Behavioural responses of off-host ticks (Ixodidae) to predators and pathogens

by

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Abstract

Ticks spend most of their life in moist off-host microhabitats, where they are protected from desiccation but susceptible to predators and entomopathogens. I investigated whether ticks avoid chemical cues indicative of ant predators and the entomopathogenic fungus *Beauveria bassiana* (*Bb*). In olfactometer bioassays, *Ixodes scapularis* ticks were significantly deterred by semiochemicals originating from the poison and Dufour's glands of *Formica oreas* thatching ants. Formic acid and hydrocarbons released from these glands deterred ticks but attracted worker ants. Contrary to my prediction, females and males of the ticks *Amblyomma americanum*, *Dermacentor variabilis*, *Ixodes ricinus, and I. scapularis* sought, rather than avoided, *Bb*. In further bioassays, *I. scapularis* oriented towards both harmful *Bb* and harmless soil-dwelling fungi, implying that fungi – regardless of their pathogenicity – signal habitat suitability to ticks. Responses to *Bb* were mediated by contact chemoreception of metabolites associated with cellulose breakdown. Ticks were deterred by the common fungal metabolite 2-methylisoborneol.

Keywords: Entomopathogens; Ixodidae; microhabitat; predation; semiochemicals; repellent

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Chapter 1.

Introduction

1.1. Hematophagy of ticks

Ticks (Order Ixodida) are obligatory blood feeders of vertebrates and are present on every continent [1]. Hard ticks (Family Ixodidae) are of great human importance, impacting the health of humans, companion animals, and livestock [2]. Ticks transmit many causative agents of diseases, including tick-borne encephalitis, Rocky Mountain spotted fever, anaplasmosis, and Lyme disease [3–6]. In the United States alone, there are an estimated 300,000 cases of Lyme disease annually [7].

Ixodid ticks have three life stages: larva, nymph, and adult [8]. Each life stage must take one bloodmeal to successfully molt to the next stage. This process commonly involves feeding on three distinct hosts. Some ixodid ticks, however, have one- or two-host lifecycles. Locating a host is achieved through questing, wherein ticks perch atop understory vegetation, with their forelegs outstretched, to ambush hosts as they pass by. Using specialized sensory receptors on their forelegs, ticks perceive physical and chemical cues associated with potential hosts, such CO_2, infrared radiation, and host odorants [9–12]. Host preferences vary by species and life stage, with immature life stages often feeding on rodents and other small vertebrates, and adults often feeding on ungulates [13]. Once a tick successfully contacts a host, it will typically select specific body regions for feeding. For example, adult *Ixodes* ticks parasitizing deer typically attach themselves to the head, neck and ears [14], whereas adult lone star ticks, *Amblyomma americanum,* parasitizing calves typically attach themselves to posterior body regions [15]. In some *Amblyomma* species, selection of attachment sites may also be affected by attraction-aggregation-attachment pheromones [16].

1.2. Off-host behavior and survival of ticks

Ticks are highly prone to desiccation because of their permeable cuticle and large surface area to volume ratio. To reduce desiccation during questing, ticks adjust questing behavior in accordance with ambient relative humidity, questing in a lower

vertical stratum [17] and for shorter periods of time [18] when the relative humidity is low. Following questing bouts, ticks take refuge in humid leaf litter and detritus to regain water [13]. Some tick species are capable of imbibing liquid water [19] but generally ticks regain moisture from humid air, using a hygroscopic solution secreted from their salivary glands [20]. Tolerance for desiccating conditions varies by species [21], and is often reflected in questing environments. For example, *A. americanum* is fairly desiccation tolerant and often quests in open fields [22], whereas black-legged ticks, *Ixodes scapularis*, are more susceptible to desiccation and quest in shaded, forest environments [23]. Regardless of desiccation tolerance, nearby humid microhabitats – typically leaf litter – are required by ixodid ticks to regain water between questing bouts [13,22,24].

Off-host distributions of ticks are affected by climatic variables, host distributions, type of vegetation, and leaf litter. Broad-scale climatic data delineate the geographic ranges of ticks. Variables such as winter temperature, humidity and precipitation, are commonly reported predictors of tick distribution ranges [25–29]. Abundance of host species correlates with tick abundance [30–32] and tick dispersal to new habitats [33]. Birds, in particular, may contribute to long-distance dispersal of ticks [33,34]. Associations between vegetation type or cover and tick presence vary with tick species. For example, American dog ticks, *Dermacentor variabilis*, are prevalent in open-canopy fields, whereas *I. scapularis* is most abundant in closed-canopy forests [35]. Generally, ticks prefer habitats with understory vegetation for questing [22,23,35–37]. Finally, leaf litter provides the high relative humidity that ticks require to regain water (see above), and thus is a key characteristic of suitable tick micro-habitats [22,24,38–40]. Leaf litter also reduces tick susceptibility to arthropod predation [41].

1.3. Predators of ticks

Ticks are preyed upon opportunistically by both vertebrate and invertebrate predators. Ticks taking refuge in leaf litter and detritus risk encounters with generalist arthropod predators which occupy the same habitat. Ants, spiders, and beetles all prey on ticks [42]. Ant populations have been correlated with decreased tick abundance [43,44] and an experimental introduction of wolf spiders (Lycosidae) reduced tick populations [41,45]. Beetles sporadically feed on ticks [42] but effects on tick populations have not yet been investigated. Vertebrates also feed on ticks but only a few species,

such as *Buphagus* oxpeckers, are known to consume ticks in large numbers [46]. Red-billed oxpeckers, *Buphagus erythrorhynchus*, obtain over 30% (by mass) of their diet from the ticks they remove from oxen [47], a foraging behavior which may, or may not, also benefit the oxen [48]. Opossums (Didelphidae) were previously thought to be significant predators of ticks [49] but more recent findings do not support this concept [50]. Other vertebrates, including toads, tortoises, lizards, and rodents, also feed on ticks, but likely only in a sporadic and opportunistic manner [51].

As ixodid ticks have finite energy stores and move slowly, their ability to escape predators is limited. Tick adaptations to evade predation may be physical, behavioral, or chemical. Tick coloration and patterning have been proposed, but not been experimentally tested, to be physical adaptations to help avoid predation. Tick coloration may serve as camouflage to reduce detection by both tick predators and tick hosts, whereas patterning may provide disruptive coloration (a form of camouflage that works by breaking up the outlines of an animal) [52]. UV fluorescence patterns displayed by many ixodid ticks [53] may also help achieve disruptive coloration. Behavioral responses to predators are similar across tick species. When ticks face a potential predator, they typically curl in their legs and cease movement. This tonic immobility (the act of feigning death or exhibiting thanatosis) is a well-documented and wide-spread anti-predation behavior of animals. In ticks, tonic immobility is typically followed by a brief period of rapid locomotion, presumably to escape the vicinity of the potential predator. Interestingly, both the exhibition and duration of tonic immobility decrease with time elapsed since the last blood meal, suggesting that ticks with their energy stores depleting prioritize host-seeking over predator avoidance [54]. *Ixodes scapularis* also reduce predation risk by shortening questing bouts in the presence of a predatory spider [45] but how ticks detect spiders is not yet known. Chemical defenses against predation have been reported only in metastriate ticks. When distressed, metastriate ticks secrete squalene from large wax glands to deter predators, particularly ants [55].

1.4. Entomopathogens of ticks

Interactions between fungi and ticks have been studied primarily in the context of biological control. Two entomopathogenic fungi have shown great promise as biological control agents of ticks: *Beauveria bassiana* and *Metarhizium brunneum* (formerly *anisopliae*) [56]. Both fungi are pathogenic to at least 11 tick species [57–65] but their

lethality varies greatly based on tick species, fungal strain, application method, and environmental conditions [56]. Infection prevalence in nature also varies in accordance with tick species, their life stage, the fungal strain, and location surveyed. Tick fungal infection rates as low as 0.15% and as high as 30.3% have been reported [66–68]. Infection of arthropods with entomopathogenic fungi typically occurs through the cuticle. Spores attach to the cuticle, germinate and form appressoria to penetrate the cuticle and colonize the hemolymph [69]. Eventually, fungal infections lead to the death of the host and to sporulation of the cadaver if environmental conditions are suitable [70].

With entomopathogenic fungi such as *B. bassiana* and *M. brunneum* being lethal to various arthropods, arthropods have developed microbial and behavioral defenses. For example, stable fly larvae, *Stomoxys calcitrans*, harbor a bacterial symbiont with anti-fungal activity in their mucus [71], and queens of red imported fire ants, *Solenopsis invicta*, preferentially nest in soil containing bacteria which inhibit the growth of entomopathogenic fungi [72]. Behavioral defenses may also be based on avoidance. For example, the predatory mites *Phytoseiulus persimilis* avoid prey infected with *B. bassiana* [73], and the termites *Macrotermes michaelseni* avoid *M. brunneum* strains proportionally to their virulence [74]. Lastly, arthropods may engage in self- or allo-grooming behavior which mitigates fungal infection following contact with entomopathogenic fungi [75–79].

Soil-dwelling arthropods, including ticks, inevitably encounter non-entomopathogenic soil fungi which significantly contribute to the structure and processes of soil ecosystems [80,81]. Soil fungi are vital for the subsistence of mycophagous and saprophagous arthropods [82,83]. Soil fungi break down cellulose and lignin which otherwise would not be digestible by many saprophytic arthropods [84]. Soil fungi also significantly shape physical characteristics of soil microhabitats [80] which, in turn, affect soil-dwelling arthropod communities. Whereas behavioral responses of arthropods to soil-dwelling non-entomopathogenic fungi have hardly been studied, behavioral responses of arthropods to soil-dwelling bacteria have been documented. For example, springtails, *Folsomia candida*, are attracted to soil colonized by edible bacteria [85], and red imported fire ant queens preferentially nest in soil colonized by bacteria that inhibit the growth of entomopathogenic fungi [72]. Although off-host ticks commonly seek soil microhabitats as refuge, it remains unknown whether ticks detect, and behaviourally respond to, soil-dwelling fungi or bacteria.

1.5. Overview of research chapters

In Chapter 2 (Research Chapter 1), I investigated whether *I. scapularis* has developed behavioural defenses against tick predators such as ants. Specifically, I explored whether *I. scapularis* responds to semiochemical cues of the thatching ant *Formica oreas*. I tested the hypotheses that (1) chemical deposits from *F. oreas* worker ants deter *I. scapularis*, (2) deterrent semiochemicals originate from the ants' poison and/or Dufour's gland(s), and (3) tick-deterrent semiochemicals serve as alarm-recruitment pheromone components in *F. oreas*. In two-choice olfactometer bioassays, filter paper soiled with ant chemical deposits significantly deterred ticks. Poison and Dufour's gland extracts in combination, but not alone, deterred ticks. Gas chromatographic-mass spectrometric (GC-MS) analyses of gland extracts revealed formic acid as the major constituent of the poison gland, and eight hydrocarbons as constituents of the Dufour's glands. Analogous to results obtained in bioassays with gland extracts, neither synthetic formic acid nor synthetic hydrocarbons affected behavioural responses of ticks but synthetic formic acid in binary combination with synthetic hydrocarbons significantly deterred ticks. According to GC-MS analysis, sprays discharged by distressed *F. oreas* workers contain formic acid and hydrocarbons, and synthetic equivalents of these compounds elicit alarm-recruitment responses from *F. oreas* workers. All data combined indicate that ticks eavesdrop on the ants' chemical communication.

In Chapter 3 (Research Chapter 2), I investigated whether the presence of harmful and harmless soil-dwelling fungi affects the selection of micro-habitats in soil or leaf litter by off-host ticks. Working with six species of ixodid ticks (*I. scapularis,* the lone star tick, *Amblyomma americanum*, American dog tick, *Dermacentor variabilis*, brown dog tick, *Rhipicephalus sanguineus*, castor bean tick, *Ixodes ricinus*, and western blacklegged tick, *Ixodes pacificus*) in olfactometer bioassays, I tested the hypothesis that ticks avoid the entomopathogenic fungus *Beauveria bassiana* (*Bb*). Contrary to my prediction, nearly all ticks sought, rather than avoided, *Bb*-inoculated substrates. In further bioassays with female *I. scapularis*, ticks oriented towards both harmful *Bb* and harmless soil-dwelling fungi (*Rhizopus stolonifer*, *Fusarium oxysporum*, *Penicillium roqueforti*), implying that fungi – regardless of their pathogenicity – signal habitat suitability to ticks. Only accessible *Bb*-inoculated substrate appealed to ticks, indicating

that they sense *Bb,* or its metabolites, by contact chemoreception. *Bb*-inoculated substrate required ≥24 h of incubation before it appealed to ticks, suggesting that they respond to *Bb* metabolites rather than to *Bb* itself. Similarly, ticks responded to *Bb*-inoculated and incubated cellulose but not to sterile cellulose, indicating that *Bb* detection by ticks hinges on *Bb* metabolism of cellulose. Finally, I tested behavioural responses of ticks to two common metabolites of soil-dwelling fungi: geosmin and 2-methylisoborneol. Ticks were indifferent to synthetic geosmin but were strongly deterred by synthetic 2-methylisoborneol. As disturbed soils have elevated levels of 2-methylisoborneol, avoiding such soils may help ticks lower their risk of physical injury.

