to remove host material. This was followed by three rounds of centrifugation (5,000 G for 5 min) and resuspension. The pelleted spores were finally re-suspended in 50% w/v sucrose solution which was used for inoculation of naïve bees.

Bees were collected, housed and inoculated as described above with high doses ($\geq 5x10^7$ spores/ml) to maintain a laboratory culture of *Vairimorpha* infected bees that were used to collect fresh spores for subsequent inocula of experimental bees (Figure 6.1).

For inoculum doses, naïve one day old (0-24 h old) groups of 10 bees from all treatments were starved for 1 hour before being batch fed an inoculum either containing 5_x10^7 spores/ml of *Vairimorpha* spp. (infected treatment), equating to 5_x10^6 spores/bee, or sterile 50% w/v sucrose (control, uninfected-smell, and infected-smell treatments; Figure 6.1). Following inoculation, bees in all treatments were housed in incubators for 7 days before being individually introduced into observation colonies (Figure 6.1).

Scent transfer

To perfume the bees in the uninfected- and infected-smell treatments, we modified a previously established smell transfer method (Ratnieks et al., 2011). Groups of 20 workers, either infected with *Vairimorpha* spp. or uninfected (using inoculation methods described above), were placed into 60 ml plastic tubes for 60 minutes to transfer scent to the tube interior. After removal of the bees, the perfumed tubes were sealed and stored at -20°C and used within 24 hours (Figure 6.1). Bees used to transfer scent to the tubes came from the same colony as the focal bee that would later be introduced – e.g., if the focal bee was from Colony A, scent donors (infected or uninfected) also came from Colony A. To create treatment animals, a single uninfected focal bee from the appropriate treatment group (infected smell or uninfected-smell) was placed into the matching perfumed tube for 15 minutes to

acquire the scent and was then immediately introduced into the observation colony and observed (Figure 6.1). Bees from non-perfumed treatments (control, infected) were similarly placed into sterile, unused tubes previously cleaned with ethanol to remove any scent prior to introduction.

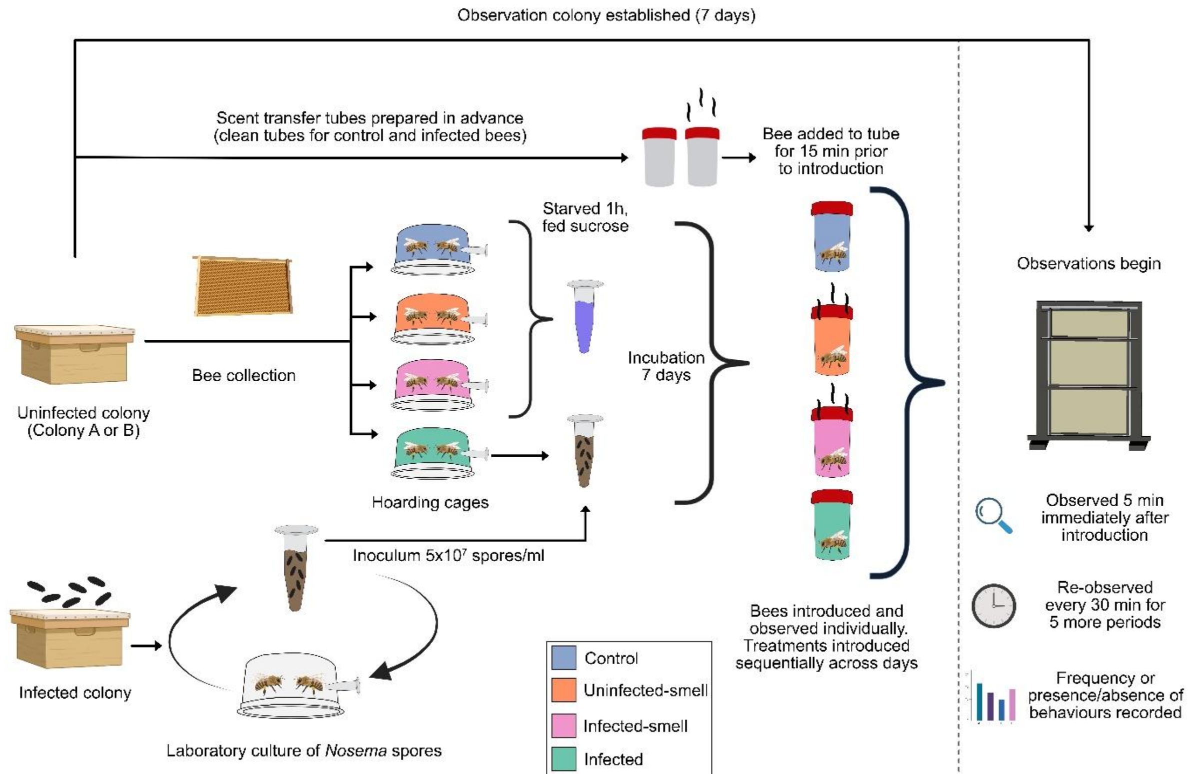

Figure 6.1: Schematic overview of experimental methods used to assess if honey bee behaviours are influenced by infection and scent cues. Newly emerged *Apis mellifera* workers were collected from uninfected colonies (A or B) and randomly assigned to one of four treatment groups: Control, uninfected-smell, infected, and infected-smell. Bees in the infected group were fed a *Vairimorpha* spp. spore solution (5×10^7 spores/ml), while all others received a sterile sucrose solution. All bees were incubated for 7 days at 33 °C and 65% relative humidity in darkness before being individually introduced into observation colonies composed of nestmates. Prior to introduction, bees in the uninfected-smell and infected-smell treatments were exposed for 15 minutes to pre-scented tubes prepared using infected or uninfected workers from the original donor colonies. Bees from non-perfumed treatments were placed into sterile tubes previously cleaned with ethanol to remove scent. Bees were observed for 5 minutes immediately after introduction and re-observed for 2 min per bee every 30 minutes for five additional periods. Observations included counts or presence/absence of 36 behaviours.

Observation colonies

Two observation colonies were established; each seeded with approximately 3,000 workers – one with bees from Colony A and one from Colony B (Figure 6.1) Colonies were provisioned with a strip of Queen Mandibular Pheromone (synthetic queen pheromone, TempQueen), along with frames containing eggs, capped and uncapped brood, honey and pollen. The colonies were given one week to acclimate before experimental work began. Colonies were provided with sugar water (50% w/v) *ad libitum*. The observation hives were custom designed to accommodate two brood frames and one super frame (Mullberry Workshop). To facilitate natural foraging, each hive was connected to the outside *via* an entrance tube (Figure 6.2). Feeders were removed on observation days to discourage clustering near the feeding area and to promote normal colony activities. This setup ensured controlled yet naturalistic conditions for observing bee behaviour.

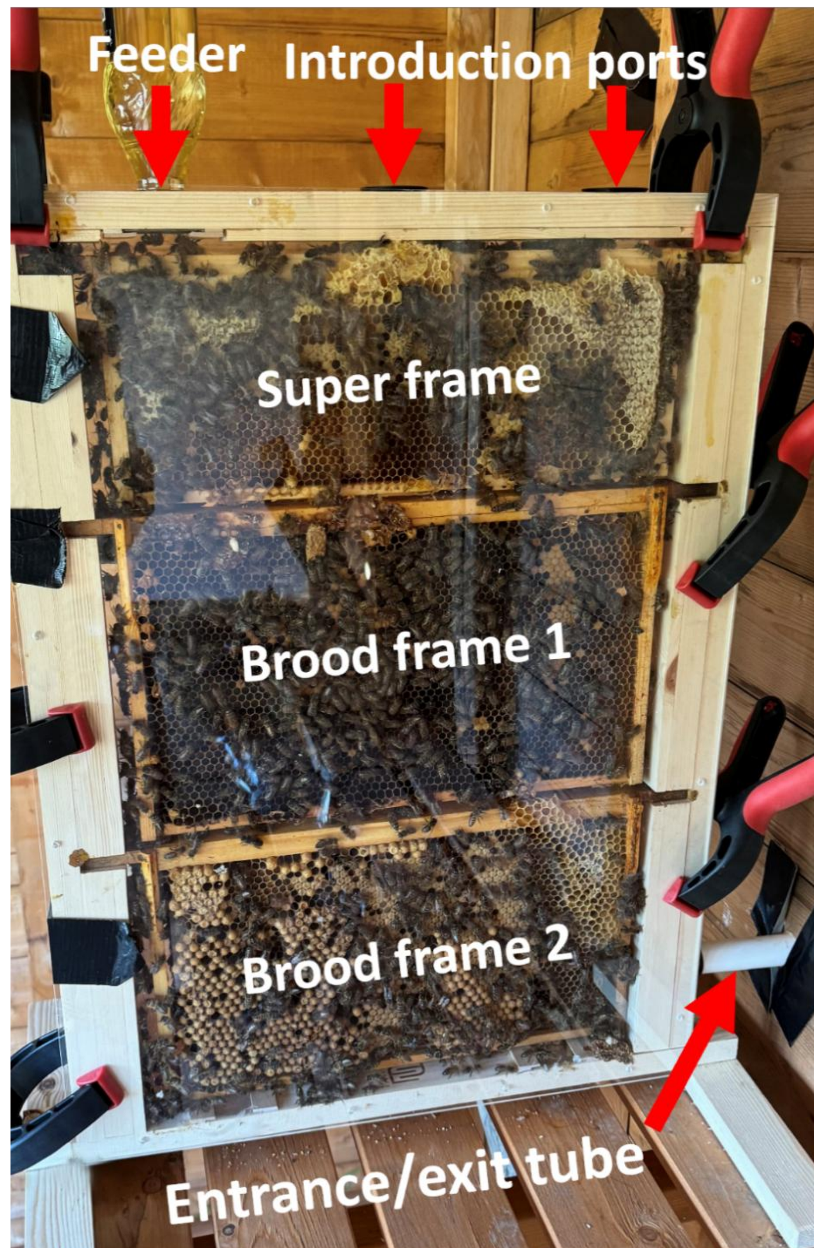

Figure 6.2: Front view of the custom observation hive used for behavioural assays. Each hive contained two brood frames and one super frame and was connected to the outside environment *via* an entrance/exit tube to allow natural foraging. Experimental bees were introduced individually through dedicated ports at the top of the hive. Colonies were provisioned with sugar syrup *via* feeders (removed during observation periods) and maintained under semi-natural conditions to facilitate normal colony behaviour while enabling controlled observations.

Observation protocol

Both sides of the observation hives were manually observed under low natural lighting conditions. For each colony, focal bees from a single treatment were introduced one at a time *via* dedicated ports in the top of the hive. Each bee was observed for 5 minutes immediately after introduction (Figure 6.1) and then another focal bee was introduced. This process was repeated until a maximum of 25 bees had been introduced, herein referred to as the 'introduction' period.

Thirty minutes after the final bee had been introduced and observed, the entire observation colony was systematically searched to locate focal bees. Forty minutes were allocated for any given observation period, which was sufficient to observe up to 20 different focal bees for 2 minutes per bee. If a bee that had been already observed in that period was encountered again, it was ignored until the next period. This was repeated a total of 5 times (periods 1-5) throughout the day between 0900 and 1900, with each observation period spaced at least 30 minutes apart, resulting in a total of 6 observation periods (introduction period and periods 1-5, Figure 6.1).

Each treatment group was introduced into the colony on sequential days, starting with the control treatment (day 1), followed by uninfected-smell (day 2), infected-smell (day 3), and finally infected (day 4). This same order of treatment introductions was then repeated in Colony B (days 5-8). This sequential approach, rather than randomising exposure to different treatment groups, was carried out to minimise colony exposure to *Vairimorpha* spp. Our goal was to introduce 25 bees from each treatment across both colonies, but due to natural mortality during the 7-day incubation period prior to introduction, our sample sizes varied between colonies and treatments. Across four treatments 158 bees were introduced individually into two observation colonies Control (Colony A: n = 22, Colony B: n = 21), uninfected-smell (Colony A: n = 25, Colony B: n = 22), Infected (Colony A: 25, Colony B: n = 10), and infected-smell (Colony A: n = 24, Colony B: n = 21). Across both colonies and all treatments, a total of 1990 minutes of behavioural data were collected.

Behavioural metrics

During observations, we recorded the frequency or presence/absence of a total of 36 distinct behaviours (Table S6.1). These included actions performed by the focal bee as well as behaviours directed toward it by host colony members. Count data were used for behaviours that could occur multiple times within a single observation and where each instance represented a distinct event (e.g. antennation, biting, trophallaxis). Prolonged behaviours were scored as counts if they could be reliably separated into distinct bouts, such as extended grooming, where one bout ended and a different host bee(s) initiated a new grooming event. By contrast, binary scoring was applied to behaviours that are continuous or inherently difficult to separate into independent events. For example, extended dragging reflected a sustained action by the same host bee(s); patrolling represents an ongoing task that cannot be meaningfully subdivided; and autogrooming, inspections, maintenance behaviours, and fanning are all state-like activities with ambiguous end points (Table S6.1). To focus on the most common and biologically relevant interactions, behaviours that were rare (≤15% of observed bees in all treatments) were excluded from subsequent analyses, resulting in a total of 20 remaining behaviours. Details of all behaviours, including those excluded due to low frequency, are provided in Table S6.2.

To assess behavioural responses at a broader scale, we grouped each of the remaining behaviours into one of seven categories: aggression, avoidance, antennation, grooming, maintenance, patrolling, and social feeding (Table S6.1). For example, trophallaxis was distinguished by direction (focal bee receiving versus providing food), so fit within ‘social feeding’, ‘aggression’ encompassed multiple behaviours such as biting, dragging, and chasing, and ‘grooming’ included both self-directed (autogrooming) and social variants (allogrooming performed or received) (Table S6.1).

