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The BiteBarrier perimeter: A passive spatial device for tick control and bite prevention

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ABSTRACT

Tick-borne diseases (TBDs) impact human and animal health on a global scale. Prevention of TBDs relies primarily on prevention of tick bites. New bite-prevention technologies are needed as an alternative to current approaches such as topical repellents and treated clothing which suffer low user compliance. To date, no passive spatial devices have been commercialized for area protection against ticks. The BiteBarrier (formerly the Personal Insect Repellent Kit, PIRK), a passive, lightweight device that emits transfluthrin, offers to fill this gap. In a previous study, we demonstrated contact toxicity of the BiteBarrier substrate to three tick species, *Ixodes scapularis*, *Dermacentor variabilis* and *Amblyomma americanum*, and reported differences in efficacy depending on species and short-range spatial efficacy against *I. scapularis* adult females. Here, we extended analyses and demonstrated modest spatial activity of the BiteBarrier substrate against *A. americanum* and *D. variabilis* adult females. Using a dual-choice behavioral assay, we showed that the three tick species preferred an area of untreated substrate. Lastly, we present a novel perimeter assay, developed to assess the efficacy of the BiteBarrier ground-based prototype against ticks. At short-range in a Peet Grady-style chamber, the BiteBarrier perimeter induced greater than 90% knockdown of *I. scapularis* adult females at 1 and 2 h post-exposure and 90% mortality at 48 h post-exposure. Taken together, study findings indicate the potential of the BiteBarrier perimeter to control ticks at near range and potentially, to protect against tick bites.

1. Introduction

Hard ticks (family Ixodidae) and the pathogens they transmit constitute a significant problem for human and animal health. The distribution and range of ticks and tick-borne diseases (TBDs) are affected by changes in climate, urbanization, habitat loss and disturbance, and host abundance (Soneshine, 2018; Diuk-Wasser et al., 2021; Tsao et al., 2021). The annual number of TBDs cases is increasing, with Lyme disease reaching a yearly estimated number of cases of 476,000 (CDC, 2024a). In addition, a new tick species, *Haemaphysalis longicornis*, first identified in New Jersey in 2017 (Rainey et al., 2018), is now present in 20 U.S. States (CDC, 2024b). Lastly, an increasing number of cases of the tick-induced alpha gal allergy, also known as red meat allergy, also represent a public health concern in the USA. The condition is thought to result from an allergic response to a sugar (galactose-α-1,3-galactose) present in the saliva of some tick species (e.g. the lone star tick, *Amblyomma americanum*). Binder and colleagues reported a six-fold increase in the number of alpha-gal IgE-positive tests in the USA from 2011 to 2018 (Binder et al., 2021).

Personal protection against tick-borne diseases relies on prevention of tick bites. CDC recommendations include the use of thorough tick checks, permethrin-treated clothing and repellants such as DEET, among others (CDC, 2024c). These measures are not in use homogeneously throughout the USA, and their adoption is low in areas of low Lyme disease (LD) incidence (Eisen, 2022). Therefore, there is a pressing need for new tick-bite prevention tools.

Highly volatile passive emanators (HVPE) containing volatile pyrethroids, such as allethrin, methofluthrin and transfluthrin, have shown efficacy against mosquitoes in outdoor, limited outdoor and indoor settings (Masalu et al., 2018, 2020; Britch et al., 2021) and against pyrethroid-resistant mosquitoes (Tambwe et al., 2021; Kim et al., 2023). HVPEs may also offer some degree of efficacy against ticks (Bibbs and Xue, 2016; Murgia et al., 2022; Siegel et al., 2022). Commercially available emanators, such as ThermaCELL, containing metofluthrin,

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have shown close-range spatial repellency and toxicity to *A. americanum* (Bibbs and Xue, 2016). The study of Siegel et al. (2022) showed spatial repellency of metofluthrin and transfluthrin to *Dermacentor variabilis* (American dog tick), *A. americanum*, and *Ixodes scapularis* (black legged tick) females, expressed as climbing deterrence, with greatest efficacy of the active ingredients (AIs) to *D. variabilis*.