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Chapter 2.

Blacklegged ticks, *Ixodes scapularis*, reduce predation risk by eavesdropping on communication signals of *Formica oreas* thatching ants[1]

2.1. Abstract

Ticks spend most of their life inhabiting leaflitter and detritus where they are protected from sun but preyed upon by ants. Ants secrete chemical communication signals to coordinate group tasks such as nest defense. Ticks that avoid ant semiochemicals – as indicators of ant presence – would reduce predation risk by ants. We tested the hypotheses that (1) chemical deposits from the thatching ant *Formica oreas* deter blacklegged ticks, *Ixodes scapularis*, (2) deterrent semiochemicals originate from the ants' poison and/or Dufour's gland(s), and (3) tick-deterrent semiochemicals serve as alarm-recruitment pheromone components in *F. oreas*. In two-choice olfactometer bioassays, filter paper soiled with ant chemical deposits significantly deterred female and male ticks. Poison and Dufour's gland extracts deterred ticks in combination but not alone. Gas chromatographic-mass spectrometric analyses of gland extracts revealed formic acid as the major constituent in the poison gland and 8 hydrocarbons as constituents in the Dufour's gland. Synthetic formic acid and hydrocarbons deterred ticks only when combined. *F. oreas* workers sprayed both formic acid and hydrocarbons when distressed. A synthetic blend of these compounds elicited alarm-recruitment responses by *F. oreas* in behavioural bioassays. All results combined indicate that ticks eavesdrop on the ants' communication system.

[1]A nearly identical version of this chapter has been published in Royal Society Open Science with the following authors: Claire E. Gooding, Charlotte Pinard, Regine Gries, Anand Devireddy, Gerhard Gries.

2.2. Introduction

Blacklegged ticks, *Ixodes scapularis,* are obligate blood-feeding ectoparasites that feed on mammalian, avian, and reptilian hosts [1]. They are most abundant in the Eastern and Central United States but can be found as far north as the Canadian maritime provinces, and as far south as the Mexican province of Coahuila [2]. Their preferred hosts are white-tailed deer, *Odocoileus virginianus,* and rodents, but they also feed opportunistically on humans [1]. Blacklegged ticks carry 16 known human pathogens including *Borrelia burgdorferi,* the causative agent of Lyme Disease [3]. In the United States alone, there are an estimated 300,000 cases of Lyme Disease annually, making *I. scapularis* a species of significant medical importance [4].

Blacklegged ticks spend most of their life taking refuge in leaf litter and detritus [5]. The high humidity and protection from sun afforded by these microhabitats are essential for the survival of ticks which are prone to desiccation [6,7]. However, ticks share these microhabitats with numerous generalist arthropod predators [8]. Opportunistic predation by ants on leaf litter-dwelling ticks, beetles and spiders [8–10] significantly impacts tick survival and/or distribution [11–13]. For example, the abundance of *Ixodes* ticks is affected by both European fire ants, *Myrmica rubra,* and red wood ants, *Formica polyctena* [12,13], and the *Amblyomma* tick burden on small mammals is reduced in areas inhabited by red imported fire ants, *Solenopsis invicta* [14]. Both ant predation on ticks, and tick avoidance of ant semiochemicals, may underlie the effects of ants on tick abundance and distribution.

Predator-derived cues can prompt predator avoidance behaviours in prey [15] but the type of cue, and its specific characteristics mediating predator avoidance behaviours by prey are often not investigated. Ants prey on many arthropods, including ticks, and often exert both consumptive and non-consumptive effects on prey species [12,13,16]. Ants use a plethora of chemical communication signals to coordinate group tasks such as nest defense, brood care, and foraging behaviour [17]. Potential prey of ants, including spiders, bees, and fruit flies, eavesdrop on these ant communication signals, and avoid areas where they have been deposited [18–22]. Whether ticks avoid ant semiochemicals has not yet been investigated.

Avoidance of ant semiochemicals – as indicators of ant presence – would be adaptive for ticks, if these semiochemicals were to (*i*) accumulate in areas inhabited or frequently visited by ants, and (*ii*) reliably signal the current or imminent presence of any species of predatory ant. Constituents in the ants' poison and Dufour's glands (for their location see Fig. 2.1A) have multiple communication functions including alarm-recruitment of nestmates [23–31], and thus are frequently secreted, and likely accumulate, in areas inhabited by ants. As gland constituents are highly conserved across formicine ants, they could be adopted by prey as generic predator recognition and avoidance cues. Poison and Dufour's gland secretions typically comprise formic acid and assorted hydrocarbons, respectively [23–26,28,29,32–35]. Whereas hydrocarbons may originate from multiple sources other than the Dufour's gland, formic acid is rather indicative of ant presence, and thus could – alone or in combination with specific hydrocarbons – reliably indicate predation risk by ants.

The thatching ant *Formica oreas* is a representative member of the *Formica rufa* species group. It occurs in various woodland and prairie habitats and is known for its conspicuous thatch-mound nests [36]. With some overlap in the geographic distribution of *I. scapularis* and *F. oreas* [36–38], and *I. scapularis* and other woodland-dwelling species in the *Formica rufa* group [39,40], it is conceivable that *I. scapularis* interacts with *F. oreas,* and with other *Formica rufa* group ants, in a predator-prey relationship. *Formica oreas* is an aggressive and competent predator of various arthropods [38,41]. It has not yet been documented to prey upon *I. scapularis* or other ticks but its congener *Formica polyctena* curtails the abundance of *Ixodes* ticks in Europe [13].

Working with *F. oreas* worker ants and *I. scapularis* ticks, we tested the hypotheses that (1) ant semiochemical deposits deter ticks, (2) tick-deterrent semiochemicals originate from the ants' poison and/or Dufour's gland(s), and (3) the tick-deterrent semiochemicals serve as alarm-recruitment pheromone components in ants.

2.3. Materials and Methods

2.3.1. Tick maintenance

Adult *I. scapularis* were acquired from BEI Resources (American Type Culture Collection) and the National Tick Research and Education Resource (Oklahoma State University). Groups of 10–12 ticks were housed in 20-mL glass scintillation vials (VWR International, PA, U.S.A) fitted with strips of paper towel as refuge and substrate for climbing, and with a mesh-covered hole (~1 cm) in the lid to enable air exchange. As ticks are prone to desiccation, vials were kept at high relative humidity (85–95%) in a vessel (d = 26 cm, h = 30 cm) containing a saturated solution of K_2SO_4 (99% purity; Alfa Aesar, ON, CA). To minimize the risk of tick escape, the vessel was retained in a plexiglass box (50 × 35 × 35 cm) which was kept at 22 °C and a 14:10 light/dark cycle. To prevent mold/fungal growth, vials were washed weekly with Sparkleen (Thermo Fisher Scientific, MA, U.S.A) and dried at 100 °C for >1 h. Monthly, the vessel was washed and sterilized with Sparkleen and 70% ethanol, respectively, and the K_2SO_4 solution was replaced.

2.3.2. Collection and maintenance of ant colonies

Colonies of *Formica oreas* were collected in Surrey, B.C., Canada (49°10'04.7"N 122°41'57.8"W) in August 2020. Colonies were housed in plastic bins (66 × 40 × 35 cm) filled halfway with nesting material from collection sites. Bins were kept in the Science Research Annex (49°16'33.5"N 122°54'55.0"W) on the Burnaby campus of Simon Fraser University exposed to a 12:12 light/dark cycle. Ants were provisioned with mealworms, *Tenebrio molitor,* German cockroaches, *Blattella germanica*, American cockroaches, *Periplaneta americana*, apple slices, and a 20-% sugar water solution *ad libitum*.

2.3.3. General experimental design

Deterrent effects of test stimuli on behavioural responses of ticks were bioassayed in still-air Pyrex glass olfactometers (Fig. 2.1B), consisting of one central chamber and two lateral chambers (each d = 9 cm, h = 5 cm), linearly interconnected by glass tubes (d = 1 cm, l = 3 cm) [42]. Treatment and control stimuli were assigned to the

two lateral chambers, alternating the position of stimuli between replicates to account for potential side bias. Both lateral chambers were also fitted with a wet cotton ball (Thermo Fisher Scientific, MA, USA) to ensure sufficiently high humidity. To initiate an experimental replicate, a single tick was introduced into the central chamber, briefly exposed to human exhale to stimulate movement, and then allowed 20 h to respond. A tick was considered a responder, if it was found in a lateral chamber, or in a connecting glass tube closer to a lateral chamber than the central chamber (Fig. 2.1). All other ticks were deemed non-responders and excluded from statistical analyses but were reported in figures. To prevent tick escape, all three chambers of the olfactometer were sealed with Parafilm (Bemis, WI, USA) for the duration (20 h) of the experiment. To minimize the potential for tick escape, and to prevent ticks from sensing cues (e.g., convective heat, infrared radiation, CO_2) originating from experimentalists that initiated or scored experiments, olfactometers (n = 10-15) were housed in a plexiglass box (112 × 24 × 14 cm). Experiments were run under a 14:10 light/dark cycle, thus enabling ticks to respond to test stimuli while maintaining a circadian rhythm. After each experiment, olfactometers were washed with Sparkleen (Thermo Fisher Scientific), thoroughly rinsed with distilled water, and dried at 100 °C for > 1 h.

2.3.4. Specific experiments

Hypothesis 1: Ant semiochemical deposits deter ticks

Collection, and behavioral effects, of chemical deposits from worker ants

Both lateral chambers of olfactometers were fitted with a piece of filter paper (d = 90 mm; Cytiva, MA, U.S.A). To collect chemical deposits of worker ants, the glass tube connecting the randomly assigned lateral treatment chamber to the central chamber was blocked with a damp cotton ball, and 20 cold-anaesthetized (-15 °C for 5 min) ants were introduced into the treatment chamber, which was then sealed with parafilm and covered with a petri dish lid, as was the control chamber. After the ants had roamed 16 h in the treatment chamber, both the treatment and the control chamber were ‘unsealed’, and the ants were allowed to leave the treatment chamber on their own accord, thus minimizing agitation. Then, the cotton ball block was removed from the connecting tube, a tick was introduced into the central chamber, the olfactometer was sealed with parafilm, and the bioassay replicate was initiated. Ant-soil filter paper (see above) was tested for avoidance responses of male ticks (Exp. 1, n = 40) and female ticks (Exp. 2, n = 40).

Extraction of ant chemical deposits from filter paper

Filter paper previously cut into squares (2.5–5 mm) and exposed to ants for 16 h (see Exps. 1, 2) was placed into 20-mL scintillation vials for extraction of ant chemical deposits. Each of four scintillation vials received filter paper squares originating from three 90-mm wide filter paper discs. To extract both polar and non-polar compounds, filter papers were sequentially extracted in hexane (7 mL/vial × 4 vials) and dichloromethane (DCM; 7 mL/vial × 4 vials), each for 20 min at room temperature. Following extractions, hexane and DCM extracts were pipetted into separate clean 20-mL scintillation vials. Prior to analyses, combined hexane extracts and combined DCM extracts were concentrated to 12 mL each under a nitrogen stream.

Hypothesis 2: Deterrent semiochemicals originate from the ants' poison and/or Dufour's gland(s).

Extractions of poison and Dufour's glands

Worker ants were collected from laboratory colonies (see above) and cold-euthanized in a -15 °C freezer, where they remained until dissection (up to 4 days). Ants were dissected in chilled, distilled water under a dissecting microscope (ZEISS Stemi 2000), using fine-tipped forceps (Almedic, FR, CH) and insect pins. In total, 310 poison glands (with reservoirs) and 315 Dufour's glands were excised and placed in separate 4-mL glass vials (VWR International, PA, U.S.A) each containing DCM (1 mL). To minimize passive emanation of gland constituents from open vials during dissections, vials were kept on ice. To facilitate gland extractions, both samples were first vortexed for 60 s to homogenize gland tissues and then kept 15 min at room temperature. Following extractions, samples were filtered through glass wool into clean 4-mL glass vials capped with Teflon-lined lids. To minimize cross-contamination between poison gland and Dufour's gland constituents, all tools were cleaned with DCM between gland excisions, and ruptured glands were omitted. Both filter paper extract and gland extracts were analysed to determine the origin of chemical constituents in filter paper extract that proved deterrent to ticks in experiments 1 and 2 (see Results; Fig. 2.2).

Behavioural effects of poison and Dufour's gland contents

Parallel experiments 3–8 (n = 40 each) tested avoidance behaviour of male and female ticks in response to poison gland extract (Exps. 3, 4), Dufour's gland extract (Exp. 5, 6), and both combined (Exps. 7, 8), all *versus* a solvent control. Treatment

stimuli were presented at one gland equivalent (Table 1) dissolved in 500 µL of DCM, whereas an equal volume of DCM was used as the control stimulus. Stimuli were applied dropwise evenly spread across the 90-mm wide filter paper disc. After 5 min of DCM evaporation, a tick was placed into the central chamber of the olfactometer, and the bioassay was initiated.