Statistical methods

All analyses were performed in R version 4.3.2 (R Core Team, 2023) and plots created using *ggplot2* (Wickham, 2011).

Preliminary model: introduction order and day effects

To assess whether colonies exhibited learning across sequential introductions of focal bees or whether behavioural responses varied among trial days, we carried out preliminary binomial GLMs (*lme4*: Bates et al., 2003) with behaviour category coded as a binary response (0/1). Models included either introduction order or experimental day together with colony as fixed effects, to test whether the likelihood of a focal bee receiving interactions from the host colony depended on the day of the trial or its position in the introduction sequence.

Does infection alter behavioural profiles?

To test whether the behavioural profiles (all 20 behaviours) of focal bees differed between treatments, we fitted multivariate generalised linear models (manyglm) using the *mvabund* package (Wang et al., 2012). A behavioural profile was defined as the cumulative set of all behaviours a focal bee performed or received across all observation periods and consisted of a response matrix, of both log-transformed frequency data (counts of repeated behaviours) and behaviours scored as present or absent. Treatment was included as the explanatory variable. Model significance was assessed using the anova.manyglm function with PIT-trap resampling (999 iterations). Pairwise comparisons between treatments were used to identify which treatment groups differed in their overall behavioural profiles. To determine which behaviours contributed to these multivariate differences, we extracted univariate test statistics from the manyglm output, applying p-value adjustment for multiple testing.

To visualise treatment effects on behavioural profiles, we performed a principal component analysis (PCA) using the dudi.mix function from the *ade4* package (Chessel et al., 2004). This ordination was chosen because it accommodates datasets containing both continuous (behavioural frequencies) and binary (presence/absence)

variables, matching the structure of our data. The ordination was used to display clustering of treatments in multivariate space, while loadings for individual behaviours were used to assess their relative contributions to variation along the first two principal components. These loadings were then compared with the univariate manyglm results to identify behaviours driving separation between treatments.

Temporal changes in behavioural responses at the categorical level

We asked whether the proportion of bees experiencing each behavioural category varied across observation periods by calculating the proportion of focal bees that experienced each category within each period. For each category (n = 7), we fitted a binomial GLM with a binary response (whether a bee ever experienced a behavioural category) as the response and treatment, observation period, colony and their interaction as explanatory variables. Likelihood ratio tests (LRTs) were used to assess the significance of explanatory terms against null models lacking treatment and lacking the interaction, and post hoc pairwise comparisons were performed using estimated marginal means (*emmeans* package; Lenth, 2025) with Tukey adjustment for multiple testing.

Apparent survival of focal bees

To assess whether infected or infected-smell bees were removed from the colony more often than control or uninfected-smell bees, we estimated apparent survival using Cormack–Jolly–Seber (CJS) models. This allowed us to distinguish between focal bees that had been removed from the colony and those that were present but not detected. These models jointly estimate the detection probability (p), defined as the probability of observing a bee if it is alive and present, and the apparent survival probability (Φ), defined as the probability of surviving from one observation period to the next. Cumulative survival over the course of the experiment was calculated as Φ^k, where k is the number of observation intervals (0–5), giving the expected proportion of bees remaining at each period after accounting for imperfect detection. To test for effects of colony, treatment, and time, we compared alternative CJS models using Akaike's Information Criterion corrected for small sample sizes (AICc),

with models within ΔAICc ≤ 2 considered equally supported. To calculate 95% confidence intervals for cumulative survival, we used the delta method on the log scale. This approach is appropriate because cumulative survival is the product of interval estimates, and the delta method correctly propagates variance across intervals (Lebreton et al., 1992).

6.4 Results

A total of 5147 individual behavioural observations were recorded throughout the study. These were distributed across the treatment groups as follows: Control (n = 1282), uninfected-smell (n = 1264), Infected (n = 967), and infected-smell (n = 1634). Introduction order ($p = 0.08$, $z = -1.78$, $\beta = -0.017$), colony ($p = 0.77$, $z = 0.28$, $\beta = 0.04$), and experimental day (all $p > 0.06$) had no significant effect on the probability that focal bees were interacted with by the host colony, indicating that these factors did not meaningfully alter the likelihood of the colony responding to a focal bee.

Does infection alter the behavioural profile of bees?

Manyglm multivariate analysis of behavioural profiles pooled across all time points revealed significant differences among treatments (Dev = 397, $p = 0.001$). Pairwise comparisons confirmed the largest differences were between healthy bees (control and uninfected-smell) and infection-associated bees (infected and infected-smell), with all four contrasts highly significant ($p = 0.001$) and associated with large deviances (Dev > 100; Table 6.1). The difference between control and uninfected-smell bees was significant but smaller in magnitude (Dev = 78.6, $p = 0.001$). The smallest difference overall was between infected and infected-smell bees (Dev = 39.8, $p = 0.019$), indicating that although their behavioural profiles were not identical, they were far more similar to one another than to the healthy groups. These statistical patterns were reflected in the PCA ordination (Figure 6.3), where control and uninfected-smell bees clustered closely, whereas infected and infected-smell bees occupied more variable positions and were clearly separated from the healthy groups along PC1 and PC2.

Table 6.1: Pairwise manyglm multivariate analysis of focal bee behavioural profiles based on the frequencies and presence/absence of behaviours received or performed across all observation periods. The test statistic (Dev) is the sum of deviances across behaviours for each contrast. Significance was assessed using PIT-trap resampling with 999 iterations, and *p*-values are adjusted for multiple testing.

Treatment contrast	Dev	Adjusted p-value
Infected smell vs uninfected smell	233.356	0.001
Infected vs uninfected smell	153.325	0.001
Control vs infected smell	126.811	0.001
Control vs infected	108.596	0.001
Control vs uninfected smell	78.587	0.001
Infected vs infected smell	39.754	0.019

Univariate tests extracted from the manyglm models identified the behaviours that contributed most strongly to differences in behavioural profiles between treatments. Aggressive behaviours, including biting, extended dragging, chasing, charging, and grabbing, significantly drove separation between focal bee behavioural profiles (all $p \leq 0.02$), alongside avoidance behaviours (rejected trophallaxis) ($p = 0.003$; Table S6.3). Antennation ($p = 0.001$), extended grooming ($p = 0.001$) and group extended grooming ($p = 0.001$) also significantly contributed to behavioural profile separation between treatments (Table S6.3). These results were consistent with the positions of each treatment group within the PCA ordination and the individual loadings of each behaviour (Figure 6.3). Infected and infected-smell bees clustered on the positive side of PC1 and PC2 were associated with higher frequencies of aggression (biting, dragging, charging), antennation rejected trophallaxis, autogrooming, and extended grooming, which loaded positively on PC1. In contrast, control and uninfected-smell bees, which clustered on the negative side of PC1 and PC2, were associated with affiliative interactions such as trophallaxis, allogrooming, and neutral maintenance behaviours including brood cell inspection. Together, these results indicate that infection and infection-associated scent shift the behavioural profiles of introduced

bees away from the uniform, affiliative responses observed in the healthy treatments, and toward more variable and socially exclusive interactions dominated by aggression, antennation, and extended grooming behaviours.

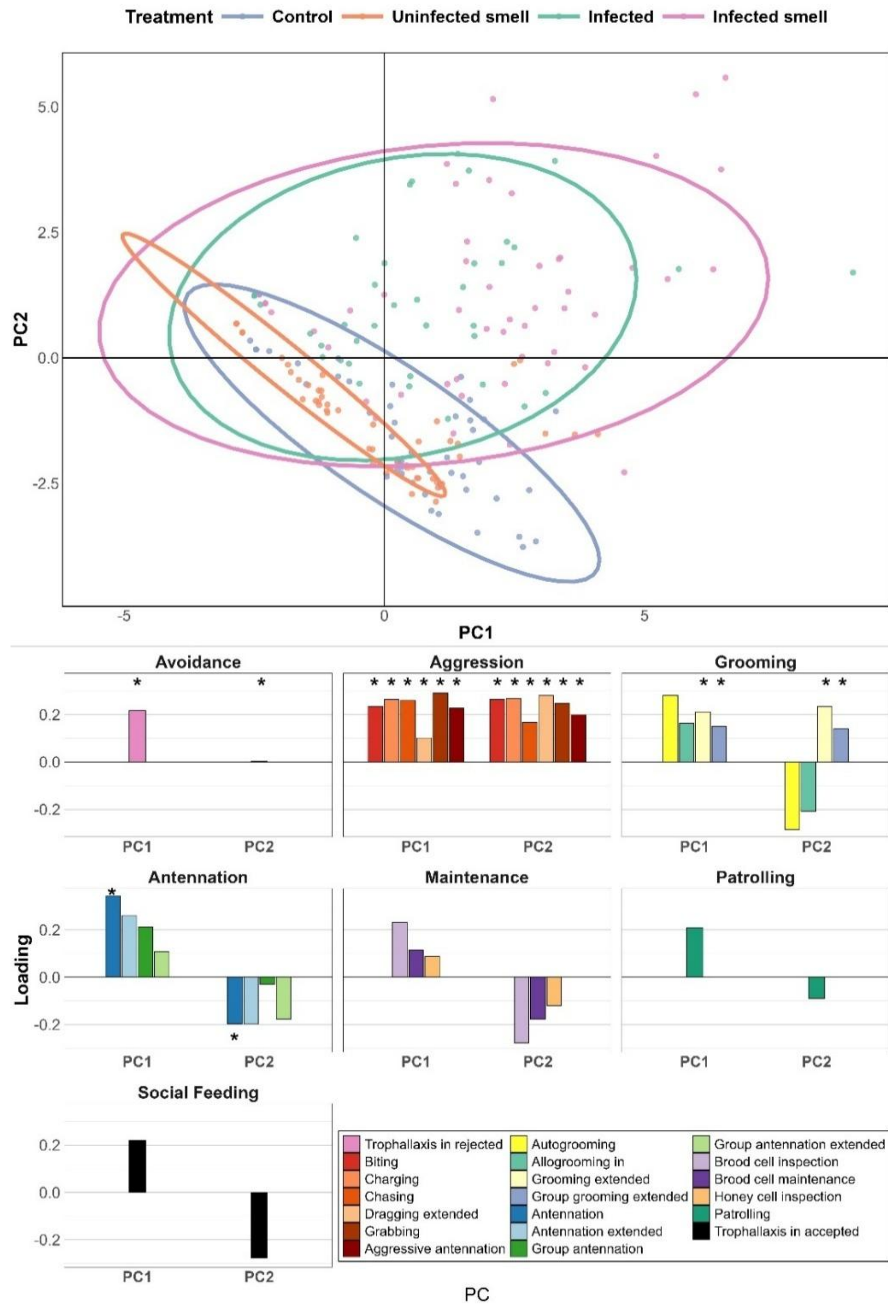

Figure 6.3: Principal component analysis (PCA) of behavioural profiles of focal bees across treatments. The ordination (top) shows the first two principal components from a dudi.mix PCA, explaining 36.8% of the total variation (PC1 = 24.2%, PC2 = 12.6%). Each point represents an individual bees behavioural profile, based on the total frequencies and presence/absence of behaviours received or performed, with ellipses showing 95% confidence intervals around treatment centroids. The loading plots (bottom) show the contribution of individual behaviours to PC1 and PC2, with bar height indicating the strength and direction of association. Stars (*) mark behaviours identified in the univariate tests from manyglm models as significantly contributing to treatment-level differences in multivariate behavioural profiles.

How do behaviours change over time, and do these trends differ by treatment?

We examined how the proportion of focal bees experiencing each behavioural category changed across observation periods (full post-hoc outputs for significant interactions in Table S6.4, S6.5). There was no evidence that treatment effects differed between colonies (all interactions between treatment and colony: $p > 0.23$). We therefore interpret the effects of treatment and its interaction with period averaged across colonies, while accounting for baseline colony differences by including colony as a fixed main effect. Aggression showed the most striking temporal pattern, varying strongly with treatment and period ($\chi^2_{15} = 54.0$, $p < 0.001$; Figure 6.4). It was concentrated in the introduction period, where nearly all infected (80.0 ± 6.9%) and infected-smell bees (97.6 ± 2.4%) experienced aggression. By contrast, controls (31.0 ± 7.2%) and uninfected-smell bees (25.6 ± 7.1%) experienced significantly less aggression (all $p < 0.001$; Figure 6.4). Aggression declined sharply by period 1 in all groups and remained consistently low thereafter (<22%), with no further differences between treatments (Figure 6.4). Similarly, avoidance also differed strongly by treatment, but unlike aggression, these differences were consistent across periods ($\chi^2_{15} = 3.5$, $p = 0.99$; Figure 6.4). Infected (45.7 ± 8.5%) and infected-smell bees (50.0 ± 7.8%) were avoided significantly more often than controls (23.8 ± 6.7%) or uninfected-smell bees (15.4 ± 5.9%; all $p < 0.01$) but did not differ significantly from one another ($p = 0.6$; Table S6.6). Controls were also avoided more than uninfected-smell bees ($p = 0.01$), which remained the least avoided group throughout (Figure 6.4; Table S6.6). A similar pattern was found for patrolling: although the proportion of bees patrolling varied across periods, this relationship was not significant ($\chi^2_{15} = 24.1$, $p = 0.06$). However, infected and infected-smell bees were more likely to patrol than controls, and uninfected-smell bees patrolled least ($p < 0.01$; Figure 6.4; Table S6.6). This suggests that bees associated with infection were more frequently walking around the hive without engaging in a distinct task. Antennation showed the opposite pattern, with no variation across periods ($\chi^2_{15} = 18.4$, $p = 0.24$) but a significantly higher percentage of controls received antennation than any other group ($p < 0.011$), while all other treatments did not differ from one another (Figure 6.4; Table S6.6).