The BiteBarrier is the first EPA-approved passive device containing transfluthrin (TF) and has shown activity against flying insects (mosquitoes, sand flies, stable flies) in a 30 m^3 area for up to three weeks. The BiteBarrier is under investigation for application in limited outdoor settings as a promising novel tool for control of ticks and tick-bite prevention. The study of Murgia et al. (2022) demonstrated contact efficacy of the BiteBarrier substrate against the nymphs of *I. scapularis*, *D. variabilis* and *A. americanum* and short-range spatial activity against *I. scapularis* adult females.

In this study, we evaluated a BiteBarrier prototype containing TF-impregnated substrate as a perimeter device for area-wide control of three hard tick species and tick-bite prevention. The spatial efficacy of the BiteBarrier substrate against *A. americanum* and *D. variabilis* adult females during and following forced exposure was demonstrated using a modular assay described by Murgia et al. (2022). The potential of the BiteBarrier prototype to contain unrestrained *I. scapularis* adult females in a 100 cm^2 area, as would occur in limited outdoor use scenarios, was assessed in an enclosed chamber using a novel ground-based perimeter assay. The impact of BiteBarrier substrate exposure on tick behaviors associated with host location was assessed for the three tick species using a dual-choice behavior assay. Here we describe an innovative perimeter assay to evaluate the spatial and contact efficacy of new actives and formulations delivered *via* a ground-based passive spatial device for control of terrestrial arthropod pests such as ticks. Further, we demonstrate the potential of the BiteBarrier prototype in a perimeter configuration to control adult ticks at near range and possibly, to disrupt tick behaviors that precede host location, attachment and feeding.

2. Materials and methods

2.1. Modular spatial assay

The spatial activity of the BiteBarrier substrate against *A. americanum* and *D. variabilis* adult females was investigated using a modular assay modified from Grieco et al. (2005) and described in Murgia et al. (2022). Briefly, the test chamber comprised of a plastic cylinder (28 cm in length and 3.8 cm in diameter), flanked by two treatment drums (10 cm in length and 3.8 cm in diameter) that contained either untreated substrate (no AI; left and right drums) for the control group, or BiteBarrier treated substrate (left drum) and BiteBarrier untreated substrate (right drum) for the treated group. The BiteBarrier treated substrate was allowed to acclimate for 2 h; five ticks were introduced to the test chamber and their position recorded every 10 min for 2 h. Tick behavior was also recorded *via* short videos on a smart phone. Knockdown (KD) in the chamber was defined as ticks being incapable of movement in a horizontal or vertical plane, or on their scutum and unable to correct to standing or exhibiting spastic movement of the legs. After exposure, ticks were transferred to surgical packets (7 × 7 cm biopsy bags made of porous paper which allows for gas exchange and the containment of the ticks), and scored for KD every 10 min up to 1 h and for mortality at 1, 24, 48, 72, and 96 h post-exposure (PE). All assays included $n = 3$ technical replicates per biological replicate, and $n = 3$ biological replicates. All components of the test apparatus were replaced, and a new BiteBarrier substrate was used for each biological replicate.

2.2. Horizontal dual-choice behavioral assay

The horizontal dual-choice assay was designed to assess the capability of the BiteBarrier treated substrate to disrupt host-seeking and bite behaviors in the presence of a host stimulus. A schematic of the assay is shown in Fig. 1. The assay consists of a Petri dish lined with either (i) a semicircle of BiteBarrier treated substrate adjacent a semicircle of untreated substrate (treated group) or (ii) semicircle of untreated substrate on both sides of the dish (negative control group). The Introduction Zone comprised a 1.5 × 1.5 cm square untreated Whatman paper #1 placed in the middle of the Petri dish on top of the substrates. The host cue (human finger) was introduced through a hole cut in the lid of the Petri dish. Five ticks were placed in the introduction zone and their location in the Petri dish: Introduction Zone, BiteBarrier treated substrate + Host Zone, BiteBarrier untreated substrate Control (no AI) Zone, and location on the finger was recorded at 1-min intervals for 5 min. The behavioral choice assay with untreated substrate (negative control group) was designed to assess BiteBarrier treated substrate impact on *I. scapularis* adult females and to exploit the "ambush-style" questing behavior typical of this species. Three volunteers, two females and one male, performed the assay and the same individuals were employed as host for each of the treated and control groups. All assays included $n = 3$ technical replicates per biological replicate, and $n = 3$ biological replicates. New substrate (BiteBarrier treated and control) was used for each biological replicate.