With experimental data demonstrating that poison gland extract and Dufour's gland extract in combination elicit avoidance responses by ticks (see Results, Fig. 2.4), experiments 9–14 tested avoidance behaviour of ticks in response to synthetic equivalents of compounds present in poison gland extract (Exps. 9, 10), Dufour's gland extract (Exps. 11, 12), and both combined (Exp. 13, 14). Like gland extracts, synthetic blends were tested at one gland equivalent, and were applied in 500 µL DCM, with the same volume of DCM serving as the control stimulus.

Hypothesis 3: The tick-deterrent semiochemicals serve as alarm-recruitment pheromone components in ants.

Collection of *F. oreas* defensive sprays

To elicit defensive behaviour by ants, a pair of fine-tipped forceps was inserted vertically into the nesting box with the tips touching the substrate. Once a single ant had bitten onto the forceps indicating defensive behaviour, the forceps – together with the ant hanging on them – were withdrawn, and a piece of filter paper (5 × 20 mm) was placed for 10 s under the ant's abdominal tip to capture her defensive spray(s). Then, the filter paper was extracted sequentially in hexane (500 µL) and DCM (500 µL) for 60 s each. This process was repeated with 20 ants randomly selected from three laboratory colonies. Prior to analyses of samples, they were concentrated to 100 µL under a nitrogen stream.

Ant recruitment to micro-locations treated with poison and Dufour's gland semiochemicals

To test alarm-recruitment behaviour of *F. oreas* worker ants in response to poison and Dufour's gland semiochemicals, we followed an established protocol [43]. For each bioassay replicate (n = 20), two filter paper discs (90 mm each) were placed in a plexiglass bioassay arena (64 × 44 × 10 cm) 41 cm apart. By random assignment, one filter paper was treated with a synthetic blend of formic acid and hydrocarbons dissolved in DCM (10 µL) at one gland equivalent (Table 1), whereas the control disc

received only DCM (10 µL). To initiate a bioassay, a 15-mL Falcon tube (Thermo Fisher Scientific, Waltham, MA, USA) containing five ants was placed into the arena such that the tube's tapered tip was flush with the arena floor and equidistant to the two filter paper discs. Then, the cotton plug was removed from the 0.7-cm-diameter hole cut in the tube's tip, thus allowing the ants to enter the arena on their own accord. Once the first ant had entered the arena, the ants' behavior was filmed (Canon EOS Rebel T7, Canon, Tokyo, Japan) for 150 s. Videos were reviewed using VLC Media Player (Version 3.0.17.4), and visits to each of the two filter paper discs were counted. Multiple visits were counted for a single ant, if she had completely left the filter paper disc between visits.

Chemical analyses of ant-soiled filter paper, gland extracts, and defensive sprays

Aliquots (2 µL) of filter paper extracts in hexane and DCM were analyzed in splitless mode (purge valve open for 0.8 min) by gas chromatography-mass spectrometry (GC-MS), using an Agilent 7890B gas chromatograph (GC) fitted with a DB-5 GC-MS column (30 m × 0.25 mm ID, film thickness 0.25 µm), and coupled to a 5977A MSD. The GC injector port was set to 250 °C, the MS source to 230 °C, and the MS quadrupole to 150 °C. With helium as the carrier gas (flow rate: 35 cm s^{-1}), the following temperature program was used: 40 °C held for 5 min, 10 °C min^{-1} to 280 °C (held for 10 min). Compounds in extracts were identified by comparing their retention indices [44] and mass spectra with those of authentic standards that were purchased or synthesised. Double bond positions in unsaturated hydrocarbons were determined by treating aliquots of extracts with dimethyl disulfide (DMDS) [45], and by analysing DMDS derivatives for double bond positions. To test for the presence of formic acid which chromatographs poorly and thus is easily missed or incorrectly quantified, further aliquots of extracts were treated with 1-decanol to derivatize formic acid to decyl formate which readily chromatographs [46].

Poison and Dufour's gland extracts were analysed using the same protocol. However, because derivatization of formic acid to decyl formate enabled detection, but not accurate quantification, of formic acid in poison gland extract, formic acid was quantified instead using a 7964 Agilent Headspace Sampler coupled to a Varian 2000 Ion Trap GC-MS fitted with a DB-FATWAX Ultra Inert GC column (30 m × 0.25 mm ID). To this end, we applied one gland equivalent of poison gland extract to filter paper

(Cytiva, MA, U.S.A) in a 20-mL vial, which was then sealed with a 20-mm OD silicon septum and a crimped cap. The vial was heated to 150 °C, and headspace volatiles were withdrawn with an automated syringe and subjected to GC-MS analysis, using the following temperature program: 40 °C (10 min), 10 °C min^{-1} until 200 °C.

Defensive sprays were analyzed using the same protocol as described for the filter paper extracts.

2.3.5. Purchase and synthesis of semiochemicals

Undecane, tridecane, heptadecane, (Z)-9-tricosene, pentadecane, and (Z)-9-heneicosene were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was purchased from Anachemia Science (Rouses Point, NY, USA). (Z)-4-Tridecene and (Z)-9-nonadecene were synthesized in our laboratory as previously described [47].

2.3.6. Statistical analysis

All data were analyzed using R-studio (Version 2022.07.2+576), and all figures were prepared using R-studio and Inkscape (Version 0.92.4). The glmmTMB [48], DHARMa [49], and ggeffects [50] packages were used to aid in analyses, and the scales package [51] was used to aid in creating figures. When we tested for potential side bias in pre-screening experiments, ticks – in the absence of any test stimuli – chose equally often either one of the two lateral chambers. Tick avoidance behavior in response to treatment stimuli was then assessed by comparing the ratio of treatment and control responses to a hypothetical response ratio of 1:1, using a two-sided exact binomial test and excluding non-responders from analyses. This statistical approach aligns with the best practices for analysis of data collected in dual-choice olfactometer bioassays [52]. A Cohen's G test was used to calculate and categorize effect sizes as 'negligible', 'small', 'medium', or 'large' based on guidelines established by Cohen (1988). Ant attraction to filter paper discs treated with synthetic ant semiochemicals was modelled using a zero-inflated generalized linear mixed model (GLMM) with a Poisson distribution, using treatment as a fixed effect and replicate as a random effect. We assessed the effect of treatment using a likelihood ratio test.

2.4. Results

2.4.1. Hypothesis 1: Ant semiochemical deposits deter ticks

Behavioural effects of chemical deposits from worker ants

When ticks in olfactometers (Fig. 2.1B) were offered a choice between filter paper with or without ant chemical deposits, female and male ticks were significantly deterred by filter paper soiled with ant chemical deposits (exact binomial tests; Exp. 1: females, n = 27, p = 0.0015; Exp. 2: males, n = 25, p = 0.015; Fig. 2.2). There was a 'large' effect size for avoidance responses by females (Cohen's g: 0.26) and males (Cohen's g: 0.31).

Identification of ant chemical deposits in filter paper extracts

GC-MS analyses of filter paper extracts in DCM and hexane, before and after chemical derivatization, revealed the presence of formic acid and hydrocarbons, respectively. The hydrocarbons consisted of undecane, tridecane, pentadecane, heptadecane, (*Z*)-4-tridecene, (*Z*)-9-tricosene, (*Z*)-9-nonadecene, and (*Z*)-9-heneicosene.

2.4.2. Hypothesis 2: Deterrent semiochemicals originate from the ants' poison and/or Dufour's gland(s)

In two-choice olfactometers (Fig. 2.1B), ticks were deterred neither by poison gland extract (exact binomial tests; Exp. 3, females, n = 18, p = 0.81; Exp. 4, males: n = 19, p = 0.36) nor by Dufour's gland extract (exact binomial tests; Exp. 5: females, n = 20, p = 0.82; Exp. 6: males, n = 26, p = 0.56; Fig. 2.4). However, ticks were significantly deterred by extract of both the poison gland and the Dufour's gland (binomial test; Exp. 7: females, n = 24, p = 0.0066; Exp. 8: males, n = 23, $p = 6.6 \times 10^{-5}$). There was a 'large' effect size for avoidance responses by females (Cohen's g = 0.29) and males (Cohen's g = 0.41) to combined gland extracts.

Identification of compounds in gland extracts

GC-MS analyses of gland extracts in DCM, before and after chemical derivatization, revealed the presence of formic acid in poison gland extracts and of hydrocarbons in Dufour's gland extracts (Fig. 2.3). Of all compounds detected, formic

acid was most abundant followed – in order of decreasing abundance – by undecane, tridecane, (Z)-4-tridecene, heptadecane, (Z)-9-tricosene, pentadecane, (Z)-9-nonadecene, (Z)-9-heneicosene (Table 1).

Behavioral responses of ticks to synthetic poison and Dufour's gland constituents

In two-choice olfactometer bioassays (Fig. 2.1B), ticks were deterred neither by formic acid (poison gland constituent) (exact binomial tests; Exp. 9: females, n = 32, p = 0.60; Exp. 10: males, n = 32, p = 0.11; Fig. 2.5) nor by hydrocarbons (Dufour's gland constituents) (exact binomial tests; Exp. 11: females: n = 32, p = 0.60; Exp. 12, males, n = 36, p = 1.0; Fig. 2.5). However, formic acid and hydrocarbons in binary combination deterred ticks (exact binomial tests; Exp. 13: females, n = 30, p = 0.043; Exp. 14: males, n = 33, p = 0.035). There was a 'medium' effect size for the avoidance responses by females (Cohen's g = 0.20) and males (Cohen's g = 0.20) to formic acid and hydrocarbons in combination.

2.4.3. Hypothesis 3: Tick-deterrent semiochemicals serve as alarm-recruitment pheromone components in ants.

Identification of compounds in defensive sprays

GC-MS analyses of filter paper sprayed upon by distressed single workers of *F. oreas* revealed formic acid and all the hydrocarbons identified in the poison and Dufour's gland extract, respectively (Table 1), indicating that the ants discharged the content of both glands in their defensive sprays that were experimentally provoked.

Ant recruitment to micro-locations treated with synthetic poison and Dufour's gland semiochemicals

Synthetic blends of poison and Dufour's gland pheromone components elicited alarm-recruitment responses by *F. oreas* workers in arena bioassays (Fig. 2.6). Workers visited micro-locations treated with formic acid and hydrocarbons significantly more often than micro-locations treated with a solvent control (likelihood ratio test; $\chi^2_{(df = 1)} = 40.5$, $p = 1.6\times10^{-9}$).

2.5. Discussion

Our findings support the hypotheses that (1) chemical deposits of *F. oreas* worker ants deter *I. scapularis* ticks, (2) the deterrent semiochemicals originate from the ants' poison and Dufour's glands, and (3) the tick-deterrent semiochemicals serve as alarm-recruitment pheromone components of *F. oreas* workers. Combined, the formic acid that ants discharge from their poison glands, and the various hydrocarbons that ants discharge from their Dufour's gland, produce the pheromone blend that attracts nestmates and deters ticks.

The deterrence of ticks in bioassays was contingent upon the presence of both poison and Dufour's gland extracts, or their respective constituents (Figs. 2.4, 2.5). Expectedly, both poison and Dufour's gland constituents were present in defensive sprays of *F. oreas* workers, indicating that distressed ants discharge the content of both glands to alarm and recruit nestmates, and that *I. scapularis* ticks eavesdrop on the ants' complete array of alarm-recruitment communication signals.

Synergism between alarm-recruitment pheromone components from the poison and the Dufour's gland is common in formicine ants. This type of synergism was first noted in the carpenter ant *Camponotus pennsylvanicus,* where distressed workers spray formic acid together with *n*-undecane. In *C. pennsylvanicus*, formic acid stimulates frenzied running behaviour in nestmates and even, by itself, attracts them, but its attractiveness is greatly increased in combination with *n*-undecane which is mildly attractive alone [53]. Similarly, workers of Western carpenter ants, *Camponotus modoc*, convey distress using two acids (formic, benzoic) and a set of alkanes from poison and Dufour's glands, respectively [43]. Formic acid was the most abundant constituent in the poison gland of *F. oreas* workers, as was undecane in the ants' Dufour's gland (Fig. 2.2). The same chemical constituents are released from poison and Dufour's glands of other formicine ants [23–26,28,29,32–35], suggesting that ticks could exploit them as generic cues indicative of predation risk by ants. That the synthetic blend of formic acid and alkanes/alkenes in this study was not as deterrent to ticks as combined extracts of the poison and Dufour's gland is attributed to contrasting release dynamics of natural and synthetic compounds, rather than missing pheromone components in the synthetic blend. We predict that exocrine gland secretions contain constituents that slow the release of volatile pheromone components, such as formic acid, comparable perhaps to

the role of major urinary proteins in urine deposits of rodents that facilitate sustained release of volatile sex pheromone components [54].