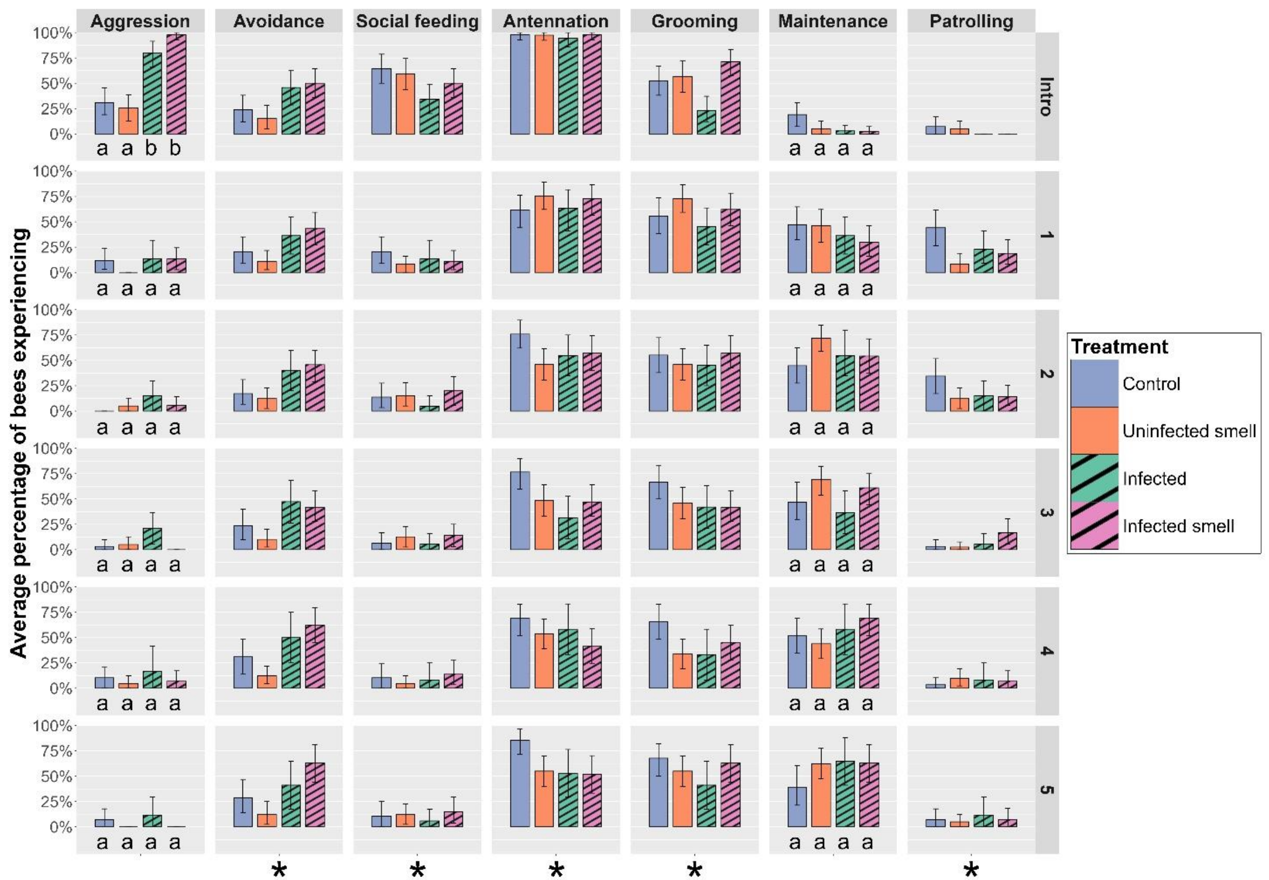

Figure 6.4: Average percentage of focal bees in each treatment that experienced at least one instance of a behaviour within each of the seven behavioural categories during each observation period. Letters are shown only when a significant interaction between treatment and period was detected (likelihood-ratio tests); different letters below bars indicate significant pairwise differences between treatments within that period (post hoc tests with Tukey correction). Stars denote significant treatment effects in additive models for categories without a significant interaction between treatment and period.

Maintenance showed weak or more transient effects, with comparisons yielding no significant differences between treatments despite significantly varying with period (LRT: $\chi^2_{15} = 32.8$, $p < 0.01$; Figure 6.4). For the remaining behaviours, there were no significant interactions with observation period (LRT: $p > 0.1$). Social feeding was highest at introduction, with controls (64.3 ± 7.5%) and uninfected-smell bees (59.0 ± 8.0%) engaging more often than infected bees (34.3 ± 8.1%), though not significantly so (Table S6.6). Social feeding then declined across all treatments to similar levels

by period 1 (Figure 6.4). Grooming also differed by treatment, with significantly fewer infected bees receiving grooming (22.9 ± 7.2%) than all other treatments (all $p < 0.033$; Table S6.6), which were all groomed at high levels (>50%) at introduction before converging from period 1 onwards (Figure 6.4).

Taken together, these results suggest that aggression, and to a lesser extent avoidance, were the main behaviours elevated toward infected and infected-smell bees compared to healthy bees, highlighting the central role of olfactory cues in mediating defensive responses within the colony.

Does infection or the smell of infection induce expulsion from the colony?

The number of focal bees observed declined across periods, which would indicate removal from the colony, but counts sometimes fluctuated as bees disappeared in one period and re-appeared in the next, indicating that raw observations alone were not a reliable measure of survival. To account for imperfect detection, we fitted Cormack–Jolly–Seber (CJS) models. The treatment-only model had the lowest AICc (951.2), with the treatment + colony model receiving similar support (ΔAICc = 1.7). Compared to the treatment-only model, the null model, assuming constant survival, was less well supported (ΔAICc = 2.1), while hive alone (ΔAICc = 3.2) and models including time effects (ΔAICc > 3.5) performed poorly. We therefore report survival estimates from the treatment-only model, which indicated a detection probability of 0.75 per observation period. Cumulative survival declined over time in all treatments, but declines were steepest for infected bees, intermediate for controls and infected-smell bees, and lowest for uninfected-smell bees (Figure 6.5).

By period 5, infected bees had the lowest survival probability (0.58, 95% CI: 0.42-0.78), with confidence intervals nearly distinct compared with uninfected-smell, which showed the highest survival probability (0.89, 95% CI: 0.78-1). Controls (0.70, 95% CI: 0.57-0.87) and infected-smell (0.70, 95% CI: 0.56-0.87) had similar survival probabilities to one another and their confidence intervals overlapped with each other and with uninfected-smell and infected bees (Figure 6.5).

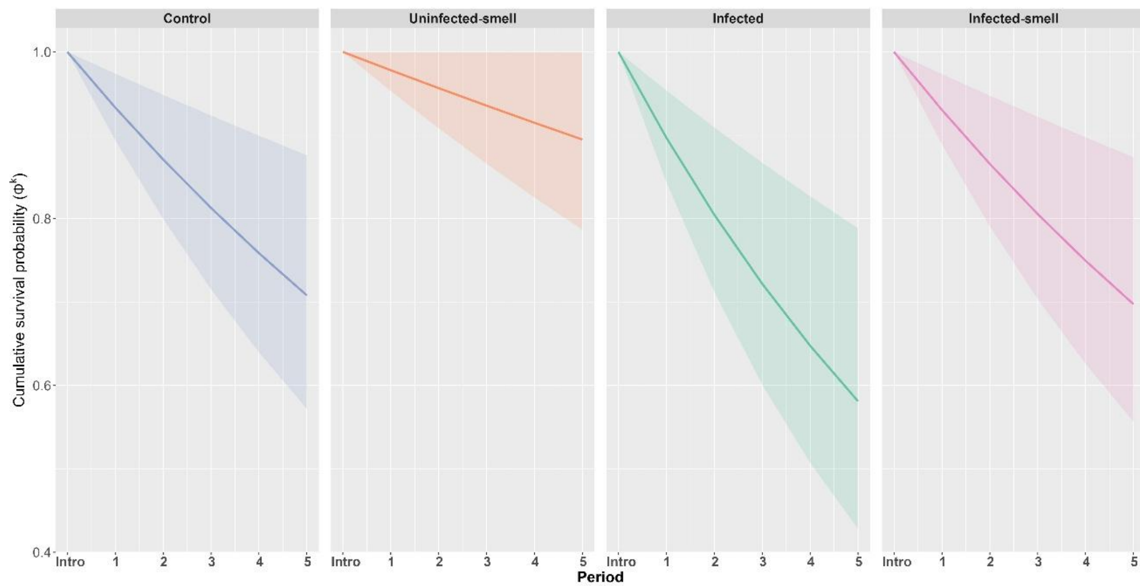

Figure 6.5: Cumulative apparent survival probabilities (Φ^k) of focal bees across observation periods for each treatment, estimated from Cormack-Jolly-Seber models with treatment as the response variable. Lines show mean survival probabilities and shaded ribbons indicate 95% confidence intervals.

6.5 Discussion

Recognising and responding to infected nestmates is a cornerstone of social immunity, yet the cues and behaviours involved remain poorly resolved for adult honey bees. Using a field-based observation hive experiment, we found that infection markedly altered the social interactions a focal bee received when compared with healthy bees. This shift was driven by heightened aggression and rejection of trophallaxis, coupled with increased inspection through antennation and elevated grooming. Uninfected bees perfumed with the scent of infection elicited nearly identical responses to truly infected bees, showing that scent cues alone are sufficient to trigger these defences. The timing of these behaviours suggests a layered strategy: aggression was concentrated at first encounter, whereas avoidance persisted when infected or infected-smell bees remained in the colony. When behaviours were considered at the categorical level, infected and infected-smell bees were nearly indistinguishable, yet both differed consistently from controls and uninfected-smell bees, underscoring the central role of olfaction in adult-focused social immunity.

Does infection alter social interaction?

Vairimorpha infection markedly altered how infected bees were treated by their nestmates. Infected individuals received more aggression and were avoided more often than healthy bees, indicating reduced social acceptance. Multivariate analyses confirmed that infected bees had distinct behavioural profiles compared with control and uninfected-smell bees, characterised by greater aggression, antennation, extended grooming, and rejection of trophallaxis. These results are consistent with infection-containment strategies described in other honey bee diseases, where infected nestmates are met with heightened aggression (Baracchi et al., 2012) and reduced trophallaxis (Geffre et al., 2020). They also align with evidence for increased aggression toward *Vairimorpha*-infected bees in cage experiments (Biganski et al., 2018), as well as broader examples of social immunity in ants, termites, and even vertebrates, where diseased conspecifics are avoided or removed (Zylberberg et al., 2013; Pereira and Detrain, 2020; Esparza-Mora et al., 2023).

One particularly notable feature of infected bees was their frequent association with extended grooming – prolonged episodes where host bees groomed a focal bee for ≥60 seconds, far longer than the maximum duration of grooming bouts of 45 seconds reported in honey bees (Božič and Valentinčič, 1995). Increased allogrooming frequency in response to ectoparasites, such as Varroa and tracheal mites, is well documented (Pettis and Pankiw, 1998; Pritchard, 2016). In contrast, we found that a smaller proportion of infected bees received grooming overall, and standard allogrooming was less associated with infection. This pattern suggests that when grooming was directed toward infected individuals, it was more often expressed as extended allogrooming and extended group allogrooming (≥ 2 host bees), both of which were strongly associated with infection. Because *Vairimorpha* is an endoparasite, these behaviours are unlikely to reduce infection intensity directly, like they would for an ectoparasite. Instead, they may parallel the intensive allogrooming observed in ants, where prolonged grooming in response to fungal pathogens induces low level infections that lead to upregulation of immune gene expression and inhibition of fungal growth (Konrad et al., 2012). Given that *Vairimorpha* is commonly found in the faeces (Bailey, 1955; Copley et al., 2012), low levels of spores could plausibly be present on the cuticle which could provide the low-level infection needed for immune priming (Konrad et al., 2012). Indeed, allogrooming is more frequent toward immune-stimulated bees (Richard et al., 2008), suggesting that bees do respond to an internal threat with allogrooming. However, from our results we cannot determine whether extended grooming reduces *Vairimorpha* transmission or inadvertently contributes to its spread.

In addition to grooming, antennation was a significant driver of treatment separation in our multivariate analyses. Both standard and aggressive antennation were associated with infected bees when overall frequencies were considered. We defined aggressive antennation as rapid antennation across the entire body directed at toward focal bees by the host colony, while being circled by the host bee, which is a common agonistic response toward non-nestmate intruders in social insects (Balas and Adams, 1996; O’Fallon et al., 2016). While aggressive antennation has not been previously reported in response to infection, Richard et al. (2008) found that antennation increased toward immune-stimulated bees. Antennation is a common evaluation behaviour in social insects (Erber and Pribbenow, 2000) and was the

most frequent interaction observed in our study. In our proportional analysis, a significantly higher percentage of controls were antennated than any other treatment, suggesting that although infected and infected-smell bees were less likely to be antennated, those that were received interactions of higher intensity. This pattern indicates that antennation may serve as a diagnostic inspection behaviour: most bees are antennated at least once, but infected bees are subject to more frequent inspections, consistent with its proposed role in triggering downstream social immune responses.

Responses to infected individuals were more variable compared to healthy bees. PCA ordinations revealed much broader confidence intervals for infected bees, suggesting host colonies differed in whether they responded to infection with exclusion, tolerance, or integration. This behavioural heterogeneity reflects the flexible nature of social immunity and is consistent with laboratory studies reporting context-dependent responses to *Vairimorpha* infection, where infection load was associated with reduced contacts from uninfected nestmates (Biganski et al., 2018). As our method used batch feeding, not all bees would have developed uniform infections. Unfortunately, we were not able to determine infection intensity post-experiment to confirm whether behavioural variation scaled with spore load.

In addition to receiving altered behavioural responses, infected bees also had the lowest cumulative survival rates over the course of the experiment, though variation was high. These reductions in survival reflect the number of bees remaining in the colony by the end of the experiment, either removed or which self-removed (Rueppell et al., 2010). Together, these results suggest that *Vairimorpha* infection can provoke social immune responses that influence the fate of infected adults within the hive.

