Chemical cues in disease recognition and their immunomodulatory role in insect immunity

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Short title:

Chemical cues affecting behaviour and immunity

**Highlights**

* Little is known about the chemical and neuronal details behind disease recognition in insects
* Chemical cues that elicit defensive behaviours have mostly been studied in honeybees and fruit flies
* Detection of parasites via olfaction or gustation is often the first line of defence

against parasites, not only modulating host behaviour, but directly mediating immunity via neuroendocrine regulation

* Combining ecological immunology and chemical ecology approaches will enhance our understanding of host-parasite interactions

**Keywords**: host-parasite interactions, sickness cues, disease recognition, volatile organic compounds (VOCs), cuticular hydrocarbons (CHCs), behavioural defence, chemical cue-immunity crosstalk, neuroendocrine immunomodulation

**Abstract**

Preventing infections is crucial for host fitness and many insects modify their behaviour upon sensing contagion. We review chemical cues that mediate insect behaviour in response to parasites, and diseased or dead conspecifics. Considering the large diversity of behavioural disease defences described, surprisingly little is known about disease-associated cues that mediate them, especially their chemoreceptor and neuronal details. Interestingly, disease cues do not only modify host behaviour, but they could play a direct role in immune system activation via neuroendocrine regulation, bypassing the need for risky immunological contact with the parasite. Such crosstalk is an exciting emerging research area in insect ecological immunology that should prove invaluable in studying host-parasite interactions by combining analytical methods from chemical ecology.

**Introduction**

Recognising cues associated with disease is critical, as timely reactions to imminent contagion can prevent infection and sickness altogether, negating the need for a costly immune response [1]. Animals can detect disease-related cues either from pathogens and parasites or infected conspecifics, and modify their behaviour to limit the risk of infection [2,3]. Avoidance of potentially hazardous cues is a simple, yet cheap and effective behavioural response to avoid disease [4\*\*,5\*], but some group-living animals may also engage in more complex behaviours, such as corpse removal from shared nests or the modulation of social interactions within the group [3,6,7]. The nervous system is specifically tuned to detect cues important for reproduction and survival, but besides affecting animal behaviour, chemical cues can also play a vital role in individual immune system function. The connection between the brain and immunity has been well-documented in vertebrate models and humans [8,9], but has been poorly explored in insects. Nevertheless, a recent example in the fruit flies showed that recognition of parasite odours can be a vital component of host defence against parasites by directly modulating cellular immunity in anticipation of the incoming threat [10\*\*]. This shows that detecting parasites via chemoreceptors and reacting promptly can be the key determinant of host fitness. We expect both behavioural and immunological mediation of disease avoidance by chemical cues to be under strong selection, however, these aspects and their evolutionary and ecological consequences on host-parasite interactions have yet received little attention in ecological immunology research.

In this review, we first provide an overview of the chemical cues that mediate insect behavioural responses to parasites. We use a broad definition of parasite that includes any organism using resources of another organism (a host) for its own growth, including microbes (viruses and bacteria) and macroparasites (worms and parasitoids) [11]. We do not include cases where parasites suppress host sickness behaviour (e.g., certain sexually-transmitted parasites [12]), and social or brood parasites that evade detection by mimicking host odours or lacking any identification cues (chemical mimicry and chemical insignificance, respectively) [13,14], but instead focus on parasite- and host-emitted compounds found or proposed to induce defensive reactions in the host (Table 1). Despite their apparent importance in modulating host-parasite interactions, research on insect disease-associated cues has been mostly limited to species with high agricultural value or as laboratory models, like the honeybee and the fruit fly [15\*–18]. Lastly, we discuss the connection between disease cues and immunity: an exciting emergent research area suggesting that olfaction or gustation may be the first line of host defence against parasites in insects, not only by directing host behaviour, but by launching immunological reactions as well [10\*\*,19\*\*]. We argue that the combination of eco-immunological approaches and chemical analytical methods to study host-parasite interactions across insects should prove invaluable in studying disease-cue mediated antagonistic interactions.

**Behavioural responses to chemical disease cues**

Disease-associated cues can induce rapid behavioural responses upon their detection and such reactions can be vital in preventing infections where timely responses are critical [20,21]. Disease-associated cues can originate either from the parasite [22,23] or from infected and non-infected (i.e. wounded or dying) conspecifics that emit chemical cues others perceive as dangerous (Table 1). Both types of cues can induce behavioural changes in the host and such responses will vary based on host ecology, life history, and whether the host lives in groups; the latter is important as diseases transmit easier within groups, placing stronger selection pressures on individuals to detect and accurately interpret disease-associated cues.