Poison and Dufour's gland secretions of ants serve both communicative and sanitary functions. In formicine ants, formic acid from the poison gland and hydrocarbons from the Dufour's gland alarm and recruit nestmates [23–29,35,55]. Moreover, workers of *Formica paralugubrius* spray formic acid – likely in combination with the Dufour's gland content – as a potent disinfectant on their nesting material [30], as do workers of the leaf-cutting ant *Acromyrmex subterraneus subterraneus* and the Southeast Asian weaver ant *Polyrhachis dives* [31]. Formic acid is effective against *Metarhizium*, a common fungal pathogen of ants [30,56]. The distinctively acidic smell of *F. oreas* nest mounts (CG, person. obs.) – even in the absence of any defensive behaviour – again implies the use of formic acid as a disinfectant, but this inference has still to be experimentally tested. While ants spray formic acid to disinfect their nesting material, ticks may eavesdrop on these disinfectant sprays to evade ant predation. Nest mounds and their immediate surroundings would have the highest concentration of formic acid and Dufour-gland hydrocarbons, and thus would signal severe predation risk. This concept would explain why the volume of *F. polyctena* nesting material was inversely correlated with tick abundance near nests [13]. Avoiding areas with significant formic acid smell would help reduce ant predation risk and thus be adaptive to *I. scapularis.* Even if some formicine ants were to use formic acid and Dufour-gland hydrocarbons only for communicative functions, their frequent use may still result in high concentrations near nests. Currently, *I. scapularis* is thought to engage in minimal (< 1 m) lateral off-host movement [57], but how far ticks may move in response to ant predatory cues to lower ant predation risk has not yet been studied in field experiments.

As ticks eavesdrop on pheromonal signals released from the poison and Dufour's glands of *F. oreas*, they may conceivably eavesdrop also on ant communication signals originating from other exocrine glands such as mandibular glands. In *F. oreas*, the functional role of mandibular gland constituents is not known but mandibular gland constituents of other ants play roles in the context of mating or alarm signaling [58,59]. The chemical composition of mandibular glands is complex, including compounds such as citronellol, citronellal, *cis*-citral, limonene, cymen, methyl salicylate, and geranial [26,60,61]. Citronellol and citronellal in mandibular glands of *Lasius umbratus* elicit alarm and defensive behaviour in nestmates [57]. Serving as mandibular gland pheromone

components of ants, these components – like poison and Dufour-gland pheromone components – could be deterrent to ticks, because some of these compounds occur in plant essential oils which are repellent to ticks [62–64]. The deterrence of plant essential oils to ticks has often been credited to their strong odor or potential toxicity at high concentration [62] but, instead, may be due to in the presence of constituents also in ant exocrine glands. Regardless, it would be interesting to test mandibular gland constituents for tick deterrence and potential synergism between poison, Dufour's and mandibular gland constituents.

In conclusion, we show that poison and Dufour's gland constituents of *F. oreas* worker ants synergistically deter female and male *I. scapularis* ticks, indicating that ticks eavesdrop on the ants' alarm communication signals. A synthetic blend of the glands' constituents – possibly in combination with other tick deterrents such as plant essential oils [64] – could be considered for development as (*i*) topical tick repellents directly applied to skin, (*ii*) tick repellents in clothing, and (*iii*) off-host tick repellents applied to areas highly frequented by humans. Woodchips are already applied along hiking trails to discourage ticks from questing on or near these trails, and could potentially be improved with the addition of deterrent ant semiochemicals [65]. Concern that formic acid – because of its acidic properties – is a dermal or ocular irritant can be dispelled because formic acid, properly formulated at low concentration, is already safely used in cosmetic products [66]. If synthetic ant semiochemicals were to be developed as tick deterrents for human protection, their deterrent effect would need be further tested in the presence of host cues that attract foraging ticks, e.g. deer-associated cues [67,68].

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2.7. Figures

Table 2.1. Mean amount (ng) of chemical constituents quantified in poison and Dufour's gland extracts of *Formica oreas* workers.

Compound	ng per gland equivalent	Gland
formic acid	10,000	poison
undecane	60,000	Dufour's
tridecane	5,100	Dufour's
(*Z*)-4-tridecene	3,600	Dufour's
heptadecane	1,140	Dufour's
(*Z*)-9-tricosene	900	Dufour's
pentadecane	840	Dufour's
(*Z*)-9-nonadecene	840	Dufour's
(*Z*)-9-heneicosene	240	Dufour's

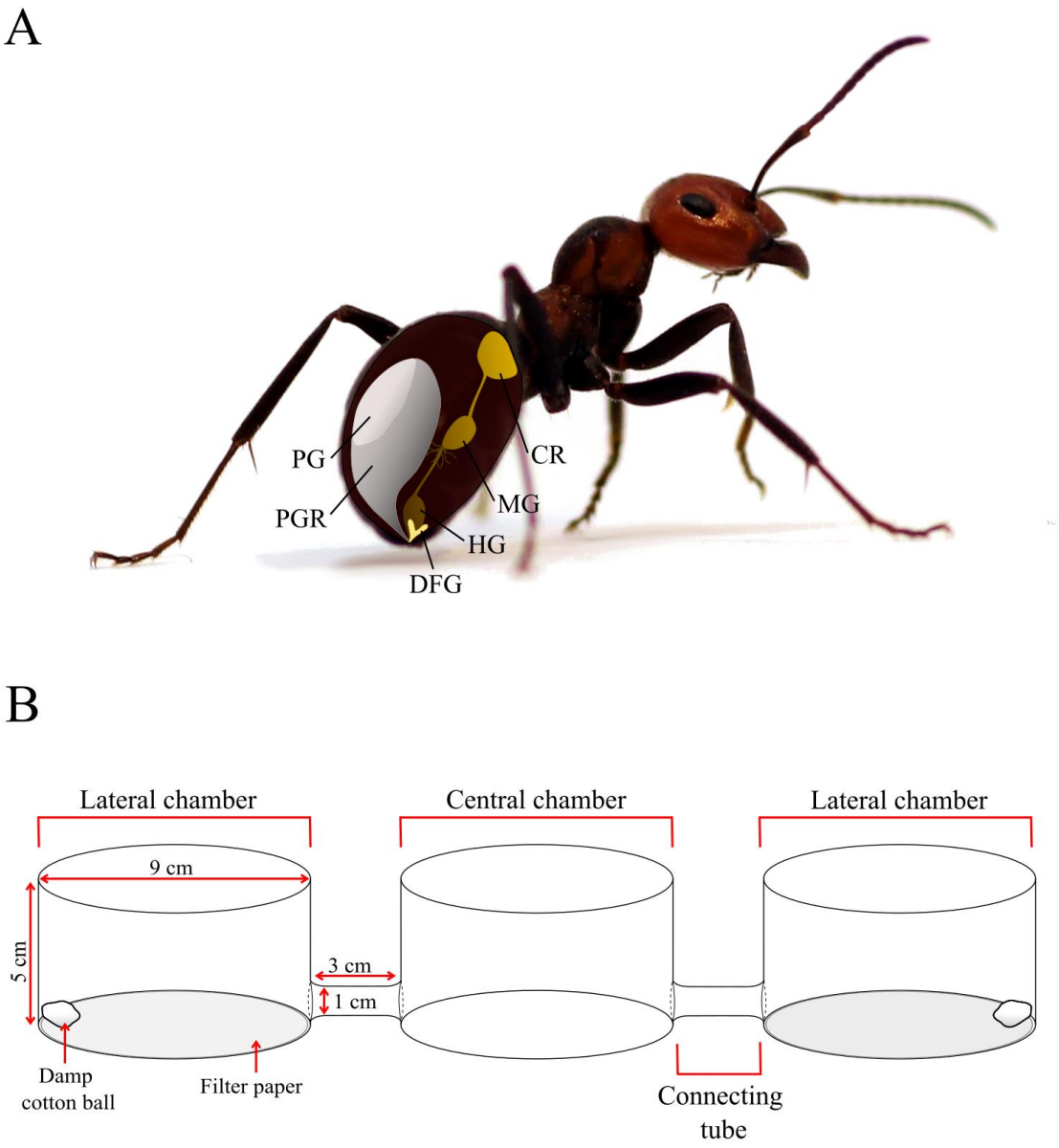

Figure 2.1. Drawings illustrating (A) the location of the poison gland and Dufour's gland in *Formica oreas* worker ants, and (B) the olfactometer used in tick bioassays. For bioassays, the lateral chambers of the olfactometer received a piece of filter paper treated with a treatment or control stimulus, and a damp cotton ball to increase relative humidity. A single bioassay tick was released into the central chamber, and was considered a responder, if it was found at the end of the bioassay in a lateral chamber, or in a connecting glass tube closer to a lateral chamber than to the central chamber. Abbreviations: PG = poison gland, PGR = poison gland reservoir, DFG = Dufour's gland, CR = crop, MG = midgut, HG = hindgut.

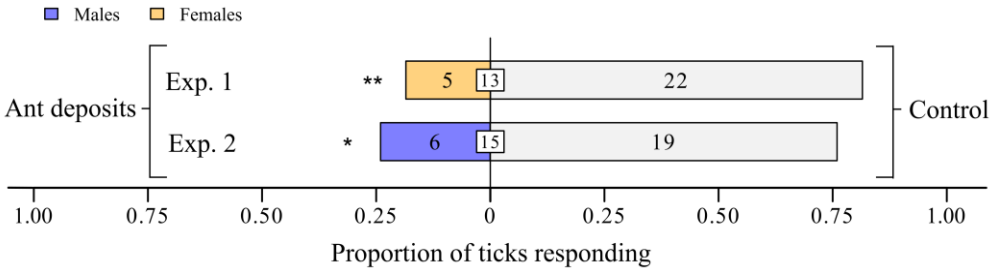

Figure 2.2. Proportion of female and male blacklegged ticks, *Ixodes scapularis,* responding in olfactometers (Fig. 2.1B) to filter paper previously soiled, or not (control), with chemical deposits of 20 *Formica oreas* worker ants. Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. Asterisks indicate significant avoidance of filter paper with ant chemical deposits (exact binomial tests; * $p < 0.05$, ** $p < 0.01$).

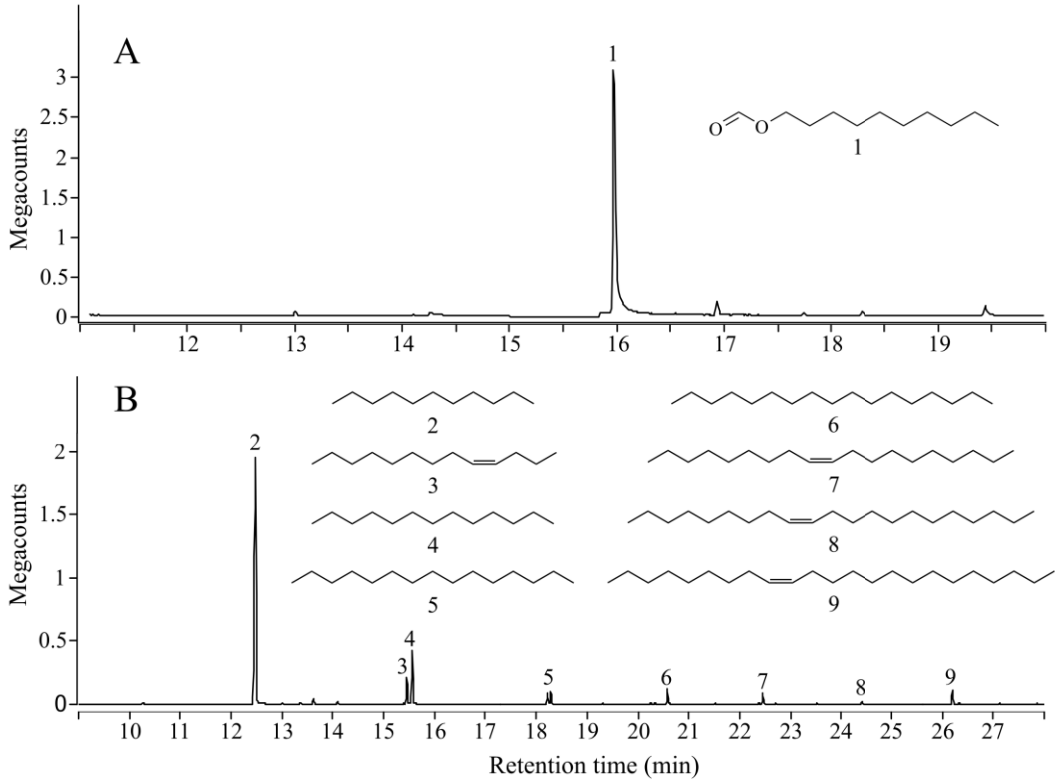

Figure 2.3. Total ion chromatograms of poison gland extract (A) and Dufour's gland extract (B) obtained from worker ants of Formica oreas. The poison gland extract was treated with 1-decanol to derivatize formic acid (which chromatographs poorly) to decyl formate (1). Constituents of the Dufour's gland are undecane (2), (Z)-4-tridecene (3), tridecane (4), pentadecane (5), heptadecane (6), (Z)-9-nonadecene (7), (Z)-9-heneicosene (8), and (Z)-9-tricosene (9). Chromatography: DB-5 column; temperature program: 40 °C (5 min), 10 °C min-1 to 280 °C (held 10 min).