Does the smell of infection alter social interaction?

In our study, smell alone was sufficient to elicit the social immune responses typically reserved for physiologically infected individuals. Bees perfumed with the scent of infected nestmates, but who were themselves uninfected, experienced aggression and avoidance behaviours comparable to those directed at genuinely infected bees.

Their behavioural profiles clustered tightly with infected individuals in multivariate analyses, associated with avoidance, antennation and aggression. Although these profiles differed significantly from those of infected bees, the deviance was far smaller than that from controls or uninfected-smell bees. This strongly suggests that olfactory cues linked to infection are not merely correlated with, but causal in triggering, behavioural exclusion.

Our findings support previous work linking *Vairimorpha* infection to altered semiochemical profiles (Dussaubat et al., 2010; McDonnell et al., 2013; Murray et al., 2015; Chapter 4) and extend these results by showing that chemical signatures of infection are sufficient to induce a social immune response. They are also consistent with Richard et al. (2008), who showed that coating bees with cuticular extracts from immune-stimulated bees could provoke aggression. By using a scent-transfer method, we isolated olfaction as the key mechanism underlying behavioural responses of host bees toward our treated focal bees, confirming its role in mediating discrimination. The association of antennation with infected-smell bees in a similar manner to infected further suggests that host workers increased evaluation in response to altered chemical cues. The fact that infected-smell bees differed significantly from uninfected-smell bees demonstrated that our responses were not driven by the perfuming process itself, but by the specific infection-related scents transferred from infected donors.

To interpret these findings in a broader context, it is helpful to draw on the theoretical models of recognition systems. Following Sherman et al. (1997), the cue was the smell of infected bees, the template was likely shaped by colony-level experiences of infection or evolved social immunity towards infection, and the response was exclusive behaviours like aggression or avoidance. Our findings are consistent with "class-level recognition" (Tibbetts and Dale, 2007; Gherardi et al., 2012) wherein individuals are sorted into behaviourally meaningful categories (e.g. infected vs. non-infected) based on phenotype matching against an internal template.

It remains unclear whether the cues responsible for triggering aggression and avoidance were primarily volatile compounds or CHCs. Bees were typically antennated prior to any behavioural escalation, suggesting evaluation based on short-range compounds such as CHCs. However, volatiles may facilitate initial

detection or orientation. The two modalities may operate synergistically, with volatiles attracting guards or nurses to the infected bee, and CHCs acting as close-range, high-resolution cues (McAfee et al., 2018; Wagoner et al., 2019). Since our method transferred both volatile and contact-based compounds, the respective roles of each remain unresolved.

Does time in the colony alter social interaction?

The way focal bees were treated by their nestmates changed over time, but these changes were not uniform across treatments. Aggression was front-loaded: infected and infected-smell bees received the highest levels of aggression immediately after introduction, with rates declining significantly by the next observation period and remaining low. This rapid drop suggests that host colonies quickly assessed these individuals – a process reflected in the high frequency of evaluative behaviours such as antennation, which also peaked during the introduction period and has been reported previously in response to immune stimulation (Richard et al., 2008). Once a bee's status was established, aggression rarely persisted. A similar temporal shift was described by Biganski et al. (2018), where aggression toward *Vairimorpha*-infected bees was high at first encounter, decreasing thereafter but surviving bees subsequently received more trophallaxis. In contrast, we found that trophallaxis by the host colony toward focal bees was more frequent for controls than infected bees, particularly at introduction, and decreased thereafter.

Previous work has shown that *V. ceranae*-infected workers are less inclined to share food with naïve nestmates (Naug and Gibbs, 2009) placing them at the periphery of the social network and potentially reducing transmission risk (Naug and Gibbs, 2009; Stroeymeyt et al., 2014). In our study, outgoing trophallaxis by focal bees was rare across all treatments (Table S6.2), perhaps because isolation in perfuming tubes prior to introduction left them with less crop content (Naug, 2008; Naug and Gibbs, 2009). Instead, we found infected and infected-smell bees that begged for food were more likely to have their trophallaxis attempts rejected than controls or uninfected-smell bees, and this difference persisted across all observation periods. While aggression declined rapidly after introduction, trophallaxis avoidance remained consistently high in infected and infected-smell bees throughout the experiment. A

similar though weaker pattern was seen in patrolling, with more infected and infected-smell bees patrolling than healthy bees suggesting they were less engaged in social tasks. Together with the sustained rejection of trophallaxis, even in the absence of overt aggression, suggests a layered recognition system: an acute response characterised by aggression, followed by a more chronic containment strategy. Such dynamics are consistent with social immunity mechanisms that limit contact with potentially infectious individuals while avoiding the costs of prolonged conflict (Cremer et al., 2007).

Comparisons across taxa highlight the complexity of trophallaxis responses. In ants, trophallaxis can increase following bacterial infection as a form of social immunisation, where antimicrobial compounds are shared during feeding (Hamilton et al., 2011). In honey bees, *Vairimorpha* infection has been linked to increased trophallaxis (Lecocq et al., 2016). However, we found evidence that infected bees trophallaxis attempts were rejected more when compared with controls, aligning with reductions in food sharing seen in response to viral infection (Geffre et al., 2020). Parasite manipulation of the host has been reported in other bee parasites (Geffre et al., 2020) and Lecocq et al. (2016) theorised that increased trophallaxis in *Vairimorpha*-infected bees may reflect manipulation to enhance transmission. Given that *Vairimorpha* is orally transmitted (Smith, 2012), avoidance of trophallaxis may be an adaptive response to reduce transmission risk that varies between host colonies and parasite strains. *Vairimorpha* infections are known to be highly diverse (Sagastume et al., 2016), and such strain-specific adaptations could explain the variation in social immune responses observed across behavioural studies on *Vairimorpha* infection (McDonnell et al., 2013; Lecocq et al., 2016; Murray et al., 2015; Biganski et al., 2018; Chapter 5), with host and parasite engaged in an evolutionary arms race that sometimes favours host defence and sometimes parasite manipulation.

Together, these temporal dynamics highlight the layered nature of social immune responses. The fact that both initial aggression and sustained avoidance was observed in infected-smell bees at the same level as truly infected bees throughout the experiment supports the conclusion that olfactory cues alone are sufficient to trigger both immediate and prolonged social defences. Colonies appear to respond quickly to abnormal or suspicious odours with aggression but often maintain longer-

term containment *via* social avoidance. This decoupling of aggression and avoidance points to a nuanced behavioural repertoire – one that allows colonies to calibrate their social defences over time in response to perceived infection risk. However, we focused on short-term behavioural outcomes across a single day of observation. It remains unclear whether bees that were tolerated in early periods were later ejected or fully reintegrated, and whether colony-level outcomes (e.g. pathogen transmission) are impacted by these individual-level interactions. Longitudinal tracking of both social behaviour and infection status over longer periods of time would address these questions.

Implications for social immunity and conclusions

Our findings illustrate a general principle of social immunity: that animals living in groups evolve collective strategies to recognise and contain infectious individuals. In this case, chemical cues associated with infection were sufficient to trigger both acute aggression and prolonged avoidance in honey bees, but the underlying logic is far broader. Across taxa, social immunity rests on the same foundation – detecting infection risk in conspecifics and modifying social interactions in ways that alter transmission dynamics.

If odour cues alone can elicit exclusion, then social immunity does not rely on overt symptoms or confirmed infectiousness but instead on sensory proxies of disease. This anticipatory quality has major consequences for transmission dynamics: groups acting on cues may prevent epidemics more effectively, even at the cost of false positives, because the fitness cost of infection is typically greater than that of unnecessary avoidance (Kouznetsova et al., 2012; Johnson et al., 2013). Such odour-driven aversion has been widely demonstrated, from mice (Boillat et al., 2015), to amphibians (Kiesecker et al., 1999) and humans (Olsson et al., 2014).

In obligate eusocial insects, these dynamics are sharpened by colony-level selection. Here, signalling infection, even at a cost to the individual, can be truly altruistic, because removing oneself from the group benefits the reproductive unit, or superorganism, as a whole (Cremer et al., 2018). Odour-based recognition in this context parallels the “find me/eat me” signals produced by infected cells in complex

multicellular organisms (Cremer and Sixt, 2009; Ravichandran, 2010; Cremer et al., 2018). Our results suggest that when infection-related odours are transferred onto uninfected individuals, they too become targets for social immune responses. These local responses (workers reacting to odour cues in one-on-one encounters) can scale up to reshape colony-wide interaction networks, just as predicted by the organisational immunity hypothesis (Stroeymeyt et al., 2014). Because similar cue-based responses occur across animal societies, from termites to vertebrates, our findings support the idea that olfactory cues are a critical mechanism shaping host-pathogen dynamics and the eco-evolutionary stability of social living.

If scent-based recognition is foundational to social immunity, then environmental stressors that disrupt olfactory perception or cue production pose a significant risk to social insect health. Stressors such as pesticides and temperature have been shown to impair insect sensory function (Williamson and Wright, 2013; Li et al., 2015; Nooten et al., 2024; Barroso et al., 2025). Such disruptions could degrade the recognition systems, increasing the likelihood that infected individuals go undetected and remain socially integrated, thereby facilitating pathogen transmission. Our results highlight the importance of considering olfaction as a sublethal effect endpoint in environmental risk assessments. Protecting the chemical communication systems that underpin social immunity may be as important as managing direct pathogen exposure in mitigating the threat of disease outbreaks in social insect populations.

6.6 References

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6.7 Supplementary material

Table S6.1: Description of tracked honey bee behaviours. Table shows behavioural category, social function, as well as the direction of interaction and which bee is performing the behaviour, alongside the metric each behaviour was recorded in either count (total frequency of behaviour during observation period) or binary (a bee performed the behaviour at any point during the observation period).

Behaviour	Behavioural category	Social function	Actor (bee performing behaviour)	Target (bee receiving behaviour)	Description	Metric
Biting	Aggression	Exclusion	Host	Focal	Bee bites any part of focal bee	Count
Charging	Aggression	Exclusion	Host	Focal	Bee runs towards focal bee at speed, often knocking into or headbutting focal bee	Count
Chasing	Aggression	Exclusion	Host	Focal	Bee pursues focal bee at speed, focal bee attempts to flee	Count
Dragging	Aggression	Exclusion	Host	Focal	Bee bites and holds onto focal bee and successfully drags or pulls focal bee	Count
Extended dragging	Aggression	Exclusion	Host	Focal	Bee bites and holds onto focal bee and drags or pulls focal bee around hive for >3 s	Binary
Grabbing	Aggression	Exclusion	Host	Focal	Bee(s) holds onto focal bee with forelegs	Count
Stinging	Aggression	Exclusion	Host	Focal	Bee uses sting or attempts to use sting	Count

Aggressive antennation	Aggression	Evaluation	Host	Focal	Bee rapidly antennates entire body while circling the focal bee	Count
Antennation	Antennation	Evaluation	Host	Focal	Bee antennates focal bee (< 2s)	Count
Extended antennation	Antennation	Evaluation	Host	Focal	Bee antennates focal bee (> 2s)	Count
Extended group antennation	Antennation	Evaluation	Host	Focal	>2 bees antennate focal bee (> 2s)	Count
Group antennation	Antennation	Evaluation	Host	Focal	>2 bees antennate focal bee (< 2s)	Count
Trophallaxis rejected (focal)	Avoidance	Exclusion	Focal	Host	Host bee opens mouthparts to present sugar droplet to focal bee and focal bee moves away/does not drink	Count
Erratic patrolling	Avoidance	Exclusion	Focal	N/A	Bee running around colony erratically with no apparent purpose, not pausing to inspect surfaces	Binary
Trophallaxis rejected (in)	Avoidance	Exclusion	Host	Focal	Focal bee extends tongue at host bees mouthparts (begging), but host bee rejects trophallaxis attempt by moving away and not offering sugar droplet	Count
Trophallaxis rejected (out)	Avoidance	Exclusion	Host	Focal	Focal bee opens mouthparts to present sugar droplet to host bee and host bee rejects trophallaxis by moving away and not drinking	Count
Honey cell feeding/deposition	Feeding	Neutral	Focal	N/A	Bee extending tongue and drinking/depositing in a full honey cell	Binary
Fanning	General	Neutral	Focal	N/A	Fanning wings	Binary

Patrolling	General	Neutral	Focal	N/A	Walking around the colony without participating in clear task	Binary
Extended grooming	Grooming	Evaluation	Host	Focal	Bee holding down and grooming focal bee ≥ 1 min	Count
Group extended grooming	Grooming	Evaluation	Host	Focal	>2 host bees holding down and grooming focal bee for ≥ 1 min	Count
Allogrooming in	Grooming	Evaluation	Host	Focal	Bee using mandibles/tongue to groom focal bee	Count
Allogrooming out	Grooming	Neutral	Focal	Host	Bee using mandibles/tongue to groom host bee	Count
Autogrooming	Grooming	Neutral	Focal	N/A	Grooming any part of self with mandibles or legs	Binary
Brood cell inspection	Maintenance	Neutral	Focal	N/A	Bee placing head inside brood cell for < 1 min	Binary
Brood cell maintenance	Maintenance	Neutral	Focal	N/A	Bee placing head inside brood cell for > 1 min	Binary
Hive maintenance	Maintenance	Neutral	Focal	N/A	Bee removing debris from nest (dead brood, dead adults, old cappings etc.)	Binary
Honey cell inspection	Maintenance	Neutral	Focal	N/A	Placing head inside honey cell for < 1 min	Binary
Honey cell maintenance	Maintenance	Neutral	Focal	N/A	Focal bee placing head inside honey cell for > 1 min	Binary

Pollen cell maintenance	Maintenance	Neutral	Focal	N/A	Focal bee placing head inside pollen cell for > 1 min	Binary
Foraging/flight	Other	Neutral	Focal	N/A	Focal bee leaves hive through entrance and is seen performing orientation flight	Binary
Tremble dance (in)	Other	Neutral	Host	Focal	Holding onto focal bee and trembling/shaking	Count
Tremble dance (out)	Other	Neutral	Focal	Host	Holding onto host bee and trembling/shaking	Count
Trophallaxis accepted (in)	Social feeding	Integration	Host	Focal	Opens mouthparts and pushes tongue forward to regurgitate sugar droplet which is consumed by focal bee	Count
Trophallaxis accepted (out)	Social feeding	Integration	Focal	Host	Opens mouthparts and pushes tongue forward to regurgitate sugar droplet which is consumed by host bee	Count

Table S6.2: Total percentage of observed bees that experienced each behaviour from each treatment. Rows highlighted in red were excluded from analysis as they were rarely observed (≤15%) in all treatments.