**Behaviours that reduce the likelihood of infection - avoidance**

Avoidance is the most cost-effective strategy against parasites and the simplest solution to prevent infection [11]. Infection avoidance is widespread in animals [24] and is known to occur in multiple insects species in relation to parasites [5\*], although the underlying chemical cues and mechanistic details are still poorly understood. Several volatile organic compounds (VOCs) emitted by harmful microbes have been found to repel insects (Table 1), including phenol [25], geosmin [17,26], fungal 3-octanone and 1-octen-3-ol [27–30]. The latter seems to repel a broader range of insects and is currently being explored for use in pest management [31]. The above compounds are easily detected from a wide distance, however, immediate avoidance can also occur upon feeding, by rapid detection of microbial components. For example, fruit flies display oviposition and feeding avoidance upon detection of specific gram-negative bacteria and bacterial cell-wall components (lipopolysaccharides, LPS) in their food, both of which depend on taste (gustation) or olfaction and the TRPA1 chemosensory cation channel [32,33]. Interestingly, when applied to the cuticle, LPS and *Escherichia coli* trigger selfgrooming response in the fruit flies – a behaviour mediated by taste receptors located at various body parts in the fly, including the legs and wings [34,35\*]. Spores of pathogenic fungi that infect insects via the cuticle also trigger selfgrooming in flies that is mediated by olfactory perception, although the exact fungal compound triggering selfgrooming is still unknown [36].

**Behaviours among conspecifics – dealing with established infections**

Avoiding infections will not always be possible, and this represents a particular problem for group-living individuals, such as the eusocial insects, which cannot simply avoid sick conspecifics, but must actively manage potential outbreaks. Eusocial insects have evolved extraordinary ways to limit disease spread within their colonies by detecting infected or contagious nestmates and providing care, or eliminating them from the colony [3,6,7]. However, studies identifying mechanisms underlying these behaviours are mostly carried out in honeybees. Honeybees perform hygienic behaviour to eliminate infected brood from the colony, by uncapping their breeding wax cells and removing affected individuals. Several compounds have been identified as activators of hygienic behaviour (Table 1). Bees use two unsaturated hydrocarbons, (*Z*)-6-pentadecene and (*Z*)-10-tritriacontene as cues to detect *Varroa* mite-infested cells, which are emitted by the brood themselves [37,38]. Two volatile compounds, ethyl hexanoate and α‐pinene, have been found to elicit similar behaviours [39], including the *Varroa*-specific ketones and fatty acid acetates: 2-tricosanone, 2-pentacosanone, 2-heptacosanone, 2-nonacosanone, tetracosyl acetate and hexacosyl acetate [22]. In contrast, *Ascosphaera apis* (chalkbrood) infections are detected via phenethyl acetate, 2-phenylethanol, and benzyl alcohol, of which phenethyl acetate triggered the most prominent response [40]. Nevertheless, comparative studies using different parasites are still too limited to make conclusions about the specificity of these cues in hygienic behaviour. Honeybees will also remove dead or dying brood when there are no parasites involved, which is mediated by an increase in β-ocimene – a brood pheromone that flags the hygienic workers’ attention – and oleic acid that acts as a necromone (death cue) and triggers removal [41].

The above studies are among a few that have identified chemical cues triggering anti-disease behaviours and confirmed their findings using a bioassay, but many more have identified potential candidate compounds. Cuticular hydrocarbons (CHCs) of infected or otherwise immune-challenged individuals are most commonly identified as candidate cues for odorant-induced behavioural changes, again almost exclusively examined in bees infected with different parasites [42–48]. Only one additional study in ants identified CHCs (alkenes and alkadienes with chain-length C33 and C35) putatively involved in *destructive disinfection* – a behaviour in which ants detect fungal-infected pupae, remove their cocoons and spray them with acidic venom to prevent further disease transmission [49]. Notably, two studies in non-eusocial insects, the flour beetles, also found differences in the CHCs upon immune challenge, one linked to male terminal investment in which they cheat the females to mate by “smelling” more attractive [50], and the other to the potential transfer of immune information from diseased to healthy individuals, which requires further validation (Lo et al. *bioRxiv Prepr* 2021).

That CHCs are the most common candidates found is perhaps not surprising considering that insects rely extensively on CHCs for communication [51]. However, this could also reflect the current methodological bias towards CHC analysis and it is likely that chemical changes will be detected using other analysis methods as well (e.g., [42]). Moreover, changes in the CHC profile of infected or immunised individuals will not always lead to a change in behaviour of healthy conspecifics [52,53]. This reinforces the need to perform specific bioassays, either to identify compound class responsible for the induced effect (e.g., CHCs [48,50,54]) or, where possible, narrow down to specific compounds [22,38].