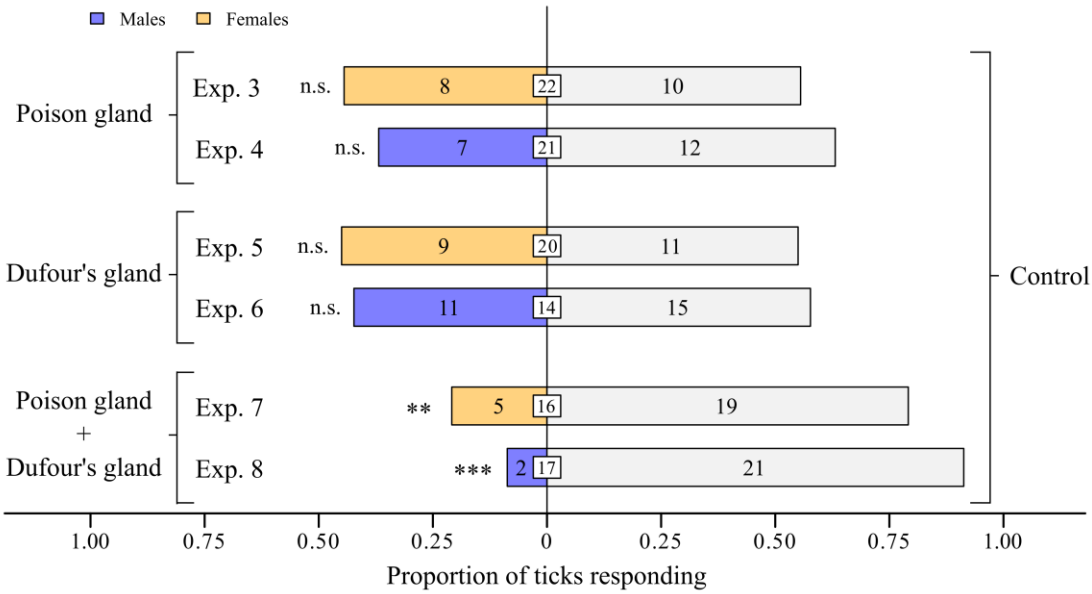

Figure 2.4. Proportion of female and male blacklegged ticks, *Ixodes scapularis*, responding in olfactometers (Fig. 2.1B) to extracts of the poison gland (Exps. 3, 4), Dufour's gland (Exp. 5, 6), or to both extracts combined (Exps. 7, 8), all obtained from worker ants of *Formica oreas* and tested at a dose of one gland equivalent. Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. Asterisks indicate significant avoidance of the treatment stimulus (exact binomial tests; ** $p < 0.01$, *** $p < 0.001$; n. s. = not significant).

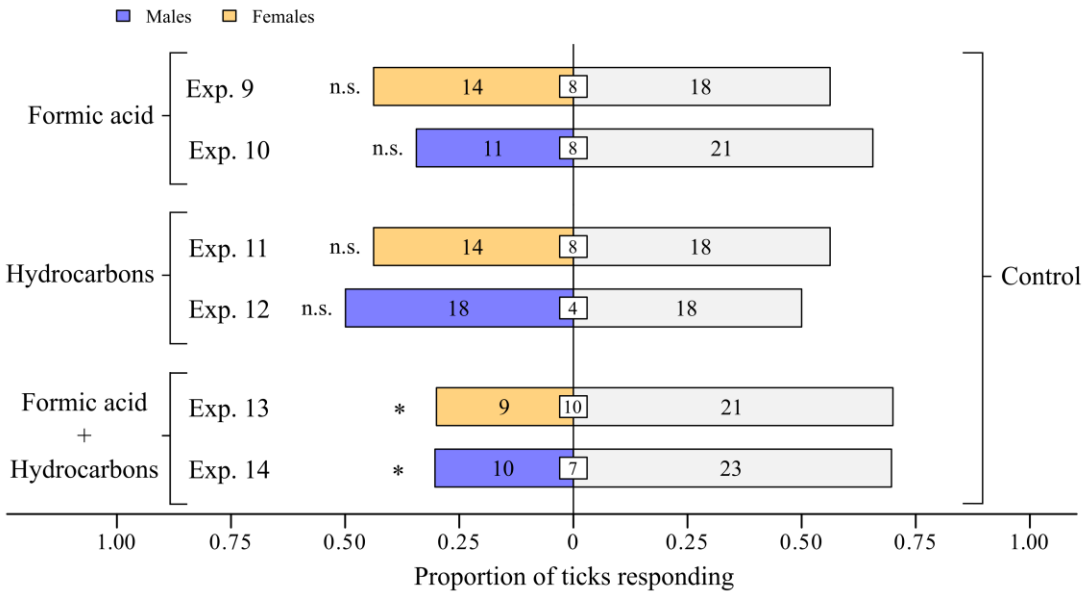

Figure 2.5. Proportion of female and male blacklegged ticks, *Ixodes scapularis*, responding in olfactometers (Fig. 2.1B) to synthetic compounds identified in the poison gland (formic acid), and in the Dufour's gland (various hydrocarbons (HCs); see Fig. 2.2) of *Formica oreas* workers ants. All compounds were tested at a dose of one gland equivalent (Table 1). Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. Asterisks (*) indicate significant avoidance of the treatment stimulus (exact binomial tests; $p < 0.05$; n. s. = not significant).

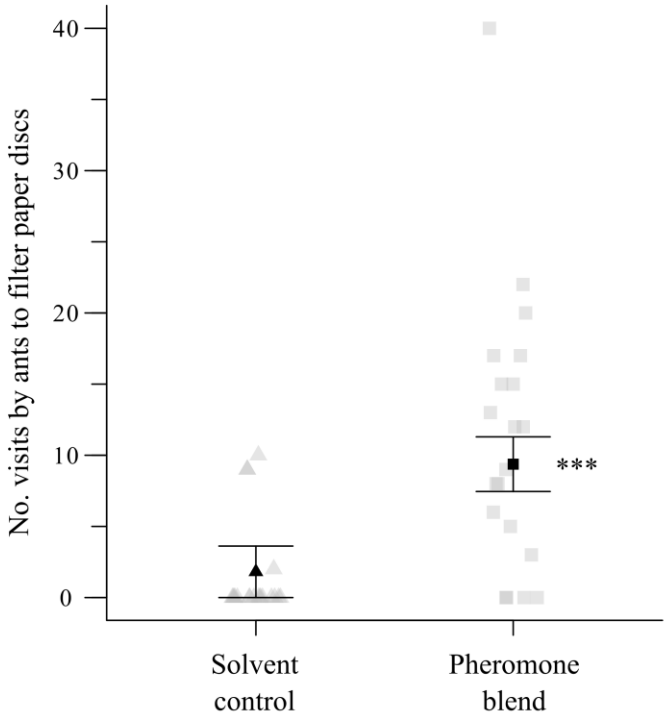

Figure 2.6. Number of visits by *Formica oreas* worker ants, tested in groups of 5 (n = 20), to paired filter paper discs placed 41 cm apart in a bioassay arena (64 × 44 × 10 cm), and treated – at one ant equivalent – with either a synthetic pheromone blend of poison and Dufour's gland components dissolved in dichloromethane (DCM) or a DCM solvent control. Grey symbols show the number of visits in each replicate and black symbols the estimated marginal means (± 95% CI). Asterisks (***) indicate significantly more visits to the disk treated with synthetic alarm-recruitment pheromone (likelihood ratio test; $p < 0.001$).

Chapter 3.

Harmful and harmless soil-dwelling fungi indicate microhabitat suitability for off-host ixodid ticks[1]

3.1. Abstract

Following blood meals or questing bouts, hard ticks (Ixodidae) must locate moist off-host microhabitats as refuge. Soil-dwelling fungi, including entomopathogenic *Beauveria bassiana* (*Bb*), thrive in moist microhabitats. Working with six species of ixodid ticks in olfactometer bioassays, we tested the hypothesis that ticks avoid *Bb*. Contrary to our prediction, nearly all ticks sought, rather than avoided, *Bb*-inoculated substrates. In further bioassays with female black-legged ticks, *Ixodes scapularis*, ticks oriented towards both harmful *Bb* and harmless soil-dwelling fungi, implying that fungi – regardless of their pathogenicity – signal habitat suitability to ticks. Only accessible *Bb*-inoculated substrate appealed to ticks, indicating that they sense *Bb*, or its metabolites, by contact chemoreception. *Bb*-inoculated substrate required ≥24 h of incubation before it appealed to ticks, suggesting that they respond to *Bb* metabolites rather than to *Bb* itself. Similarly, ticks responded to *Bb*-inoculated and incubated cellulose but not to sterile cellulose, indicating that *Bb* detection by ticks hinges on *Bb* metabolism of cellulose. 2-Methylisoborneol – a common fungal metabolite with elevated presence in disturbed soils – strongly deterred ticks. Off-host ticks that avoid disturbed soil may lower their risk of physical injury. Synthetic 2-methylisoborneol could become a commercial tick repellent, provided its repellency extends to ticks in diverse taxa.

[1]A nearly identical version of this chapter has been published in *Microorganisms* with the following authors: Claire E. Gooding, Layla Gould, Gerhard Gries.

3.2. Introduction

Ticks (Ixodida) are ectoparasites of vertebrates with diverse life histories, host-seeking strategies, and habitat preferences [1]. Most soft ticks (Argasidae) are nidicolous and remain within nests or burrows of a host throughout their entire life, repeatedly feeding on the same host species [2]. Hard ticks (Ixodidae) are typically non-nidicolous, seek hosts outside burrows, and typically engage in a 'questing' ambush strategy, climbing onto hosts as they pass by [3–7]. Alternatively, some species actively pursue hosts [8]. Unlike nidicolous ticks, non-nidicolous ticks must locate suitable off-host microhabitats to rest following questing attempts or blood-meals. Selection of these microhabitats is affected by tick-intrinsic physiological factors, arrestment pheromones, and microhabitat characteristics [9,10].

Behaviour, survival, and distribution of ixodid ticks are all affected by the availability and quality of questing locations and off-host microhabitats. The timing and duration of questing are dependent upon ambient relative humidity and sunlight exposure [11–13]. While questing, ticks lose water and periodically must replenish it in humid leaf litter and detritus [11]. High humidity, low sunlight exposure, and sufficient organic matter (e.g., leaf litter) to provide refuge are key requisites of suitable off-host microhabitats for ticks [14–19].

The microbiome of resource sites informs foraging decisions of arthropods both directly and indirectly. For example, nectar-dwelling microbes affect nectar-foraging decision of mosquitoes (direct effect) [20,21], whereas the root microbiome of plants affects above-ground herbivory (indirect effect) [22,23]. Moreover, many dipterans including mosquitoes and stable flies select oviposition sites based on their microbial community [24–27]. Attraction of *Ixodes* ticks to their vertebrate hosts is mediated, in part, by volatiles emitted from host skin microbiota [28]. However, whether the microbiota in soil and leaf litter informs selection of off-host microhabitats by ticks is largely unknown. Other soil-dwelling arthropods select microhabitats based on the presence of certain microorganisms. For example, springtails, *Folsomia candida*, are attracted to soil colonized by edible bacteria [29], whereas red imported fire ant queens, *Solenopsis invicta,* preferentially nest in soil colonized by bacteria that inhibit the growth of entomopathogenic fungi [30]. Both springtails and queen red imported fire ants are

attracted to the sesquiterpenoid geosmin and the monoterpene 2-methylisoborneol (2-MIB) emitted by cyanobacteria [25,31,32], actinobacteria [29,30,33], and fungi [34–36].

Moist off-host microhabitats offer not only abiotic benefits to ticks but also present biotic threats from pathogens and predators. To reduce predation risk, many arthropods, including *Ixodes scapularis* ticks [37], exploit chemical cues indicative of predator presence [37–44]. Entomopathogenic fungi, such as *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) (*Bb*) dwelling in soil and detritus [45–47], are lethal to ticks and other arthropods [48–50]. To reduce the risk of fungal infections, some arthropods avoid sites colonized by harmful fungi [51–56] but occasionally may even be attracted to them [57,58]. Arthropods may detect harmful fungi by sensing their volatile metabolites (olfaction) or by recognizing specific chemicals on the fungal surface (contact chemoreception). Ticks sense semiochemicals (message-bearing chemicals) using sensory receptors on their front legs and/or on their palps [59]. Conversely, harmless fungi present in shaded, damp litter and detritus [60–63] may be valuable indicators of suitable moist microhabitats that off-host ticks seek for refuge. The behavioural responses of ticks to harmless and harmful (entomopathogenic) fungi have not yet been investigated.