Treatment	Behaviour	Category	Percentage of bees experiencing
Control	Aggressive antennation	Aggression	13
Uninfected smell	Aggressive antennation	Aggression	6
Infected	Aggressive antennation	Aggression	25
Infected smell	Aggressive antennation	Aggression	20
Control	Allogrooming (in)	Grooming	35
Uninfected smell	Allogrooming (in)	Grooming	15
Infected	Allogrooming (in)	Grooming	10
Infected smell	Allogrooming (in)	Grooming	19
Control	Allogrooming (out)	Grooming	5
Uninfected smell	Allogrooming (out)	Grooming	3
Infected	Allogrooming (out)	Grooming	10
Infected smell	Allogrooming (out)	Grooming	1
Control	Antennation	Antennation	81
Uninfected smell	Antennation	Antennation	50
Infected	Antennation	Antennation	87
Infected smell	Antennation	Antennation	70
Control	Extended antennation	Antennation	62
Uninfected smell	Extended antennation	Antennation	31
Infected	Extended antennation	Antennation	35
Infected smell	Extended antennation	Antennation	38

Treatment	Behaviour	Category	Percentage of bees experiencing
Control	Autogrooming	Grooming	67
Uninfected smell	Autogrooming	Grooming	49
Infected	Autogrooming	Grooming	55
Infected smell	Autogrooming	Grooming	58
Control	Biting	Aggression	13
Uninfected smell	Biting	Aggression	6
Infected	Biting	Aggression	42
Infected smell	Biting	Aggression	35
Control	Brood cell inspection	Maintenance	45
Uninfected smell	Brood cell inspection	Maintenance	34
Infected	Brood cell inspection	Maintenance	25
Infected smell	Brood cell inspection	Maintenance	38
Control	Brood cell maintenance	Maintenance	45
Uninfected smell	Brood cell maintenance	Maintenance	61
Infected	Brood cell maintenance	Maintenance	52
Infected smell	Brood cell maintenance	Maintenance	50
Control	Charging	Aggression	3
Uninfected smell	Charging	Aggression	6
Infected	Charging	Aggression	55
Infected smell	Charging	Aggression	51
Control	Chasing	Aggression	18
Uninfected smell	Chasing	Aggression	4
Infected	Chasing	Aggression	35

Treatment	Behaviour	Category	Percentage of bees experiencing
Infected smell	Chasing	Aggression	43
Control	Dragging	Aggression	7
Uninfected smell	Dragging	Aggression	0
Infected	Dragging	Aggression	7
Infected smell	Dragging	Aggression	12
Control	Extended dragging	Aggression	0
Uninfected smell	Extended dragging	Aggression	0
Infected	Extended dragging	Aggression	22
Infected smell	Extended dragging	Aggression	11
Control	Erratic patrolling	Rejection	5
Uninfected smell	Erratic patrolling	Rejection	1
Infected	Erratic patrolling	Rejection	10
Infected smell	Erratic patrolling	Rejection	6
Control	Fanning	General	13
Uninfected smell	Fanning	General	7
Infected	Fanning	General	10
Infected smell	Fanning	General	6
Control	Foraging flight	Other	0
Uninfected smell	Foraging flight	Other	0
Infected	Foraging flight	Other	2
Infected smell	Foraging flight	Other	0
Control	Grabbing	Aggression	13
Uninfected smell	Grabbing	Aggression	5

Treatment	Behaviour	Category	Percentage of bees experiencing
Infected	Grabbing	Aggression	40
Infected smell	Grabbing	Aggression	45
Control	Grooming extended	Grooming	11
Uninfected smell	Grooming extended	Grooming	6
Infected	Grooming extended	Grooming	10
Infected smell	Grooming extended	Grooming	38
Control	Group antennation	Antennation	22
Uninfected smell	Group antennation	Antennation	8
Infected	Group antennation	Antennation	25
Infected smell	Group antennation	Antennation	22
Control	Extended group antennation	Antennation	20
Uninfected smell	Extended group antennation	Antennation	5
Infected	Extended group antennation	Antennation	5
Infected smell	Extended group antennation	Antennation	9
Control	Extended group grooming	Grooming	5
Uninfected smell	Extended group grooming	Grooming	0
Infected	Extended group grooming	Grooming	0
Infected smell	Extended group grooming	Grooming	17
Control	Hive maintenance	Maintenance	0
Uninfected smell	Hive maintenance	Maintenance	1

Treatment	Behaviour	Category	Percentage of bees experiencing
Infected	Hive maintenance	Maintenance	0
Infected smell	Hive maintenance	Maintenance	0
Control	Honey cell feeding	Feeding	9
Uninfected smell	Honey cell feeding	Feeding	3
Infected	Honey cell feeding	Feeding	2
Infected smell	Honey cell feeding	Feeding	4
Control	Honey cell inspection	Maintenance	22
Uninfected smell	Honey cell inspection	Maintenance	5
Infected	Honey cell inspection	Maintenance	5
Infected smell	Honey cell inspection	Maintenance	12
Control	Honey cell maintenance	Maintenance	1
Uninfected smell	Honey cell maintenance	Maintenance	1
Infected	Honey cell maintenance	Maintenance	7
Infected smell	Honey cell maintenance	Maintenance	14
Control	Patrolling	General	37
Uninfected smell	Patrolling	General	12
Infected	Patrolling	General	27
Infected smell	Patrolling	General	25
Control	Pollen cell maintenance	Maintenance	0
Uninfected smell	Pollen cell maintenance	Maintenance	0
Infected	Pollen cell maintenance	Maintenance	2
Infected smell	Pollen cell maintenance	Maintenance	0
Control	Tremble dance (in)	Other	1

Treatment	Behaviour	Category	Percentage of bees experiencing
Uninfected smell	Tremble dance (in)	Other	3
Infected	Tremble dance (in)	Other	0
Infected smell	Tremble dance (in)	Other	1
Control	Tremble dance (out)	Other	15
Uninfected smell	Tremble dance (out)	Other	1
Infected	Tremble dance (out)	Other	7
Infected smell	Tremble dance (out)	Other	9
Control	Stinging	Aggression	0
Uninfected smell	Stinging	Aggression	0
Infected	Stinging	Aggression	0
Infected smell	Stinging	Aggression	1
Control	Trophallaxis accepted (in)	Social feeding	64
Uninfected smell	Trophallaxis accepted (in)	Social feeding	34
Infected	Trophallaxis accepted (in)	Social feeding	42
Infected smell	Trophallaxis accepted (in)	Social feeding	46
Control	Trophallaxis rejected (in)	Rejection	18
Uninfected smell	Trophallaxis rejected (in)	Rejection	6
Infected	Trophallaxis rejected (in)	Rejection	40
Infected smell	Trophallaxis rejected (in)	Rejection	33
Control	Trophallaxis rejected (in, focal)	Other	9
Uninfected smell	Trophallaxis rejected (in, focal)	Other	6

Treatment	Behaviour	Category	Percentage of bees experiencing
Infected	Trophallaxis rejected (in, focal)	Other	5
Infected smell	Trophallaxis rejected (in, focal)	Other	0
Control	Trophallaxis accepted (out)	Social feeding	11
Uninfected smell	Trophallaxis accepted (out)	Social feeding	12
Infected	Trophallaxis accepted (out)	Social feeding	15
Infected smell	Trophallaxis accepted (out)	Social feeding	6
Control	Trophallaxis rejected (out)	Rejection	0
Uninfected smell	Trophallaxis rejected (out)	Rejection	2
Infected	Trophallaxis rejected (out)	Rejection	10
Infected smell	Trophallaxis rejected (out)	Rejection	1

Table S6.3: Univariate manyglm analysis of focal bee behavioural profiles, based on the frequencies and presence/absence of behaviours received or performed across all observation periods. For each behaviour, the test statistic (Dev) represents the change in deviance associated with treatment. Significance was assessed using PIT-trap resampling with 999 iterations, and p-values were adjusted for multiple testing.

Behaviour	Dev	P value
Antennation	22.345	0.001
Grooming extended	38.274	0.001
Group grooming extended	26.542	0.001
Biting	30.814	0.001
Dragging extended	29.799	0.001
Chasing	38.562	0.001
Charging	76.933	0.001
Grabbing	35.739	0.001
Trophallaxis in rejected	17.501	0.003
Aggressive antennation	14.173	0.019
Antennation extended	10.941	0.070
Patrolling	9.934	0.102
Honey cell inspection	9.685	0.107
Allogrooming in	9.008	0.134
Trophallaxis in accepted	7.166	0.247
Group antennation	6.153	0.253
Group antennation extended	6.886	0.253
Brood cell inspection	2.821	0.538
Autogrooming	2.012	0.645
Brood cell maintenance	1.535	0.652

Table S6.4: Post-hoc Tukey contrasts from models with a significant interaction between treatment and period comparing the proportion of focal bees experiencing each behavioural category within each observation period. Significant *p* values are in bold. N.D. indicates non-estimable standard errors due to absence or near-absence of counts in one or both groups.

Contrast	Period	Category	Estimate	S.E.	Z-ratio	P value
Control - uninfected smell	Intro	Aggression	0.292	0.511	0.571	0.941
Control - infected	Intro	Aggression	-2.538	0.564	-4.504	**<0.001**
Control - infected smell	Intro	Aggression	-4.694	1.076	-4.361	**<0.001**
Uninfected smell - infected	Intro	Aggression	-2.830	0.587	-4.825	**<0.001**
Uninfected smell - infected smell	Intro	Aggression	-4.986	1.088	-4.581	**<0.001**
Infected - infected smell	Intro	Aggression	-2.155	1.102	-1.955	0.205
Control - uninfected smell	1	Aggression	17.439	N.D.	0.010	1.000
Control - infected	1	Aggression	-0.212	0.833	-0.255	0.994
Control - infected smell	1	Aggression	-0.143	0.729	-0.196	0.997
Uninfected smell - infected	1	Aggression	-17.651	N.D.	-0.010	1.000
Uninfected smell - infected smell	1	Aggression	-17.583	N.D.	-0.010	1.000
Infected - infected smell	1	Aggression	0.069	N.D.	0.086	1.000
Control - uninfected smell	2	Aggression	-16.759	N.D.	-0.009	1.000
Control - infected	2	Aggression	-17.925	N.D.	-0.009	1.000
Control - infected smell	2	Aggression	-16.827	N.D.	-0.009	1.000
Uninfected smell - infected	2	Aggression	-1.166	0.972	-1.201	0.627
Uninfected smell - infected smell	2	Aggression	-0.068	N.D.	-0.065	1.000
Infected - infected smell	2	Aggression	1.099	0.974	1.128	0.672
Control - uninfected smell	3	Aggression	-0.541	1.257	-0.431	0.973

Contrast	Period	Category	Estimate	S.E.	Z-ratio	P value
Control - infected	3	Aggression	-2.115	1.174	-1.801	0.273
Control - infected smell	3	Aggression	16.176	N.D.	0.009	1.000
Uninfected smell - infected	3	Aggression	-1.574	0.932	-1.688	0.330
Uninfected smell - infected smell	3	Aggression	16.718	N.D.	0.010	1.000
Infected - infected smell	3	Aggression	18.292	N.D.	0.010	1.000
Control - uninfected smell	4	Aggression	0.762	0.957	0.796	0.856
Control - infected	4	Aggression	-0.606	1.005	-0.603	0.931
Control - infected smell	4	Aggression	0.454	0.964	0.470	0.966
Uninfected smell - infected	4	Aggression	-1.368	1.077	-1.270	0.582
Uninfected smell - infected smell	4	Aggression	-0.308	1.04	-0.296	0.991
Infected - infected smell	4	Aggression	1.060	1.084	0.978	0.762
Control - uninfected smell	5	Aggression	16.868	N.D.	0.010	1.000
Control - infected	5	Aggression	-0.807	1.068	-0.756	0.874
Control - infected smell	5	Aggression	16.917	N.D.	0.008	1.000
Uninfected smell - infected	5	Aggression	-17.675	N.D.	-0.011	1.000
Uninfected smell - infected smell	5	Aggression	0.050	N.D.	0.000	1.000
Infected - infected smell	5	Aggression	17.724	N.D.	0.009	1.000
Control - uninfected smell	Intro	Maintenance	1.492	0.829	1.801	0.273
Control - infected	Intro	Maintenance	1.966	1.091	1.803	0.272
Control - infected smell	Intro	Maintenance	2.299	1.088	2.113	0.149
Uninfected smell - infected	Intro	Maintenance	0.474	1.25	0.379	0.981
Uninfected smell - infected smell	Intro	Maintenance	0.807	1.248	0.647	0.917
Infected - infected smell	Intro	Maintenance	0.333	1.435	0.232	0.996