**Host-derived cues and prophylactic behaviours in the absence of parasites**

Published data on the identity of VOCs and host-derived disease cues (Table 1) are still too limited for comparative approaches. However, cues emitted from hosts in the absence of parasites, such as from dead or wounded conspecifics, seem to have a more conserved alarm function in prophylactic disease avoidance. For example, two host-derived unsaturated fatty acids show a high degree of conservation as necromones in insects and crustaceans. Oleic and linoleic acids are emitted from dying or dead individuals and function as cues primarily associated with injury and death. Upon detection, these fatty acids repel isopods, crickets, and beetles [55–57] and induce undertaking in ants and termites, i.e. burial or transfer of conspecific cadavers to the refuse pile [58–63]. Oftentimes a blend of oleic acid and other compounds work additively, e.g., in the combination with β-ocimene in the freeze-killed brood removal in honey bees [41,64] or indole and phenol in the burial behaviour of termites [65]. Interestingly, termites associate this blend with a longer post-mortem time that induces burial, whereas 3‐octanone and 3‐octanol emitted shortly after death induce cannibalism, possibly allowing the termites to differentiate between decaying and fresh corpses, and so recycle the scarce nutrients of the wood log they live in [61,66]. Environmentally-degraded unsaturated CHCs emitted from dead individuals as VOCs can also induce avoidance, as shown in cockroaches [67], and other times it is the absence of specific compounds that elicits prophylaxis, as is the case in *Linepithema humile* ants where the lack of iridomyrmecin and dolichodial on the nestmate cuticle induces undertaking [68]. The above-mentioned behaviours reduce the likelihood of potential re-infections from dead conspecifics, even though the presence of parasites or disease were not detected. Nevertheless, many insects are scavengers of organic matter, including other insect cadavers, such that individuals will always need to balance the risk of potential infection, with starvation from avoiding a meal. Repellence or attraction to necromones should, therefore, depend on several factors, like their association with other cadaver-derived compounds, concentration, and whether or not they are emitted from different species or conspecifics. The latter could also be perceived as a cue for predator presence, especially in animals that share a common shelter [55].

**The connection between disease cues, nervous system, and immunity**

A rapid reaction of the immune system is essential for the infection outcome [20,21], but what if immunity can be modulated before the actual infection occurs? Exciting recent evidence shows that insects use olfactory information to prepare their immune system against imminent infections, similar to classical examples of immune priming in invertebrates [69]. *Drosophila* larvae reared with parasitoid wasp odours during development show increased inactivation – encapsulation and melanisation – of wasp eggs upon their deposition [10\*\*]. Wasps deposit their eggs into fly larvae that are subsequently consumed by the parasitoid and it is estimated that up to 80 % of *Drosophila* populations succumb to parasitism [70]. Sensing wasps in the environment is, therefore, highly beneficial, and mediated by a dedicated olfactory receptor Or49a in larvae [16] that promotes lamellocyte differentiation - the principal blood cell population usually activated in defence against parasitoid eggs [10\*\*]. The above example is intriguing because it demonstrates that immunological priming can be achieved not only upon immunological contact with the parasite but also via the olfactory nervous system, which is a faster and potentially more cost-effective strategy for the host.

Whether or not disease cues will modulate immunity will probably depend on the evolutionary history of host and parasite, and the frequency of their encounters because immunomodulation without subsequent parasite attack could be too costly. Similarly, costs will arise if immunity is upregulated in response to any microbial cues that do not pose a significant threat to fitness. This raises questions about the specificity of such responses, the other compounds that induce immunomodulation (e.g., mentioned in Table 1) and how is this mechanistically regulated? So far, comparative approaches are possible due to a limited number of studies investigating odour-mediated immunomodulation in other host-parasite systems. A study on tobacco cutworm showed that plant defensive volatiles, most notably (E)-β-ocimene, linalool, and (Z)-3-hexenyl acetate, also upregulate insect cellular immune responses, allowing the insects to resist subsequent parasitoid attack [19\*\*]. The connection between the plant, wasp, and herbivore is intriguing; these volatiles are damage-induced plant volatiles but they are also used by the wasp as an indirect cue of herbivore host presence [71]. It is hypothesised that insects anticipate parasitoid attack by modulating their immunity in the presence of these volatiles [19\*\*], however, this needs to be confirmed. Herbivore-induced volatiles are defensive compounds that are costly for the herbivore and induce increased mortality [19\*\*,72,73], such that evolutionary causes of immune modulation by plant volatiles need to be closely examined. Another immunomodulation example comes from the honeybees, where cues emitted by the immune-challenged workers induce upregulation of antimicrobial peptide genes in the queen [54]. LPS-injected workers exhibit altered CHC profiles that the queen perceives via direct contact to workers, although the mechanistic details behind such immunomodulation in the queen remain so far elusive.