Here, we worked with females and males of six species of ixodid ticks that are taxonomically diverse and of medical and/or veterinary importance: the lone star tick, *Amblyomma americanum*, American dog tick, *Dermacentor variabilis*, brown dog tick, *Rhipicephalus sanguineus*, castor bean tick, *Ixodes ricinus*, western black-legged tick, *Ixodes pacificus*, and the black-legged tick, *Ixodes scapularis*. We tested the hypothesis that these ticks avoid substrate inoculated with *Bb*. With our data revealing that ticks seek, rather than avoid, *Bb*-inoculated substrate, we then studied underlying mechanisms for their behavioral responses, working with female *I. scapularis* as representative model organisms. Specifically, we tested whether preferential responses by female *I. scapularis* to *Bb*-inoculated substrate is dependent upon (*i*) the *Bb* incubation period, (*ii*) olfactory or contact-chemoreceptive recognition of *Bb* or its metabolites, and (*iii*) the presence of cellulose as a *Bb* growth medium. We further investigated whether female *I. scapularis* also seek harmless soil-dwelling fungi as indicators of suitable off-host refuges, and whether common volatiles of soil-dwelling fungi attract ticks to off-host refuges.

3.3. Materials and Methods

3.3.1. Tick maintenance

Adult males and females of *I. scapularis*, *I. ricinus*, *I. pacificus*, *D. variabilis*, *A. americanum*, and *R. sanguineus* were obtained from BEI Resources (American Type Culture Collection), and additional *I. scapularis* adults were purchased from the National Tick Research and Education Resource (Oklahoma State University). We tested adult ticks because – based on our experience – they tolerate laboratory conditions better than immature ticks. Ticks were maintained at 22 °C under a 14:10 light:dark cycle and housed singly in 1.5-mL microcentrifuge tubes (Corning Inc., Reynosa, MX) with a mesh-covered 5-mm hole in the lid for ventilation. Sets of 10–20 tubes were held in 150-mL plastic cups with lids which, in turn, were enclosed in clear plastic bins (46 × 32 × 18 cm). Damp cotton rounds (Dollarama, QC, CA) placed into cups provided sufficient humidity (80–90% RH). Weekly, cotton rounds were replaced, and any deceased ticks were removed to prevent potential infections.

3.3.2. Propagation of fungi and collection of conidia

Beauveria bassiana (GHA) was provided by the Cory-lab at Simon Fraser University, and *Fusarium oxysporum*, *Penicilium roqueforti*, and *Rhizopus stolonifer* were purchased from Merlan Scientific (Toronto, ON, CA). Fungi were propagated on Sabouraud dextrose agar (SDA) plates (90 × 15 mm) which were sealed with Parafilm (Bemis, WI, USA) and incubated 7 days at 26–28 °C. To harvest conidia, each plate was flooded with 50 mL of sterile 0.05% Tween80 (Sigma Aldrich, MO, USA) and gently scrubbed using a sterile inoculating loop to create a conidial suspension. This suspension was then filtered through sterile glass wool to minimize mycelial or agar debris, and the conidial suspension was vortexed 30 s to homogenize. Concentrations of conidial suspensions were determined using a Neubauer improved Hemocytometer (Superior Marienfeld, BW, DE), and diluted to a concentration of 10^7 conidia/mL for all experiments. The dose of 10^7 conidia/mL elicited the strongest behavioural responses in a preliminary dose-response experiment (see Supplementary Materials).

3.3.3. Preparation of experimental substrates

Sterile dry coconut fibre (PetSmart, Arizona, USA) was placed in 5-mL aliquot doses into 50-mL sterile centrifuge tubes (Cornell, Tamaulipas, MX) which were inoculated with either a 1-mL conidial suspension (treatment), or a 1-mL sterile 0.05% Tween80 solution (control). Centrifuge tubes were incubated 24 h at 26–28 °C, unless otherwise noted. Coconut fibre was used as a fungal growing medium because it comprises constituents (cellulose, lignin, hemicelluloses [64]) which are common in partially decomposed plant matter, where ticks are likely to take refuge [65].

To produce substrate texturally similar to coconut fibre and based only on cellulose, filter paper discs (90 mm diam.) were shredded for 5 min in an Osterizer 10-speed blender (Sunbeam Products, Fl, USA), producing 2- to 5-mm pieces. Aliquots (5 mL) of shredded filter paper were placed into centrifuge tubes and inoculated with either a 1-mL conidial suspension (treatment), or a 1-mL sterile 0.05% Tween80 solution (control).

3.3.4. General experimental design

All behavioral responses of ticks to experimental substrates were tested in two-choice still-air olfactometers (150 × 50 × 17 mm; Fig. 3.1). We used still-air, instead of moving-air, olfactometers because off-host ticks encounter fungi in soil microhabitats such as leaf litter where there is typically little, if any, air movement. Each olfactometer had three inset circular chambers (ID = 28 mm) inter-connected with inset linear paths (24 × 10 × 7 mm). The central chamber had a depth of 7 mm, whereas the lateral chambers had a depth of 16 mm with a 2-mm wide lip of 9-mm depth to accommodate a 9-mm watch glass. Olfactometers were modeled and 3D-printed using Autodesk Fusion 360 (Version 13.2.0.9150), Creality Slicer (Version 4.8.2), and an Ender-3 Pro 3D printer (Creality, Shengzhen, CN). Olfactometers were printed using translucent 1.75 mm (± 0.03 mm dimensional accuracy) polylactic acid (PLA) filament (GIANTARM, OH, USA). To reduce the porosity of 3D-printed olfactometers, we applied XTC-3D brush-on epoxy coating (Smooth-On Inc., PA, USA). The stereolithography (STL) file used for 3D printing is included in the Supplementary Materials.

Treatment and control experimental substrates were assigned to lateral chambers, alternating the position of stimuli between replicates to account for potential side bias. Substrates were poured into the wells of lateral chambers and leveled to create a uniformly flat surface. To initiate an experimental replicate, a single tick was introduced into the central chamber, briefly exposed to human exhale to stimulate movement, and then allowed 30 min to respond. A 30-min bioassay time was deemed sufficient because in pre-screening tests 80% of female *I. scapularis* left the central olfactometer chamber within 30 min. Olfactometers were sealed with parafilm and a rectangular lid (150 × 50 × 3 mm). A tick was considered a responder, if it was found in a lateral chamber after 30 min. All other ticks were deemed non-responders and excluded from statistical analyses but were reported in figures. After each experiment, olfactometers were cleaned with 70% ethanol and hexane. At the end of each experimental day, olfactometers were washed with Sparkleen (Thermo Fisher Scientific, MA, U.S.A), rinsed with distilled water, and air-dried.

3.3.5. Specific experiments

Effect of Bb-inoculated substrate on behavioral responses of diverse tick taxa

To investigate whether harmful *Bb* is a generic deterrent to diverse taxa of ticks, experiments 1–12 tested behavioural responses of adult female and male *A. americanum, D. variabilis, R. sanguineus, I. ricinus*, *I. pacificus*, and *I. scapularis* to coconut fibre inoculated, or not (control), with a *Bb* conidial suspension. Thirty replicates were run for each sex of each species, except that 40 replicates were run for female and for male *R. sanguineus* due to a high rate of non-responding ticks.

Effect of Bb-incubation period on behavioral responses of ticks

With evidence that ticks – unexpectedly – sought, rather than avoided, *Bb*-inoculated coconut fibre (see Results), we then investigated whether the ticks' preferential responses to *Bb*-inoculated fibre were contingent upon the *Bb*-incubation period. To this end, experiments 13–17 (n = 30 each) tested behavioural responses of female *I. scapularis* to fibre inoculated, or not (control), with a conidial suspension of *Bb* incubated at 26–28 °C for 0, 24, 48, 72, and 96 h prior to bioassays.

Olfactory or contact-chemoreceptive recognition of Bb (or its metabolites) by ticks

To investigate whether preferential responses of ticks to *Bb*-inoculated coconut fibre (see Results) were mediated by olfaction (i.e., receptors sensing airborne semiochemicals) or by contact chemoreception (i.e., receptors sensing substrate-borne stimuli through physical contact), parallel experiments 18–19 (n = 30 each) tested responses of ticks to coconut fibre which was physically accessible (Exp. 18) or not (Exp. 19). Coconut fibre was made inaccessible by placing a mesh screen (28 mm diam.) in each lateral chamber and by sealing it around its edge with plasticine to prevent ticks from passing under the screens. Test stimuli in both experiments consisted of fibre inoculated, or not (control), with *Bb*.

Effect of cellulose, or its fungal metabolites, on behavioral responses of ticks

Because ticks favourably responded to *Bb*-inoculated coconut fibre only after incubation/metabolism for at least 24 h (see Results), it was conceivable that ticks responded to *Bb* metabolites of cellulose, a constituent in experimental coconut fibre (cellulose, lignin, hemicelluloses), and a major component of plant cell walls which fungi commonly metabolize. To investigate this concept, parallel experiments 20 and 21 tested the responses of female *I. scapularis* to cellulose-only substrate (Exp. 20), and to coconut fibre (Exp. 21), with either substrate in both experiments inoculated (treatment), or not (control), with *Bb*.

Effect of various soil-dwelling fungi on behavioral responses of ticks

To investigate whether not only *Bb* but also other soil-dwelling fungi elicit preferential responses by ticks, experiments 20–23 (n = 30 each) offered female *I. scapularis* a choice between coconut fibre inoculated, or not, with a 1-mL 10^7conidia/mL suspension of *R. stolonifer* (Exp. 22), *F. oxysporum* (Exp. 23), *P. roqueforti* (Exp. 24), and *B. bassiana* (Exp. 25).

Effect of fungus-derived volatiles on attraction of ticks

As ticks sought substrate inoculated with various species of soil-dwelling fungi (see Results), and drawing on reports that springtails and *S. invicta* queen and worker ants are attracted to 2-methylisoborneol (2-MIB) and geosmin, which are commonly

emitted by fungi [34–36], we further investigated whether ticks, as well, are attracted to 2-MIB and geosmin. To this end, we placed a watch glass (28 mm diam.) fitted with a congruent piece of filter paper in lateral olfactometer chambers, and applied 2-MIB dissolved in 25 µL of methanol at doses of 1.0 ng (Exp. 26), 0.1 ng (Exp. 27), and 0.01 ng (Exp. 28) to the treatment filter paper, and 25 µl of methanol to the corresponding control filter paper. Similarly, we tested geosmin dissolved in 25 µL of methanol at doses of 1.0 ng (Exp. 29), 0.1 ng (Exp. 30), and 0.01 ng (Exp. 31), using 25 µL of methanol as the control stimulus. In each experimental replicate, methanol was allowed 5 min to evaporate before a tick was introduced into the central olfactometer chamber. The 2-MIB and geosmin dose range of 0.01–1 ng was deemed ecologically relevant, because *S. invicta* worker and queen ants were attracted to geosmin at a dose of 2 ng [30], and analyses of above-soil headspace volatiles yielded 0.2–9.0 ng/mL of 2-MIB and 0.01–0.7 ng/mL of geosmin over 20–30 min [66]. Geosmin and 2-MIB were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

All data were analyzed using R-studio (2023.03.1+446), and all figures were prepared using R-studio and Inkscape (Version 1.2.2). The scales package [67] was used to aid in creating figures. The ticks' responses to bioassay stimuli were analyzed by comparing the ratio of treatment and control responses to a hypothetical response ratio of 1:1, using a two-sided exact binomial test, and excluding non-responders from analyses.

3.4. Results

3.4.1. Effect of *Bb*-inoculated substrate on behavioral responses of ticks

All ticks – except for female and male *R. sanguineus* and female *I. pacificus* – tested in experiments 1–12 (Fig. 3.2) preferred *Bb*-inoculated coconut fibre to sterile (control) fibre [*A. americanum*; Exp. 1 (females): n = 27, p = 0.0059; Exp. 2 (males): n = 27, p < 0.0001; *D. variabilis;* Exp. 3 (females): n = 23, p = 0.035; Exp. 4 (males): n = 21, p = 0.027; *R. sanguineus;* Exp. 5 (females): n = 24, p = 0.54; Exp. 6 (males): n = 18, p = 0.096; *I. ricinus;* Exp. 7 (females): n = 28, p = 0.00091; Exp 8 (males): n = 25, p = 0.00091; *I. pacificus;* Exp. 9 (females): n = 28, p = 0.18; Exp. 10 (males): n = 17, p =

0.013; *I. scapularis;* Exp. 11 (females): n = 24, p = 0.0026; Exp. 12 (males): n = 26, p = 0.0025]. Altogether, the data indicate a widespread failure of ticks in diverse taxa to avoid a harmful entomopathogenic fungus.