Contrast	Period	Category	Estimate	S.E.	Z-ratio	P value
Control - uninfected smell	1	Maintenance	-0.014	N.D.	-0.029	1.000
Control - infected	1	Maintenance	0.432	0.568	0.760	0.872
Control - infected smell	1	Maintenance	0.773	0.504	1.535	0.416
Uninfected smell - infected	1	Maintenance	0.446	0.559	0.796	0.856
Uninfected smell - infected smell	1	Maintenance	0.787	0.495	1.591	0.384
Infected - infected smell	1	Maintenance	0.341	0.578	0.591	0.935
Control - uninfected smell	2	Maintenance	-1.250	0.523	-2.391	0.079
Control - infected	2	Maintenance	-0.474	0.592	-0.800	0.854
Control - infected smell	2	Maintenance	-0.444	0.511	-0.869	0.821
Uninfected smell - infected	2	Maintenance	0.776	0.58	1.338	0.538
Uninfected smell - infected smell	2	Maintenance	0.806	0.497	1.620	0.367
Infected - infected smell	2	Maintenance	0.030	N.D.	0.052	1.000
Control - uninfected smell	3	Maintenance	-1.015	0.511	-1.988	0.192
Control - infected	3	Maintenance	0.408	0.608	0.671	0.908
Control - infected smell	3	Maintenance	-0.593	0.507	-1.170	0.646
Uninfected smell - infected	3	Maintenance	1.423	0.596	2.387	0.079
Uninfected smell - infected smell	3	Maintenance	0.422	0.493	0.856	0.827
Infected - infected smell	3	Maintenance	-1.001	0.593	-1.688	0.330
Control - uninfected smell	4	Maintenance	0.284	0.493	0.575	0.940
Control - infected	4	Maintenance	-0.295	0.702	-0.420	0.975
Control - infected smell	4	Maintenance	-0.747	0.554	-1.350	0.531
Uninfected smell - infected	4	Maintenance	-0.579	0.673	-0.860	0.825
Uninfected smell - infected smell	4	Maintenance	-1.031	0.516	-1.999	0.188

Contrast	Period	Category	Estimate	S.E.	Z-ratio	P value
Infected - infected smell	4	Maintenance	-0.452	0.718	-0.630	0.923
Control - uninfected smell	5	Maintenance	-1.036	0.513	-2.019	0.181
Control - infected	5	Maintenance	-1.201	0.647	-1.858	0.247
Control - infected smell	5	Maintenance	-1.028	0.563	-1.826	0.261
Uninfected smell - infected	5	Maintenance	-0.165	0.61	-0.270	0.993
Uninfected smell - infected smell	5	Maintenance	0.009	N.D.	0.017	1.000
Infected - infected smell	5	Maintenance	0.174	0.652	0.266	0.993

Table S6.5: Post-hoc Tukey contrasts from models with a significant interaction between treatment and period, comparing the proportion of focal bees experiencing each behavioural category across observation periods within treatments. Significant *p* values are in bold. N.D. indicates non-estimable standard errors due to absence or near-absence of counts in one or both groups.

Category	Contrast	Treatment	Estimate	SE	Z-ratio	P value
Aggression	1 - intro	Control	-1.233	0.642	-1.921	0.389
Aggression	1 - intro	Uninfected smell	-18.380	N.D.	-0.011	1.000
Aggression	1 - intro	Infected	-3.559	0.775	-4.591	**<0.001**
Aggression	1 - intro	Infected smell	-5.783	1.133	-5.105	**<0.001**
Aggression	1 - 2	Control	17.587	N.D.	0.009	1.000
Aggression	1 - 2	Uninfected smell	-16.611	N.D.	-0.010	1.000
Aggression	1 - 2	Infected	-0.126	0.9	-0.141	1.000
Aggression	1 - 2	Infected smell	0.903	0.883	1.024	0.910
Aggression	1 - 3	Control	1.369	1.156	1.184	0.845
Aggression	1 - 3	Uninfected smell	-16.611	N.D.	-0.010	1.000
Aggression	1 - 3	Infected	-0.534	0.857	-0.623	0.989
Aggression	1 - 3	Infected smell	17.689	N.D.	0.010	1.000
Aggression	1 - 4	Control	0.157	0.821	0.191	1.000
Aggression	1 - 4	Uninfected smell	-16.521	N.D.	-0.010	1.000
Aggression	1 - 4	Infected	-0.237	1.014	-0.234	1.000
Aggression	1 - 4	Infected smell	0.754	0.887	0.850	0.958
Aggression	1 - 5	Control	0.628	0.917	0.685	0.984
Aggression	1 - 5	Uninfected smell	0.056	N.D.	0.000	1.000
Aggression	1 - 5	Infected	0.033	0.994	0.033	1.000

Category	Contrast	Treatment	Estimate	SE	Z-ratio	P value
Aggression	1 - 5	Infected smell	17.689	N.D.	0.009	1.000
Aggression	2 - intro	Control	-18.819	N.D.	-0.010	1.000
Aggression	2 - intro	Uninfected smell	-1.769	0.823	-2.148	0.263
Aggression	2 - intro	Infected	-3.432	0.779	-4.404	**<0.001**
Aggression	2 - intro	Infected smell	-6.687	1.258	-5.316	**<0.001**
Aggression	2 - 3	Control	-16.218	N.D.	-0.008	1.000
Aggression	2 - 3	Uninfected smell	0.000	1.035	0.000	1.000
Aggression	2 - 3	Infected	-0.408	0.862	-0.473	0.997
Aggression	2 - 3	Infected smell	16.785	N.D.	0.010	1.000
Aggression	2 - 4	Control	-17.430	N.D.	-0.009	1.000
Aggression	2 - 4	Uninfected smell	0.091	1.034	0.088	1.000
Aggression	2 - 4	Infected	-0.111	1.018	-0.109	1.000
Aggression	2 - 4	Infected smell	-0.150	1.043	-0.143	1.000
Aggression	2 - 5	Control	-16.959	N.D.	-0.009	1.000
Aggression	2 - 5	Uninfected smell	16.668	N.D.	0.010	1.000
Aggression	2 - 5	Infected	0.160	0.998	0.160	1.000
Aggression	2 - 5	Infected smell	16.785	N.D.	0.008	1.000
Aggression	3 - intro	Control	-2.602	1.079	-2.411	0.152
Aggression	3 - intro	Uninfected smell	-1.769	0.823	-2.148	0.263
Aggression	3 - intro	Infected	-3.025	0.729	-4.147	**<0.001**
Aggression	3 - intro	Infected smell	-23.472	N.D.	-0.013	1.000
Aggression	3 - 4	Control	-1.212	1.194	-1.015	0.913
Aggression	3 - 4	Uninfected smell	0.091	1.034	0.088	1.000

Category	Contrast	Treatment	Estimate	SE	Z-ratio	P value
Aggression	3 - 4	Infected	0.297	0.98	0.303	1.000
Aggression	3 - 4	Infected smell	-16.935	N.D.	-0.010	1.000
Aggression	3 - 5	Control	-0.741	1.262	-0.587	0.992
Aggression	3 - 5	Uninfected smell	16.668	N.D.	0.010	1.000
Aggression	3 - 5	Infected	0.567	0.96	0.591	0.992
Aggression	3 - 5	Infected smell	0.000	N.D.	0.000	1.000
Aggression	4 - intro	Control	-1.390	0.708	-1.961	0.365
Aggression	4 - intro	Uninfected smell	-1.859	0.822	-2.261	0.210
Aggression	4 - intro	Infected	-3.322	0.909	-3.653	**0.004**
Aggression	4 - intro	Infected smell	-6.537	1.262	-5.179	**<0.001**
Aggression	4 - 5	Control	0.471	0.965	0.488	0.997
Aggression	4 - 5	Uninfected smell	16.577	N.D.	0.010	1.000
Aggression	4 - 5	Infected	0.270	1.102	0.245	1.000
Aggression	4 - 5	Infected smell	16.935	N.D.	0.008	1.000
Aggression	5 - intro	Control	-1.861	0.818	-2.275	0.204
Aggression	5 - intro	Uninfected smell	-18.436	N.D.	-0.011	1.000
Aggression	5 - intro	Infected	-3.592	0.883	-4.069	**<0.001**
Aggression	5 - intro	Infected smell	-23.472	N.D.	-0.012	1.000
Maintenance	1 - intro	Control	1.382	0.528	2.619	0.093
Maintenance	1 - intro	Uninfected smell	2.888	0.802	3.601	**0.004**
Maintenance	1 - intro	Infected	2.917	1.111	2.626	0.091
Maintenance	1 - intro	Infected smell	2.908	1.077	2.701	0.075
Maintenance	1 - 2	Control	0.120	0.514	0.233	1.000

Category	Contrast	Treatment	Estimate	SE	Z-ratio	P value
Maintenance	1 - 2	Uninfected smell	-1.116	0.49	-2.276	0.204
Maintenance	1 - 2	Infected	-0.786	0.639	-1.229	0.823
Maintenance	1 - 2	Infected smell	-1.097	0.501	-2.191	0.242
Maintenance	1 - 3	Control	0.011	0.509	0.022	1.000
Maintenance	1 - 3	Uninfected smell	-0.990	0.484	-2.046	0.316
Maintenance	1 - 3	Infected	-0.013	0.658	-0.020	1.000
Maintenance	1 - 3	Infected smell	-1.355	0.502	-2.698	0.076
Maintenance	1 - 4	Control	-0.187	0.513	-0.365	0.999
Maintenance	1 - 4	Uninfected smell	0.111	0.462	0.240	1.000
Maintenance	1 - 4	Infected	-0.914	0.743	-1.229	0.823
Maintenance	1 - 4	Infected smell	-1.708	0.545	-3.131	**0.022**
Maintenance	1 - 5	Control	0.365	0.524	0.696	0.982
Maintenance	1 - 5	Uninfected smell	-0.658	0.47	-1.400	0.727
Maintenance	1 - 5	Infected	-1.268	0.682	-1.860	0.427
Maintenance	1 - 5	Infected smell	-1.436	0.543	-2.642	0.087
Maintenance	2 - intro	Control	1.262	0.548	2.304	0.192
Maintenance	2 - intro	Uninfected smell	4.005	0.814	4.922	**<0.001**
Maintenance	2 - intro	Infected	3.703	1.113	3.326	**0.011**
Maintenance	2 - intro	Infected smell	4.006	1.071	3.741	**0.003**
Maintenance	2 - 3	Control	-0.109	0.53	-0.206	1.000
Maintenance	2 - 3	Uninfected smell	0.126	0.502	0.251	1.000
Maintenance	2 - 3	Infected	0.773	0.663	1.166	0.853
Maintenance	2 - 3	Infected smell	-0.258	0.488	-0.529	0.995

Category	Contrast	Treatment	Estimate	SE	Z-ratio	P value
Maintenance	2 - 4	Control	-0.307	0.534	-0.575	0.993
Maintenance	2 - 4	Uninfected smell	1.227	0.48	2.553	0.109
Maintenance	2 - 4	Infected	-0.128	0.747	-0.172	1.000
Maintenance	2 - 4	Infected smell	-0.610	0.532	-1.147	0.861
Maintenance	2 - 5	Control	0.245	0.545	0.450	0.998
Maintenance	2 - 5	Uninfected smell	0.458	0.488	0.939	0.936
Maintenance	2 - 5	Infected	-0.483	0.685	-0.704	0.982
Maintenance	2 - 5	Infected smell	-0.339	0.53	-0.639	0.988
Maintenance	3 - intro	Control	1.371	0.543	2.525	0.117
Maintenance	3 - intro	Uninfected smell	3.879	0.81	4.791	**<0.001**
Maintenance	3 - intro	Infected	2.930	1.124	2.606	0.096
Maintenance	3 - intro	Infected smell	4.264	1.072	3.979	**<0.001**
Maintenance	3 - 4	Control	-0.198	0.528	-0.375	0.999
Maintenance	3 - 4	Uninfected smell	1.101	0.474	2.323	0.184
Maintenance	3 - 4	Infected	-0.901	0.764	-1.179	0.847
Maintenance	3 - 4	Infected smell	-0.352	0.533	-0.660	0.986
Maintenance	3 - 5	Control	0.354	0.54	0.656	0.987
Maintenance	3 - 5	Uninfected smell	0.333	0.482	0.691	0.983
Maintenance	3 - 5	Infected	-1.255	0.704	-1.783	0.477
Maintenance	3 - 5	Infected smell	-0.081	0.531	-0.152	1.000
Maintenance	4 - intro	Control	1.569	0.547	2.869	**0.047**
Maintenance	4 - intro	Uninfected smell	2.778	0.795	3.492	**0.006**
Maintenance	4 - intro	Infected	3.831	1.176	3.256	**0.014**

Category	Contrast	Treatment	Estimate	SE	Z-ratio	P value
Maintenance	4 - intro	Infected smell	4.616	1.092	4.225	**<0.001**
Maintenance	4 - 5	Control	0.552	0.544	1.015	0.913
Maintenance	4 - 5	Uninfected smell	-0.768	0.459	-1.674	0.549
Maintenance	4 - 5	Infected	-0.354	0.784	-0.452	0.998
Maintenance	4 - 5	Infected smell	0.272	0.572	0.475	0.997
Maintenance	5 - intro	Control	1.017	0.557	1.826	0.449
Maintenance	5 - intro	Uninfected smell	3.546	0.801	4.430	**<0.001**
Maintenance	5 - intro	Infected	4.185	1.138	3.677	**0.003**
Maintenance	5 - intro	Infected smell	4.344	1.091	3.980	**<0.001**

Table S6.6: Post hoc pairwise contrasts between treatments for behavioural categories that showed no significant interaction with observation period. Contrasts and estimates are reported from the main-effect treatment model. Significant *p*-values are in bold. Significant *p* values are in bold.