How could immune modulation by chemical cues be achieved mechanistically? In *Drosophila*, sensing wasp odours via Or49a olfactory receptor mediates systemic release of the neurotransmitter GABA, which binds to blood progenitors in the lymph gland and promotes lamellocyte differentiation [10\*\*]. This specifically shows that olfaction affects immunity via neuroendocrine modulation in flies. Such phenomenon is by now well-established in vertebrates [74,75], but has only recently been appreciated in invertebrates [5\*,76–78]. Neurotransmitters, like octopamine, serotonin and dopamine regulate numerous functions in insects, including olfaction, behaviour, and memory [79], but they also have a vital role in immunity. For example, neurotransmitters control the activity of insect blood cells via a specific type of receptors the cells carry on their surface, but they are also synthesised by the blood cells themselves, facilitating direct immunomodulation upon infection, such as enhanced nodulation and phagocytosis [80–84]. Interestingly, immune receptors, like PGRP-LC and PGRP-LE are present in octopaminergic neurons, by which the neurons directly sense microbial cues and modulate fly avoidance of bacteria-contaminated food [85] or oviposition upon bacterial infection [86,87].

The neuroendocrine modulation of immunity is an evolutionarily ancient and widespread phenomenon, occurring in vertebrate and invertebrate taxa [77,78]. The above examples show that detection of disease-associated cues via olfactory or gustatory receptors could directly promote immune modulation via neuroendocrine regulation. For the host, this can be highly advantageous because it enables speedy reactions upon detection of parasites in the environment and circumvents the need for a direct immunological contact to modulate immunity. Such crosstalk between the brain and immunity needs to be integrated into the study of behavioural and immunological responses in insects.

**Conclusions and outstanding questions**

Both ecological immunology and disease ecology are intertwined in studying ecological parameters that shape the evolution of host immunity (e.g. heterogeneity in disease susceptibility, costs of immunity, defence strategies) and parasite spread in natural populations [88] - processes that should be strongly affected when hosts recognise disease-associated cues and react behaviourally or immunologically to prevent infections in the first place. For example, host defensive behaviour was shown to alter parasite communities in coinfections [89\*\*] and it can have direct or indirect consequences on evolution of both of the antagonists [90\*\*], but these aspects received little research attention so far. We expect disease cues emitted by parasite or infected conspecifics, and recognition of those cues to be under strong evolutionary selection. Understanding the underlying mechanistic details of how parasite odours affect host behaviour and immunity is hence essential for our interpretation of host-parasite interactions and parasite epidemiology.

Critical questions to be addressed:

1) A large body of literature has documented the manifold behavioural disease defences in insects that range from avoidance and grooming to dynamic anti-disease behaviours seen so far in eusocial insects [3,5\*]. However, chemical cues and mechanistic neuronal details behind such behavioural modulation are still largely unknown. The first step is identifying the exact compounds that trigger specific phenotypes by confirming their roles using functional tests, which have so far rarely been used.

2) What type of parasites can be detected by host olfaction or gustation, and how specific are immune responses induced by these cues? Are humoral and cellular immunity equally likely to be activated by chemical cues and how specific is this response? For example, can sensing gram-negative bacteria in the environment prime immunity specifically against the same bacteria, or more broadly against other strains or gram-positive bacteria as well? Similarly, do healthy conspecifics recognise the type of parasite (e.g., bacteria, virus, or microsporidia) their group member is infected with based on their chemical profile, and does this then influence their immunity accordingly?

3) What other factors influence insect sensitivity to chemical disease cues? For example, if individuals are starved, they may be less aversive to contaminated food sources. Conversely, enhanced immune status (e.g., by immune priming) will make individuals less susceptible to parasites and potentially less aversive to disease cues as well. Therefore, individuals may react differently to disease cues depending on their physiological status.

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**Figure legends**

**Table** **1. Disease-associated cues in host-parasite interactions and their influence on host behaviour and immunity**. The table shows studies identifying parasite and host-emitted cues found to induce behavioural or immunological defensive reactions in the host. We specifically differentiate whether studies used a functional test to verify the influence of specific compounds and whether the compounds were tested together as a blend (compounds separated by a comma), or each compound separately (placed underneath each other). Abbreviations: NA – not applicable, LPS – lipopolysaccharides, PGN – peptidoglycans, VOC – volatile organic compounds, CHC – cuticular hydrocarbons, DWV – Deformed wing virus.

Table 1.