2.3. Perimeter barrier assay

A BiteBarrier prototype containing TF impregnated substrate was employed in a ground-based perimeter configuration to investigate barrier potential against *I. scapularis* adult females in a Peet Grady-style chamber (see Fig. 2). The BiteBarrier prototype perimeter configuration (12.5 cm in width, 125 × 125 cm square) was placed on top of a white ground sheet. The test arena comprised three areas: an inner area (100 × 100 cm), BiteBarrier prototype perimeter (125 × 125 cm), and an outer area (25 cm in width). For each biological replicate, the BiteBarrier prototype with treated substrate was allowed to acclimate for 2 h, and ten ticks were then placed inside the perimeter, at 1 cm from the edge of the BiteBarrier prototype, in the lower left corner, and equidistant from its edges. The location of ticks in the test arena (inner area, BiteBarrier prototype perimeter, outer area) was recorded at the end of the exposure period, following which, ticks were transferred to surgical packets and scored for KD/mortality at 1, 2, 24, 48, 72, and 96 h PE. The control comprised BiteBarrier prototype with untreated substrate (no transfluthrin). The assay was performed at a minimum room temperature of 75 °F (24 °C) and a relative humidity > 70% and included $n = 3$ biological replicates.

3. Results

3.1. Modular spatial assay

A modular assay was used to test spatial activity and repellency of the BiteBarrier treated substrate to *A. americanum* and *D. variabilis* adult females. Fig. 3 shows bar graphs representing the percent of ticks located in each of the four quadrants of the test arena at 30-min intervals over the course of a 120 min forced exposure period for both the treated and control groups. For BiteBarrier treated substrate-exposed *A. americanum*, statistically significant differences were observed in the distribution of ticks located in quadrant 4 *vs* quadrants 1–3, starting at 10 min exposure (Supplementary Table S1). No statistically significant difference was detected between the distribution of the ticks in the control *vs* treated group at any time point during exposure. Treated *A. americanum* were less active overall as compared to controls during the 2-h exposure period. KD behaviors were observed in the treated group starting from 20 min exposure and in 40% of the ticks by the end of the 2 hours (Fig. 4B). Limited KD and no mortality were observed in *A. americanum* post-exposure (data not shown).

The distribution of BiteBarrier treated substrate-exposed (treated) and control *D. variabilis* among the four quadrants is shown in Fig. 3C and D. For the control group, statically significant differences were

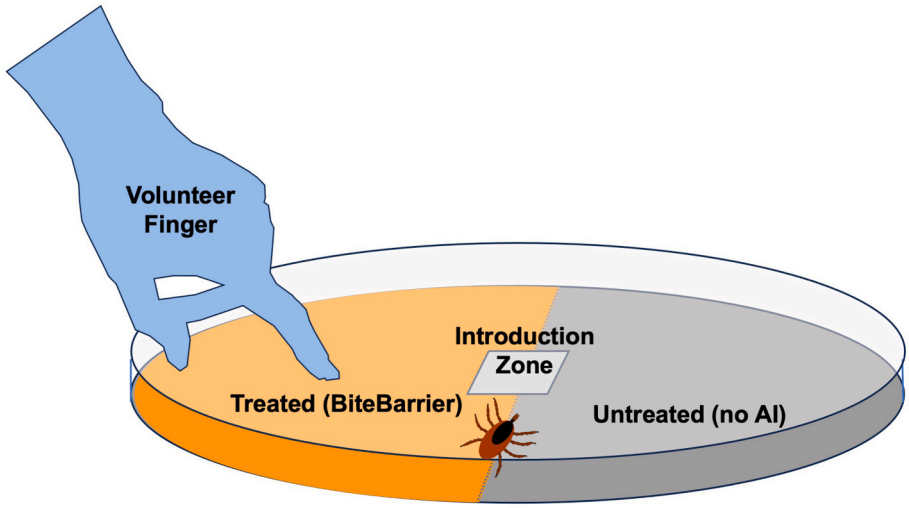

Fig. 1. Schematic of the horizontal behavioral choice assay to assess the capability of the BiteBarrier substrate to disrupt host-seeking and bite behaviors of adult female *I. scapularis*, *A. americanum,* and *D. variabilis,* in the presence of a host. The assay consists of a Petri dish lined with treated substrate and untreated (control) substrate (no AI). The Introduction zone comprises a 1.5 × 1.5 cm square of untreated filter paper placed on the substrates in the center of the dish. The finger of a volunteer is introduced *via* a hole in the lid of the Petri dish. The location of ticks (Introduction zone, BiteBarrier treated substrate + Host Zone, BiteBarrier substrate Control (no AI) Zone) is recorded at 1-min intervals for up to 5 min. All assays comprise $n = 3$ technical replicates per biological replicate, with $n = 3$ biological replicates (three individual volunteers/biological replicate, and same volunteers for control and treated ticks).