Contrast	Category	Estimate	SE	Z-ratio	P value
Control - uninfected smell	Patrolling	0.817	0.262	3.116	**0.010**
Control - infected	Patrolling	-0.913	0.250	-3.651	**0.001**
Control - infected smell	Patrolling	-1.180	0.221	-5.351	**<0.001**
Uninfected smell - infected	Patrolling	-1.729	0.272	-6.367	**<0.001**
Uninfected smell - infected smell	Patrolling	-1.997	0.245	-8.160	**<0.001**
Infected - infected smell	Patrolling	-0.268	0.230	-1.163	0.651
Control - uninfected smell	Social feeding	0.195	0.264	0.741	0.881
Control - infected	Social feeding	0.853	0.334	2.552	0.052
Control - infected smell	Social feeding	0.106	0.266	0.397	0.979
Uninfected smell - infected	Social feeding	0.658	0.332	1.980	0.196
Uninfected smell - infected smell	Social feeding	-0.090	0.263	-0.341	0.986
Infected - infected smell	Social feeding	-0.747	0.333	-2.242	0.112
Control - uninfected smell	Grooming	0.310	0.199	1.557	0.404
Control - infected	Grooming	0.937	0.240	3.907	**<0.001**
Control - infected smell	Grooming	0.103	0.206	0.500	0.959
Uninfected smell - infected	Grooming	0.627	0.230	2.721	**0.033**
Uninfected smell - infected smell	Grooming	-0.207	0.195	-1.066	0.710
Infected - infected smell	Grooming	-0.834	0.236	-3.536	**0.002**
Control - uninfected smell	Avoidance	0.817	0.262	3.116	**0.010**
Control - infected	Avoidance	-0.913	0.250	-3.651	**0.001**

Contrast	Category	Estimate	SE	Z-ratio	P value
Control - infected smell	Avoidance	-1.180	0.221	-5.351	**<0.001**
Uninfected smell - infected	Avoidance	-1.729	0.272	-6.367	**<0.001**
Uninfected smell - infected smell	Avoidance	-1.997	0.245	-8.160	**<0.001**
Infected - infected smell	Avoidance	-0.268	0.230	-1.163	0.651
Control - uninfected smell	Antennation	0.723	0.235	3.071	**0.011**
Control - infected	Antennation	0.937	0.280	3.343	**0.005**
Control - infected smell	Antennation	0.806	0.244	3.307	**0.005**
Uninfected smell - infected	Antennation	0.214	0.255	0.838	0.836
Uninfected smell - infected smell	Antennation	0.083	0.215	0.386	0.981
Infected - infected smell	Antennation	-0.131	0.262	-0.500	0.959

Chapter 7: General discussion

7.1 Overview

Infectious disease shapes the ecology and evolution of animals across the tree of life (Wilson et al., 2019). From solitary species to those with the most complex social systems, avoiding or responding to infection is a fundamental challenge. In social insects, this challenge is particularly acute: high relatedness, dense living conditions, and frequent contact promote rapid transmission of pathogens (Schmid-Hempel, 2021). Colonies counter these risks through social immunity, a suite of collective behavioural and chemical defences that reduce infection and its costs (Cremer et al., 2007). Because insect communication is dominated by chemical cues (Leonhardt et al., 2016), infection often leaves a detectable semiochemical signature that can mediate social immune responses. Honey bees provide a powerful model for investigating these processes, given their ecological importance, experimental tractability, and diverse parasite community (Hung et al., 2018; Fikadu, 2019; Lemanski et al., 2019; Pasho et al., 2021). Semiochemical signals of infection are well documented across social insects (Qiu et al., 2015; Esparza-Mora et al., 2023; Dawson et al., 2024), but there are gaps in our understanding about whether there is a consistent change in VOCs across different parasites and life stages.

This thesis examined whether infection produces a consistent and functionally significant 'smell of infection' in honey bees, and whether such semiochemical cues underpin social immune responses. In doing so, I addressed three core questions: (i) whether VOCs are associated with infection in adults as well as brood (Chapter 3, Chapter 4); (ii) when and how VOCs change during infection (Chapter 4, 5, 6) and (iii) whether olfaction mediates social immune responses between adults (Chapter 5, 6). Throughout, I discuss the broader implications of these findings for non-invasive disease surveillance in managed insect systems and social immunity in general. I addressed these core questions across four chapters.

Chapter 3 (*Volatile Organic Compounds as Indicators of Infection in Honey Bees (Apis mellifera): A meta-analysis*) investigated whether volatile organic compounds (VOCs) are consistently associated with honey bee infections across parasites and studies. A meta-analysis of 23 articles was conducted, and the presence/absence of

279 VOCs was used in a network analysis to identify communities of compounds associated with honey bee infections. Distinct VOC clusters reliably separated infected from uninfected bees for three major pathogens — *Varroa destructor*, *Paenibacillus larvae* (American foulbrood), and *Ascosphaera apis* (chalkbrood) — and included both pathogen-derived volatiles and host stress signals, showing that infection reflects contributions from both host and pathogen metabolism. The analysis demonstrated that overall profiles and co-occurrence patterns may provide robust signatures that can distinguish infected from uninfected bees and even differentiate between pathogen types. Crucially, the synthesis also highlighted that no study to date had examined VOCs associated with adult honey bee infections.

Chapter 4 (*Wake Up and Smell the Infected Bees: Volatile cues of Vairimorpha infection in honey bees*) addressed whether adult honey bee infections alter volatile organic compound (VOC) emissions, focusing on the gut parasite *Vairimorpha* spp. Volatiles from infected and uninfected workers were characterised over a 14-day time series using dynamic headspace sampling and GC×GC-MS. Infected bees showed consistent shifts in their overall volatile profiles, with the clearest separation from controls at six and twelve days post-infection. These differences were driven by subsets of compounds that varied in their abundance at different stages of infection. While no single biomarker defined infection, the pattern and relative abundance of putative VOCs changed during infection. This chapter provides the first evidence that *Vairimorpha* infection alters the volatile profile of adult honey bees.

Chapter 5 (*Smell as a mechanism regulating the social response of honey bees to infection with Vairimorpha ceranae*) tested whether infection-related volatiles are sufficient to trigger behavioural responses between pairs of adult bees. Behavioural assays comparing the interactions between infected and uninfected bees when they could only detect volatiles were compared with those that could also make physical contact, thereby allowing exposure to both volatiles and cuticular cues. These were then compared to dyads containing two uninfected bees to assess whether infection and the volatiles associated with it altered grooming, contact frequency, and spatial proximity. No evidence that infected bees elicited different responses from nestmates under either condition was found, supporting previous studies that found no social immune response toward *Vairimorpha* infection (McDonnell et al., 2013; Murray et al., 2015), but contradicting evidence that there are strong aggressive responses

(Biganski et al., 2018). Under controlled laboratory conditions, infection-related volatiles alone do not reliably induce social immune behaviours in adults. Instead, they point to the likelihood that colony-level context and social dynamics are required for infection cues to become behaviourally relevant.

Chapter 6 (*The smell of infection as a mechanism underlying social immunity in social insects*) tested whether semiochemicals associated with infection are sufficient to trigger social immune responses towards adult honey bees in a field setting. Marked workers from four treatments (uninfected controls, uninfected bees perfumed with healthy scent, *Vairimorpha*-infected bees, and uninfected bees perfumed with infection scent) were introduced into observation colonies and monitored across multiple time periods. Whether truly infected or perfumed with infection scent, bees received heightened aggression, avoidance, and intensive grooming compared with healthy bees. Similarities between infected and infection-perfumed bees extended to the overall behavioural profile level, where both groups were strongly aligned and distinct from healthy treatments, showing that olfactory cues alone can reproduce the colony response to infection. By isolating olfaction using scent transfer, these findings provided the first direct evidence that olfaction mediates adult-focused social immunity in honey bees.

7.2 From Smell to Response: Infection Cues in Honey Bees

7.2.1 Are volatile organic compounds associated with honey bee infections across life stages?

This thesis provides evidence that infection consistently alters volatile organic compounds (VOCs) in honey bees in a variety of contexts, not only through single diagnostic markers but through shifts in the overall volatile profile. In honey bee larvae, infections were associated with bouquets of VOCs that can distinguish between both uninfected and infected brood (Chapter 3). This thesis also demonstrates, for the first time, that adult honey bees show comparable changes through variation in the emissions of 71 VOCs (Chapter 4), in line with previous studies on adult cuticular hydrocarbons (CHCs) (McDonnell et al., 2013; Murray et al., 2015; Geffre et al., 2020). In a similar manner to CHCs, *Vairimorpha*-infected

adults did not produce novel compounds but instead showed changes in the ratios of constitutively emitted semiochemicals (Murray et al., 2015, McDonnell et al., 2013, Chapter 4).

The common theme across life stages is that infections produce detectable and discriminable changes in volatiles, but the way these changes manifest (unique compounds versus shifts in abundance) may vary with the host-pathogen system (Chapter 3, Chapter 4). Infection-related volatiles in honey bees are therefore best understood as bouquets that shift in composition and relative abundance, rather than as single compounds acting in isolation. This is consistent with the broader principle that semiochemicals often act synergistically, with blends providing the biologically relevant signal (Wyatt, 2014).

Whilst some volatiles that discriminated *Vairimorpha*-infected adults in Chapter 4, such as tetradecane and dodecane, were also found in brood infections (Chapter 3), most compounds emitted by infected adults were absent from larval studies. This indicates that adults produce distinct volatile profiles during infection and not generalised sickness cues used by all life stages. By demonstrating that infection-associated volatile changes occur in both brood and adults, this thesis broadens the scope of infection semiochemistry in honey bees and establishes adults as a previously overlooked source of cues. This new perspective provides a foundation for investigating how volatile shifts inform social immunity (Cremer et al., 2007; Cremer et al., 2018) and opens avenues for applied research into how these cues might be harnessed for disease detection and management.

7.2.2 When during infection can semiochemical changes be detected?

Across this thesis, a picture emerges that VOC changes in adults are detectable at particular stages of *Vairimorpha* spp. infection. In Chapter 4, VOC profiles of infected bees were distinct from uninfected at six and twelve days post-infection, coinciding with periods of rapid replication reported between four and eight days, and again between ten and fourteen days (Forsgren and Fries, 2010; Huang and Solter, 2013; Fan et al., 2024). At intervening time points the profiles converged, suggesting that parasite growth and host metabolism influence the dynamics of volatile emissions.

Behavioural evidence shows that bees themselves can detect infection-derived semiochemical differences at a comparable stage of infection (Chapter 6). When *Vairimorpha*-infected adults were introduced into colonies seven days post-infection (during the initial exponential growth phase) the host colony responded with higher levels of aggression, intensive grooming, and avoided the bees more than controls. This same response could be elicited simply by perfuming an uninfected bee with the scent of infected nestmates, showing that nestmates can detect and act on infection-derived semiochemical cues by this infection stage. Because the perfuming process transferred both VOCs and low volatility compounds (LVCs), such as CHCs, Chapter 6 does not isolate the specific class of cues, but their timing is informative. Studies on *Vairimorpha* infection have shown that CHC profiles do not diverge from uninfected bees until ten days post-infection or later (McDonnell et al., 2013; Murray et al., 2015). In contrast, distinct VOC profiles were identified by day six post-infection (Chapter 4). When considered alongside the behavioural evidence from Chapter 6, which shows that colonies respond to infection using olfaction at seven days-post infection, this thesis provides evidence that bees detect and respond behaviourally to semiochemical cues at a stage when CHCs remain unchanged. This comparison suggests that volatiles are the more likely drivers of detection during this stage of infection.

Infection cues may also be detectable at the earliest stages of infection. While at the profile level VOCs were similar in the early stages of infection, tetradecane strongly contributed to separating VOC profiles of uninfected and infected bees almost immediately following exposure (Chapter 4). Given that this compound is known to strongly trigger hygienic behaviour *Varroa*-infested brood when applied in isolation (Noël et al., 2025), bees may be able to detect infection even at earliest stages of infection. Taken together, these findings show that infection alters the VOCs emitted by adult bees at multiple stages of infection and provides evidence that bees themselves can also detect and respond to semiochemicals at these intervals.

7.2.3 Does olfaction mediate social immune responses between adults?

Behavioural assays designed to isolate olfaction were used to determine whether olfactory cues regulate social immune responses toward infected adults. In dyadic

laboratory assays, we found no evidence supporting that this was the case, regardless of whether nestmates could physically touch (and therefore detect LVCs such as CHCs) or were restricted to VOCs alone (Chapter 5). In contrast, when bees were introduced into colonies in semi-field conditions, both infected bees and uninfected bees perfumed with the scent of infected nestmates elicited nearly identical responses: increased aggression, intensive grooming, and avoidance compared with healthy bees (Chapter 6). The findings support the idea that there is an adult social immune response towards infection, and that olfaction plays a key role in regulating behavioural responses towards infected adults, but that such responses are only expressed in group contexts. Honey bee behaviour is strongly modulated by social environment (Petrov et al., 2022): colonies rely on pheromones and other chemical signals to regulate both physiological development and task allocation (Bortolotti and Costa, 2014), and in other social insects, chemical cues of infection are only produced in the presence of nestmates (Dawson et al., 2024). For example, pupae of ants emit infection-linked CHCs only when caged with adults, suggesting social cross-talk is necessary for signal production (Dawson et al., 2024). Therefore, complex signalling between workers may be required to elicit social immune responses, or to even produce the cues associated with infection at all. Although further analysis is required (discussed in section 7.3.2) to elucidate the relationships between group contexts, semiochemical signalling and social immunity, this thesis provides the basis for future studies to assess how group contexts alter social immunity and semiochemical cues.