3.4.2. Effect of *Bb*-incubation period on behavioral responses of ticks

Without prior incubation of *Bb*-inoculated coconut fibre, female *I. scapularis* responded equally to *Bb*-inoculated coconut fibre and to sterile fibre (Exp. 13: n = 20, p = 0.15; Fig. 3.3). In contrast, after an incubation period of 24, 48, 72, and 96 h, female *I. scapularis* invariably preferred *Bb*-inoculated coconut fibre to sterile fibre [24 h (Exp. 14): n = 26, p < 0.0001; 48 h (Exp. 15): n = 19, p < 0.0001; 72 h (Exp. 16): n = 25, p < 0.0001; 96 h (Exp. 17): n = 25, p = 0.0002; Fig. 3.3]. The data suggest that the responses of ticks are likely mediated, in part, by fungal metabolites produced during incubation.

3.4.3. Olfactory or contact-chemoreceptive recognition of *Bb* (or its metabolites) by ticks

Physical access to coconut fibre inoculated (treatment), or not (control), with *Bb* determined the ticks' behavioral responses (Fig. 3.4). When access to coconut fibre was blocked, female *I. scapularis* responded equally to treatment and control fibres (Exp. 18: n = 24, p = 0.31). However, when access was not blocked, females preferred *Bb*-inoculated fibre to sterile fibre (Exp. 19: n = 25, p = 0.015), indicating that recognition of *Bb* is based on contact chemoreception rather than olfaction.

3.4.4. Effect of cellulose, or its fungal metabolites, on behavioral responses of ticks

Cellulose as a *Bb* culture medium was sufficient to elicit preferential responses by ticks (Fig. 3.5). Cellulose inoculated with *Bb* (Exp. 20), and coconut fibre (consisting of cellulose, lignin, and hemicelluloses) inoculated with *Bb* (Exp. 21), both elicited stronger behavioural responses from female *I. scapularis* than corresponding sterile cellulose (Exp. 20: n = 25, p = 0.043) or sterile coconut fibre (Exp 25: n = 29, p = 0.024). These data, coupled with those presented in figure 3, suggest that *Bb* breakdown of cellulose contributes to the preferential foraging responses of ticks.

3.4.5. Effect of various soil-dwelling fungi on behavioral responses of ticks

All four species of fungi tested elicited preferential responses by ticks (Fig. 3.6). Relative to sterile coconut fibre, female *I. scapularis* preferred coconut fibre inoculated with *R. stolonifer* (Exp. 22: n = 26, p = 0.0094), *F. oxysporum* (Exp. 23: n = 23, p = 0.011), *P. roqueforti* (Exp. 24: n = 25, p = 0.0041), and *Bb* (Exp. 25: n = 19, p = 0.019).

3.4.6. Effect of fungus-derived volatiles on attraction of ticks

2-Methylisoborneol (2-MIB), but not geosmin, affected behavioural responses of ticks (Fig. 3.7). Female *I. scapularis* were strongly deterred by 2-MIB at a dose of 1 ng (Exp. 26: n = 21, p = 0.0002), moderately deterred at a dose of 0.1 ng (Exp. 27: n = 24, p = 0.064), but not deterred at a dose of 0.01 ng (Exp. 28: n = 17, p = 1). Conversely, irrespective of the dose tested, female *I. scapularis* ticks were neither deterred by, nor attracted to geosmin [1 ng (Exp. 29): n = 19, p = 0.65; 0.1 ng (Exp. 30): n = 23, p = 1; 0.01 ng (Exp. 31): n = 23, p = 0.21].

3.5. Discussion

Our data do not support the hypothesis that ixodid ticks (*A. americanum, D. variabilis, R. sanguineus, I. ricinus*, *I. pacificus*, *I. scapularis*) avoid substrate inoculated with the harmful entomopathogen *Beauveria bassiana* (*Bb*). Contrary to our prediction, all ticks tested – except for female and male *R. sanguineus* and female *I. pacificus* – preferred *Bb*-inoculated coconut fibre to sterile fibre, implying that the benefits accruing from these preferential responses outweigh the potential harm inflicted on ticks by *Bb*. That female and male *R. sanguineus,* which are known to inhabit dry human dwellings [68], were indifferent to the presence of *Bb* may be attributed to their reduced reliance on moist micro-habitats, as typically indicated by the presence of fungi.

Ticks orienting towards harmful *Bb,* and towards harmless *R. stolonifer*, *F. oxysporum*, and *P. roqueforti*, may accrue benefits in that all these fungi – regardless of their pathogenicity – might signal habitat suitability for ticks. High humidity, low sunlight exposure, and the presence of organic matter (e.g., leaf litter) for refuge are all essential requisites of favourable off-host microhabitats for ticks [14–19]. As fungal biomass in soil

is positively correlated with soil moisture [63], and negatively correlated with sunlight exposure [61,62], fungal presence or high relative fungal biomass could be a measure of sufficiently moist and shaded microhabitats that ticks require for their well-being and survival. Through both hygroreception and photoreception ticks are able to gauge the current suitability of a microhabitat [69] but they cannot readily gauge sustained microhabitat suitability. However, fungal breakdown products of plant cellulose could reliably indicate long-term habitat suitability, because metabolism of plant cellulose by fungi [70] and the ensuing accumulation of a high fungal biomass are supported by relatively persistent moisture and shade [71].

Preferential responses of ticks to *Bb*-inoculated fibre only when it was physically accessible indicate that ticks sense *Bb,* or its metabolites, mainly by contact chemoreception. Compounds sensed by contact chemoreceptors typically have high molecular weight and low volatility, and thus cannot readily be detected by olfaction [72]. It is plausible, however, that the ticks' preferential responses to *Bb*-inoculated coconut fibre is mediated by both contact chemoreception and olfaction, because – numerically – more ticks oriented to *Bb*-inoculated fibre than to sterile fibre, even when access to coconut fibre was blocked.

Without at least 24-h incubation of *Bb*-inoculated coconut fibre, ticks responded equally to *Bb*-inoculated fibre and to sterile fibre. These findings imply that ticks respond to products of *Bb* metabolism rather than to *Bb* itself. That ticks responded equally to *Bb*-inoculated coconut fibre (consisting of cellulose, lignin, and hemicelluloses) and to *Bb*-inoculated cellulose, further implies that it is the breakdown products of cellulose that mediate *Bb* recognition.

There is no obvious explanation as to why ticks avoided 2-MIB but responded indifferently to geosmin. Both 2-MIB and geosmin are emitted by numerous microorganisms including cyanobacteria [25,31,30], actinobacteria [29,30,33], and fungi [34–36], and both compounds commonly co-occur in above-soil headspace [66]. Based on current literature, there are no consistent behavioural responses of arthropods to 2-MIB and geosmin. Geosmin attracts the yellow fever mosquito *Aedes aegypti* to oviposition sites [25,29,30]. Sporulating streptomyces bacteria emit geosmin and 2-MIB, thereby attracting springtails that then aid spore dispersal [25,29,30]. Newly mated queens of *S. invicta* are attracted to geosmin and 2-MIB produced by actinobacteria as

indicators of suitable soil nesting sites with reduced risk of entomopathogenic fungal infections [30]. Conversely, *Drosophila melanogaster* vinegar flies sense and avoid geosmin as an indicator of feeding or oviposition sites containing harmful bacteria [73], and the bacteriophagous nematode *Caenorhabditis elegans* avoids grazing on geosmin-producing bacteria [74]. As 2-MIB was strongly deterrent to ticks only at elevated levels, microhabitats with elevated 2-MIB levels may signal potential threats that ought to be avoided. Ticks would encounter elevated 2-MIB levels in disturbed soils, which produce relatively large amounts of 2-MIB and geosmin [66,75]. As any form of current or future soil disturbance may physically harm off-host ticks, it follows that avoidance of sites with a strong 2-MIB odor is adaptive to ticks.

In conclusion, the cues that moisture-dependent hard ticks exploit to locate and select suitable off-host microhabitats were previously not known. We present data showing that the presence of soil-dwelling fungi (or their metabolites) – irrespective of their pathogenicity – inform decisions of ticks that seek suitable off-host microhabitats. Avoiding sites with elevated 2-MIB levels may help off-host ticks reject sites prone to significant disturbance that is harmful to ticks. That 2-MIB is deterrent to ticks was an unexpected and serendipitous finding in our study. Synthetic 2-MIB alone, or in combination with other tick deterrents [37,76,77], may become a highly effective commercial tick repellent, provided that the repellent effect extends to ticks in diverse taxa. This line of research is currently ongoing.

3.6. References

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3.7. Figures

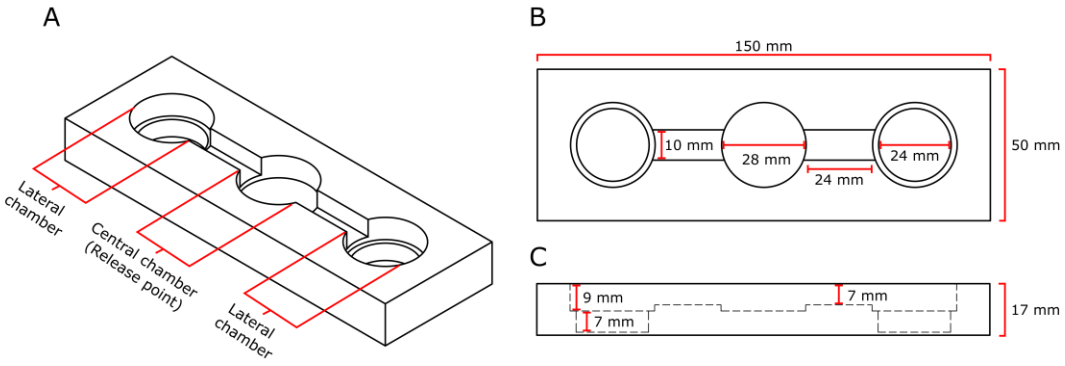

Figure 3.1. Graphical illustrations of lateral (A), overhead (B) and cross-section (C) views of the olfactometer used in tick bioassays. For bioassays, the lateral chambers of the olfactometer were filled with (i) treatment or control substrate (5 mL each; Exps. 1–25) or (ii) fitted with a watch glass holding a piece of filter paper treated with a test or a control stimulus (Exps. 26–31). Olfactometers were sealed with parafilm and a rectangular lid (150 × 50 × 3 mm). For each bioassay replicate, a single tick was released into the central chamber, and was considered a responder, if it was present in a lateral chamber at the end of the bioassay.

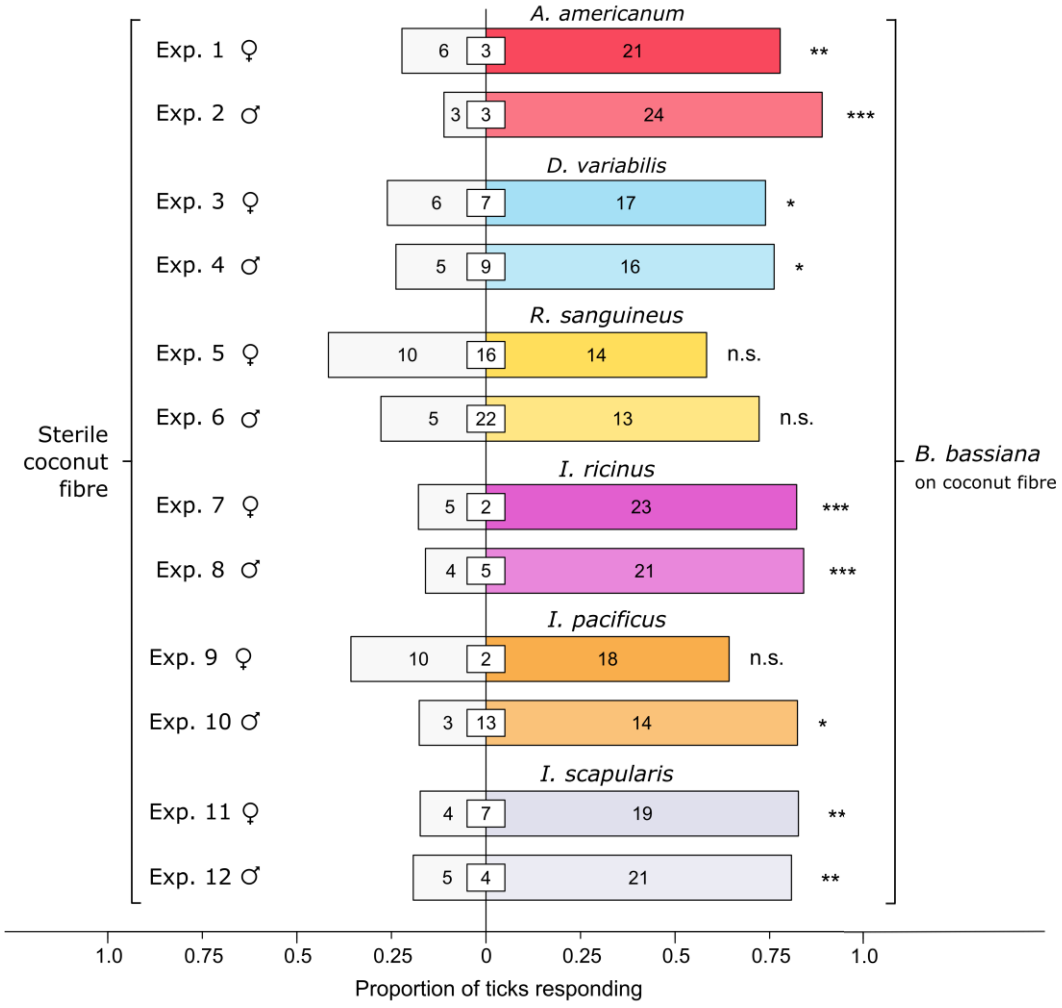

Figure 3.2. Effect of a *Beauveria bassiana* spore suspension on behavioral responses of ticks in six species: *Amblyomma americanum*, *Dermacentor variabilis*, *Rhipicephalus sanguineus*, *Ixodes ricinus*, *Ixodes pacificus*, and *Ixodes scapularis*. In olfactometer bioassays (Fig. 3.1), ticks were offered a choice between coconut fibre treated, or not (control), with a 1-mL *B. bassiana* spore suspension (10^7conidia/mL; 0.05% Tween80) incubated for 24 h. The control coconut fibre was treated with sterile 0.05% Tween80 (1 mL). Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. Asterisks indicate significant arrestment behavior on coconut fibre treated with the fungal spore suspension (exact binomial tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n. s. = not significant).

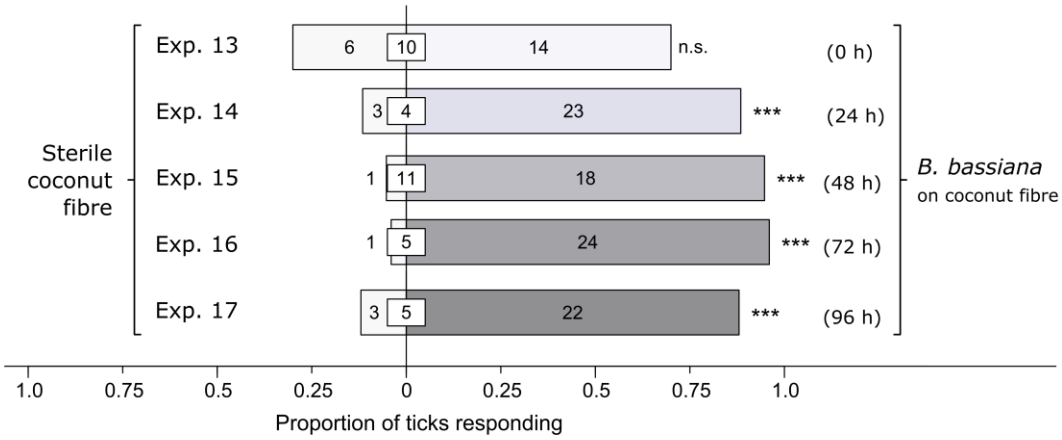

Figure 3.3. Effect of incubation time of a *Beauveria bassiana* spore suspension on behavioral responses of female black-legged ticks, *Ixodes scapularis.* In olfactometers bioassays (Fig. 3.1), ticks were offered a choice between coconut fibre treated, or not (control), with a 1-mL *B. bassiana* spore suspension (10^7conidia/mL; 0.05% Tween80) incubated for 0, 24, 48, 72, or 96 h before bioassays. The control coconut fibre was treated with sterile 0.05% Tween80 (1 mL). Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. Asterisks indicate significant arrestment behavior on coconut fibre treated with the fungal spore suspension (exact binomial tests; *** $p < 0.001$; n. s. = not significant).

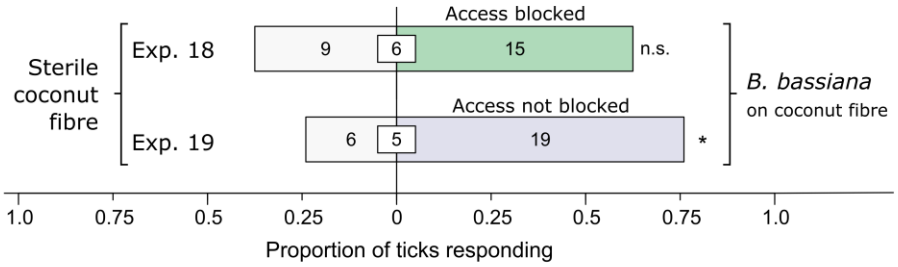

Figure 3.4. Effect of stimulus accessibility on behavioral responses of female blacklegged ticks, *Ixodes scapularis.* In olfactometer bioassays (Fig. 3.1), ticks were offered a choice between coconut fibre treated, or not (control), with a 1-mL *Beauveria bassiana* spore suspension (10^7conidia/mL; 0.05% Tween80) incubated for 24 h before bioassays. The control coconut fibre was treated with sterile 0.05% Tween80 (1 mL). Stimuli in lateral chambers were accessible (Exp. 18) and inaccessible (Exp. 19), respectively. Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. The asterisk indicates significant arrestment behaviour on accessible coconut fibre treated with a fungal spore suspension (exact binomial tests; * $p < 0.05$; n. s. = not significant).

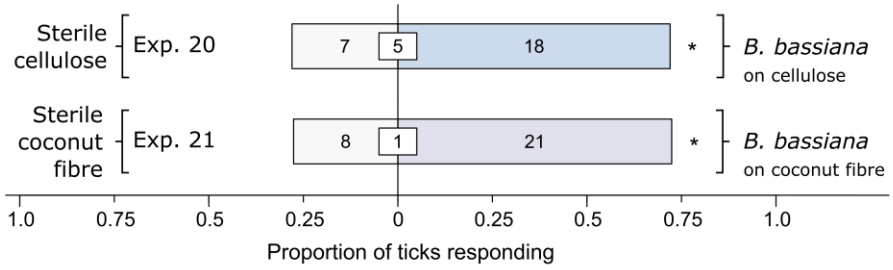

Figure 3.5. Effect of substrate (cellulose or coconut fibre), treated with a *Beauveria bassiana* spore suspension and incubated for 24 h, on behavioural responses of female black-legged ticks, *Ixodes scapularis*. In olfactometer bioassays (Fig. 3.1), ticks were offered a choice between substrate treated, or not (control), with a 1-mL spore suspension of *B. bassiana* (10^7conidia/mL; 0.05% Tween80) incubated for 24 h. The control substrate was treated with sterile 0.05% Tween80 (1 mL). Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. Asterisks indicate significant arrestment behavior on the substrate treated with the fungal spore suspension (exact binomial tests; * $p < 0.05$; n. s. = not significant).

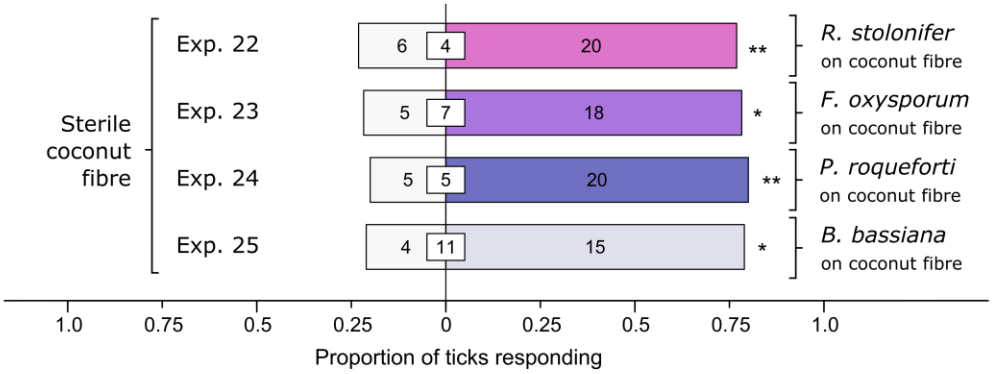

Figure 3.6. Effect of fungal species on arrestment behavior of female black-legged ticks, *Ixodes scapularis*. In olfactometer bioassays (Fig. 3.1), ticks were offered coconut fibre treated, or not (control), with a 1-mL spore suspension of *Rhizopus stolonifer*, *Fusarium oxysporum*, *Penicillium roqueforti*, or *Beauveria bassiana* (each 10^7conidia/mL; 0.05% Tween80) incubated for 24 h. The control coconut fibre was treated with sterile 0.05% Tween80 (1 mL). Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. Asterisks indicate significant arrestment behavior on coconut fibre treated with a fungal spore suspension (exact binomial tests; * $p < 0.05$, ** $p < 0.01$; n. s. = not significant).

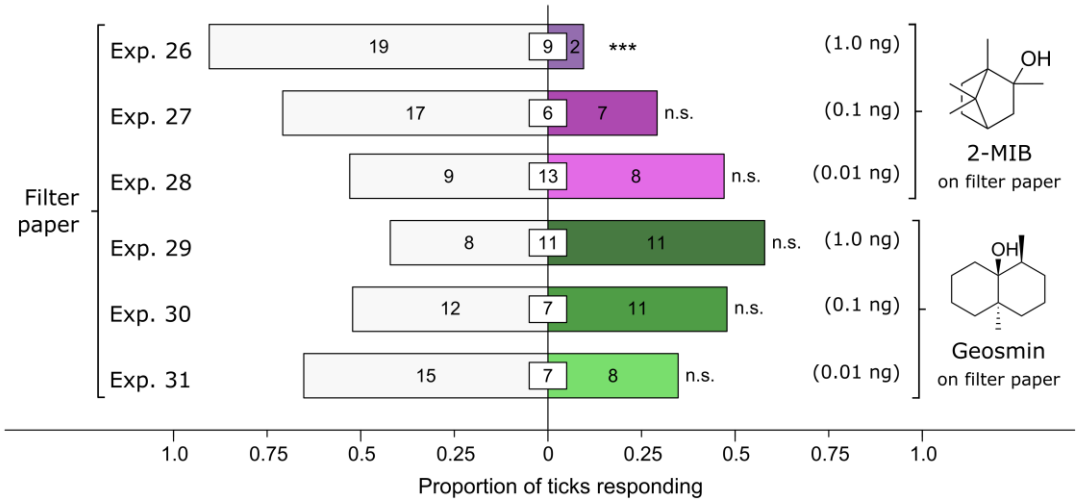

Figure 3.7. Effect of test chemicals applied on filter paper on behavioural responses of female black-legged ticks, *Ixodes scapularis.* In olfactometer bioassays (Fig. 3.1), ticks were offered a choice between filter papers treated with (*i*) 2-methylisoborneol (2-MIB) dissolved in methanol and methanol (control), and (*ii*) geosmin in methanol and methanol. Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. Asterisks indicate significant avoidance of filter paper treated with 2-MIB at 1 ng (exact binomial tests; *** $p < 0.001$; n. s. = not significant).

Appendix A. Supplementary Materials

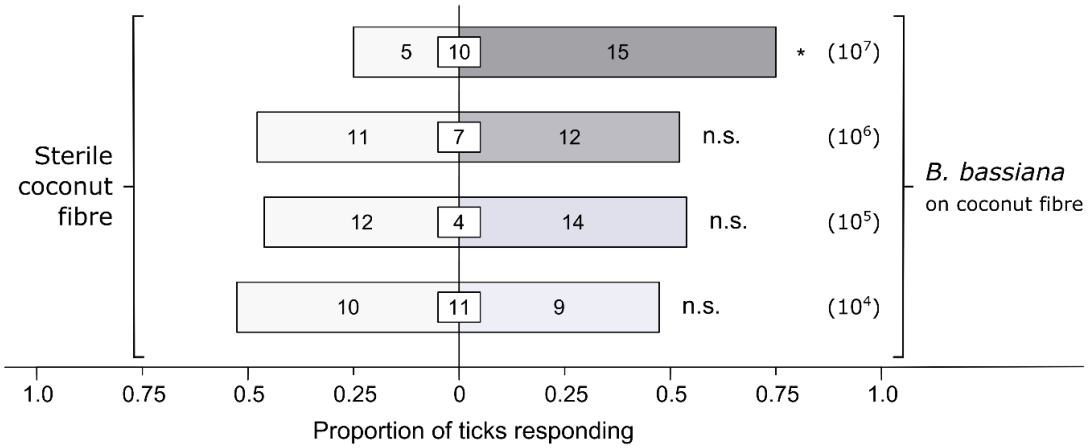

Figure A.1. Effect of dose (conidia/mL) of a *Beauveria bassiana* spore suspension on behavioral responses of female black-legged ticks, *Ixodes scapularis.* In olfactometers bioassays (Fig. 1), ticks were offered a choice between coconut fibre treated, or not (control), with a 1-mL *B. bassiana* spore suspension of 10^4, 10^5, 10^6, or 10^7 conidia/mL incubated 24 h before bioassays. The control coconut fibre was treated with sterile 0.05% Tween80 (1 mL). Numbers in bars represent the total number of ticks choosing a stimulus, and numbers in white inset boxes represent the total number of non-responding ticks. Asterisks indicate significant arrestment behavior on coconut fibre treated with the fungal spore suspension (exact binomial tests; * $p < 0.05$; n. s. = not significant).