7.3 Implications and future directions

7.3.1 Volatiles as cues for individual and collective immunity

A key first step for future research is to confirm the absolute identities of the VOCs putatively identified in this thesis and establish how sensitive bees are to the compounds here. While retention indices and ion fragment patterns mean the VOCs identifies are very likely true, the only definitive way to confirm the compound identities is to test them against reference standards. Sourcing of individual VOCs and assessing them alongside VOC samples would confirm their identities in subsequent GC-MS analyses and electroantennography could be used to assess

olfactory sensitivity. Previous studies show that bees of different ages vary in their olfactory sensitivity toward VOCs associated with larval infections and that applications of synthetic VOCs can elicit hygienic behaviour when tested in the field (Swanson et al., 2009; Lee et al., 2020). This is likely also true for VOCs associated with adult infections. Electroantennogram assays would allow direct measurement of antennal responses to infection-associated volatiles, as well as revealing the amplitude by which these volatiles are sensed. This could change how we interpret the meanings behind of the shifts in VOC profiles seen in this thesis: while the overall profile of VOCs change, perhaps only certain VOCs are biologically active in bees, and the relative shifts and patterns of those compounds are what truly drives social immune responses. If bees do perceive these compounds, the next question is how specific cues shape their physiology. Semiochemicals may not only mediate social responses but could also act as priming cues for both individual and collective immunity. Immune priming is a key aspect of insect physiology, where pre-exposure to low levels of a pathogen protects against future infections (Sheehan et al., 2020). Given that social insect physiology and behaviour can be heavily influenced by VOCs (Bortolotti and Costa, 2014), future research could test whether exposure to infection-associated VOCs can act as an immune-priming signal. This could be examined by exposing naïve insects to either synthetic isolations of VOCs identified as infection-associated, such as those identified in Chapter 4, or to volatiles emitted by infected individuals, and assessing antimicrobial responses. A design similar to that used in Chapter 5, where bees were restricted to perceiving only the volatiles of infected nestmates, could provide a useful framework. While no behavioural differences were detected in those assays, immunological responses of the naïve conspecifics were not measured, and future studies could determine whether volatile exposure alone alters immune gene expression or antimicrobial activity.

The same principle may apply to other forms of social immunity that do not directly target and exclude infected individuals but instead act prophylactically. Cremer et al. (2018) proposed that volatiles could function as colony-wide signals, coordinating social defences beyond the scale of individual interactions. This idea can be tested experimentally by introducing synthetic VOC mixtures into colonies or by placing infected bees inside cages that prevent contact but allow volatiles to diffuse into the hive atmosphere. Such designs would allow researchers to assess whether colony-

wide exposure to infection volatiles alters collective behaviour. This could include measuring antimicrobial propolis production, which provides broad protection against pathogens, and is known to increase during chalkbrood infection (Simone-Finstrom and Spivak, 2012; Simone-Finstrom et al., 2017), as well as the behaviour of the colony itself. Guard bees become less accepting of returning nestmates and non-nestmates during robbing events (Couvillon et al., 2008); a similar shift in defensiveness may occur if colonies detect infection within the nest *via* volatile cues. Testing such colony-level responses, and whether they translate into reduced infection prevalence within the colony, would clarify whether volatiles serve as collective signals that trigger prophylactic or defensive behaviours, thereby clarifying the role of semiochemicals beyond the targeted removal of infected individuals.

Low-volatility compounds (LVCs), including CHCs, are well-established cues for recognition and social immunity in social insects (Bortolotti and Costa, 2014; Geffre et al., 2020; Wagoner et al., 2020). Now that we know VOCs are distinctive in both brood infections (Chapter 3) and adult infections (Chapter 4), considering both VOCs and LVCs as blends rather than in isolation may be the most informative way to understand infection signalling. Evidence from other systems supports this: in aphids, individual plant volatiles that elicit negative responses can generate positive responses when presented together as blends (Webster et al., 2010), showing that mixtures can fundamentally alter perception. The same principle is likely to apply to infection cues in social insects. If both VOCs and LVCs contribute to recognition, their interaction may produce characteristic shifts in the total semiochemical profile that better reflect how infection is detected. LVCs require antennation to be perceived (Wang et al., 2016) but VOCs would also be encountered during this process, meaning both classes could act together to shape the behavioural outcome. However, no studies to date have examined infection-associated changes in both VOCs and LVCs simultaneously in the context of infection. Future work should therefore aim to integrate methods: dynamic headspace sampling can be paired with solvent extractions to measure volatiles and cuticular compounds from the same individuals. Analysing the complete semiochemical phenotype would allow synthetic mixtures to be reconstructed and tested in behavioural assays, revealing whether combined cues generate distinctive responses compared to VOCs or CHCs alone.

7.3.2 Context and complexity in infection signalling

A key theme emerging from this thesis is that social immunity and VOC emissions appear to be context dependent, with both social environment, pathogen, and infection stage shaping whether cues are produced and behavioural responses are expressed. In Chapter 5, dyadic assays revealed no change in social interactions towards infected bees, whereas in Chapter 6, strong behavioural responses were observed when assays were scaled up to colonies in semi-field conditions. It is possible that infection cues are only produced when in a group context – in ants infection cues are only produced when individuals are housed with nestmates (Dawson et al., 2024). Future studies could build on this by sampling infected bees embedded within colonies, using tagged infected individuals reintroduced to hives and then collected for VOC analysis as infection progresses. This would provide a more realistic picture of which volatiles are produced in natural contexts and whether colony environment alters cue expression. It would also help to address a limitation of all laboratory-based assays: maintaining adults in cages is known to induce stress-related alterations in physiology and metabolism (Alburaki et al., 2019; Lattorff, 2022), raising the possibility that some of the oscillatory patterns in VOC emissions observed in Chapter 4, and absence of behavioural response in Chapter 5, reflected stress induced changes in semiochemical emissions as well as infection dynamics. Sampling individuals in colonies would help disentangle pathogen-driven changes from caging artefacts and clarify how infection stage and social environment interact to shape semiochemical cues.

This approach could also be combined with direct assessments of spore load at each infection stage, strengthening the link between parasite burden and volatile emissions. VOCs in Chapter 4 appeared to be more distinct during periods of active parasite replication (Forsgren and Fries, 2010; Huang and Solter, 2013; Fan et al., 2024). Infection development was assessed in parallel, but quantification of the spore loads directly following VOC sampling could not be conducted due to a limitation in how many bees were available during the experiment. Pairing VOC profiling in colony conditions with post-sampling dissections to quantify spore load would allow direct tests of how volatiles scale with infection intensity by incorporating these data into multivariate analyses. This could be paired with behavioural studies to assess if infection load correlates alters the social response of conspecifics, as

seen in termites (Davis et al., 2018). Such work would reveal whether volatiles provide quantitative information about pathogen burden, as well as serving as binary cues of infection status.

Another possible explanation for the variation in behavioural responses between Chapter 5 and Chapter 6 is the parasite strains used in each experiment. A strain of *Vairimorpha* spp. isolated from colonies in Cardiff, Wales, was used in Chapters 4 and 6, where both volatile shifts and behavioural responses were detected. By contrast, the strain used in Chapter 5 was collected from Silwood Park, England, the previous year, but assessments of its induced volatile profile were not possible. *Vairimorpha* infections are genetically heterogeneous: individuals and colonies often harbour multiple strains, and the prevalence of infections can vary across years (Gomez-Moracho et al., 2014; Sagastume et al., 2016; Gisder et al., 2017). This may explain the wide range of social responses to *Vairimorpha* reported in the literature (McDonnell et al., 2013; Murray et al., 2015; Biganski et al., 2018). Mechanistically, *Vairimorpha* has demonstrated the ability to suppress cuticle genes, suggesting it can manipulate host scent (Badaoui et al., 2017) – a phenomenon also observed in other honey bee pathogens (Geffre et al., 2020). This raises the possibility that signalling is not consistent between parasite strains. Chapter 3 showed that different pathogens are associated with distinct VOC profiles, and the same may be true for strains within a single pathogen species. However, even studies reporting no behavioural responses to infected adults still identified CHC differences compared to uninfected bees (McDonnell et al., 2013; Murray et al., 2015), suggesting that cues are consistently produced, but the type of cue, its relative abundance, and how it is perceived may vary between strains. Comparative experiments across strains of the same pathogen, and across different pathogens, combining chemical, electrophysiological, and behavioural assays, would address whether pathogens evolve to modify or suppress host signals, and whether hosts differ in their ability to perceive and act on those cues.

7.3.1 Semiochemical signalling beyond colonies

If infection alters volatile profiles and these cues mediate social immunity within colonies, they may also play a role in disease transmission outside the hive.

Semiochemicals are already implicated in intercolonial transmission, where parasites can manipulate host cues to enhance their transmission between colonies (Geffre et al., 2020). Flowers represent a particularly important context: they are major transmission hubs for pathogens across insects and are recognised as hotspots for emerging infectious diseases (Graystock et al., 2015; Nicholls et al., 2022). Evidence from bumble bees (*Bombus terreestris*) shows that foragers can avoid flowers contaminated with pathogens without touching them (Fouks and Lattorff, 2011). These behaviours cannot be explained by scent marks deposited by conspecifics (Fouks and Lattorff, 2013), suggesting that VOCs released by the pathogens themselves enable detection. This raises a broader question: does floral contamination alter pollinator social networks at the field scale? If foraging insects can detect pathogen volatiles, visitation rates on contaminated flowers may decline, with cascading effects on pollinator community structure, crop visitation, and ultimately pollination efficiency and yield.

The evolutionary context is also important. If semiochemical cues are pathogen-specific, as suggested in Chapter 3, then hosts are most likely to evolve responses to long-associated parasites, in an evolutionary arms race between host detection and parasite avoidance (Marques and Carthew, 2007). By contrast, emerging infectious diseases or invasive parasites may evade detection by naïve hosts, giving them an advantage in establishing infections and spreading across taxa (Daszak et al., 2000). Honey bees are considered a major source of emerging infectious diseases for other pollinators, spilling over into other Hymenopterans, as well as Diptera, Lepidoptera, Blattodea, Dermaptera, and Coleoptera (Graystock et al., 2013; Manley et al., 2015; Gomez-Moracho et al., 2022). This highlights the potential for semiochemical-mediated detection (or its absence) to shape cross-species transmission. Future studies could therefore test whether volatile cues of pathogens on flowers influence flower visitation in field settings, and whether these cues differ between co-evolved and novel pathogens. This would clarify the role of infection volatiles in shaping pollinator behaviour, spillover dynamics, and crop pollination services.

7.3.2 The Future of Disease Surveillance

Volatile organic compounds provide a promising foundation for non-invasive disease surveillance. Recent studies have shown that gas sensor arrays can be trained to detect shifts in colony headspace associated with brood diseases, even in the absence of specific biomarkers (Bak et al., 2020; König, 2021). Such approaches rely on recognising overall patterns of volatile change, and the results from this thesis provide guidance on which groups of compounds may be most informative for monitoring both adult and brood infections. A key next step is to test whether the characteristic shifts in volatile profiles associated with *Vairimorpha* infections identified in Chapter 4 can be detected directly at the colony level using gas sensors, and whether infected and uninfected colonies can be reliably distinguished under field conditions. This should be extended to assess the robustness of gas sensor surveillance when different strains of the same pathogen are present, given the heterogeneity of infections (Sagastume et al., 2016; Gomez-Moracho et al., 2014; Gisder et al., 2017). VOC analysis, similar what was conducted in Chapter 4, would be valuable in linking chemical changes to sensor outputs.

Expanding this framework to other pathogens is also essential. Many adult honey bee diseases, including common viral infections such as deformed wing virus (DWV) and Kashmir bee virus (Chen and Siede, 2007), have not been characterised for volatile changes and may also present detectable shifts in emissions. Beyond honey bees, surveying VOCs associated with infection could support disease monitoring in other commercially important insects, such as silk moth (*Bombyx mori*) (Chopade et al., 2021) and feeder crickets (Gryllidae) (Szelei et al., 2011; Weissman et al., 2012). Extending VOC-based surveillance in this way could provide a powerful, non-invasive tool for safeguarding insect health across managed systems, as discussed in Chapter 2 (Asiri et al., 2024).

7.3.3 Implications for environmental policy

It is now possible to understand how environmental stressors that impair natural olfaction may disrupt social immune responses in adults. Pesticides are a prime example: they are known to suppress innate immune responses (James and Xu, 2012) and reduce olfactory sensitivity (Williamson and Wright, 2013; Li et al., 2015;

Nooten et al., 2024; Barroso et al., 2025). Hygienic behaviour towards brood has already been shown to decline under pesticide exposure (Morfin et al., 2019; Gashout et al., 2020), and it is likely that similar impairments extend to adult-focused social immunity. Therefore, pesticides may not only directly cause mortality, but they may indirectly increase colony mortality through exacerbating pathogen prevalence by suppressing behavioural defences against both adult and larval infections. Yet current UK and European regulatory frameworks only assess endpoints such as mortality, brood development, and generic behavioural abnormalities (OECD, 1998; EFSA, 2013; OECD, 2017; HSE, 2025), with no consideration of olfactory or learning impairments in adults despite their central role in colony defence. Future work should address this gap by testing whether pesticide exposure suppresses social immune responses to infected adults. Field-based assays similar to those developed in Chapter 6 could be combined with controlled pesticide treatments to assess whether pesticide exposed colonies can still detect and respond to infection. This would establish whether suppression of olfaction can lead to a reduced social immune response and help guide future developments in environmental policy and conservation in general.

7.4 Concluding remarks

Through the integration of chemical and behavioural evidence, this thesis provides the first demonstration that volatile cues of infection are present in adult honey bees and play a role in social immunity. The identification of infection-associated volatile bouquets across life stages shows that infections consistently alter semiochemical profiles, not just through single diagnostic biomarkers but through shifts in the overall volatile profile.

By linking semiochemical cues associated with infection to colony-level behaviours, this work highlights the importance of olfaction in regulating social immunity and shows that responses are context-dependent. These findings extend the concept of the “smell of infection” beyond brood to adults, providing a basis for comparative work across pathogens and pollinator species. More broadly, they demonstrate that infection volatiles have the potential to shape social interactions, transmission

dynamics, and opportunities for non-invasive surveillance, with direct implications for pollinator health and disease management.

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