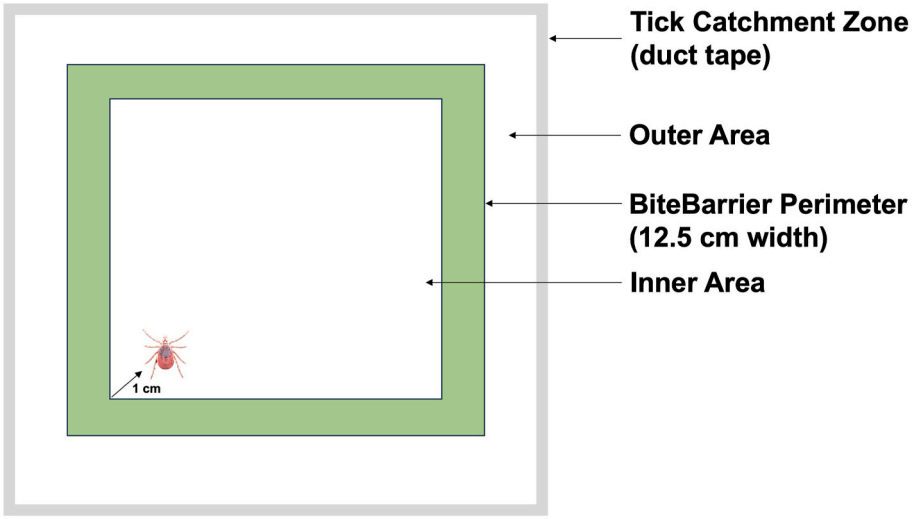

Fig. 2. Schematic of the perimeter assay to assess the spatial activity of the BiteBarrier perimeter to *I. scapularis* adult females. The BiteBarrier perimeter comprising either untreated substrate (control) or treated BiteBarrier, dimensions: 12.5 cm width; 125 cm length × 125 cm height, was placed on a white ground sheet, edged with duct tape, which had the sticky surface exposed to prevent the escape of ticks. The BiteBarrier was acclimated for 2 h, and 10 adult female ticks were introduced and placed in the lower-left corner at 1 cm equidistance from the edge of the substrate.

detected between ticks located in distant quadrants (Q1 and Q4) and adjacent quadrants (Q2 and Q3). No statistically significant difference was observed in the distribution of ticks between Q1 and Q4 (Supplementary Table S2). For the treated group, a statistically significant difference was observed in the distribution of ticks between Q4 (located farthest from the BiteBarrier treated substrate) and the remaining quadrants (Supplementary Table S2). In addition, a statistically significant difference was observed in the distribution of control and treated ticks between Q1 and Q4 at 20-, 30-, 70-, and 80-min exposure, with 51% of treated ticks located in Q4 at 20-min exposure compared to 29% of control ticks in the same quadrant at this time point (Fig. 4A and Supplementary Fig. S1). Differences in the behaviors of control and ticks exposed to treated substrate were observed, with ticks in the control group exhibiting movement and questing, and occasionally resting during the exposure period. Conversely, ticks in the group exposed to treated substrate were less active and spent most of the time resting or exhibited alternatingly movement and resting, and by 60 min, most of the ticks were resting. When tested post-exposure, less than 20% of ticks in the group exposed to treated substrate exhibited KD at 1 h, and no mortality was recorded at any time point post-exposure (data not shown).

3.2. Horizontal dual-choice behavioral assay in presence of human host

The behavioral choice assay revealed preferential location of ticks on the untreated substrate, independent of species (Fig. 5). The location of *A. americanum* on the untreated substrate *versus* the BiteBarrier treated substrate + Host was statistically significant at 1- and 4-min exposure (Supplementary Table S3). The assay revealed a statistically significant difference in the location of *D. variabilis* at 5-min exposure between

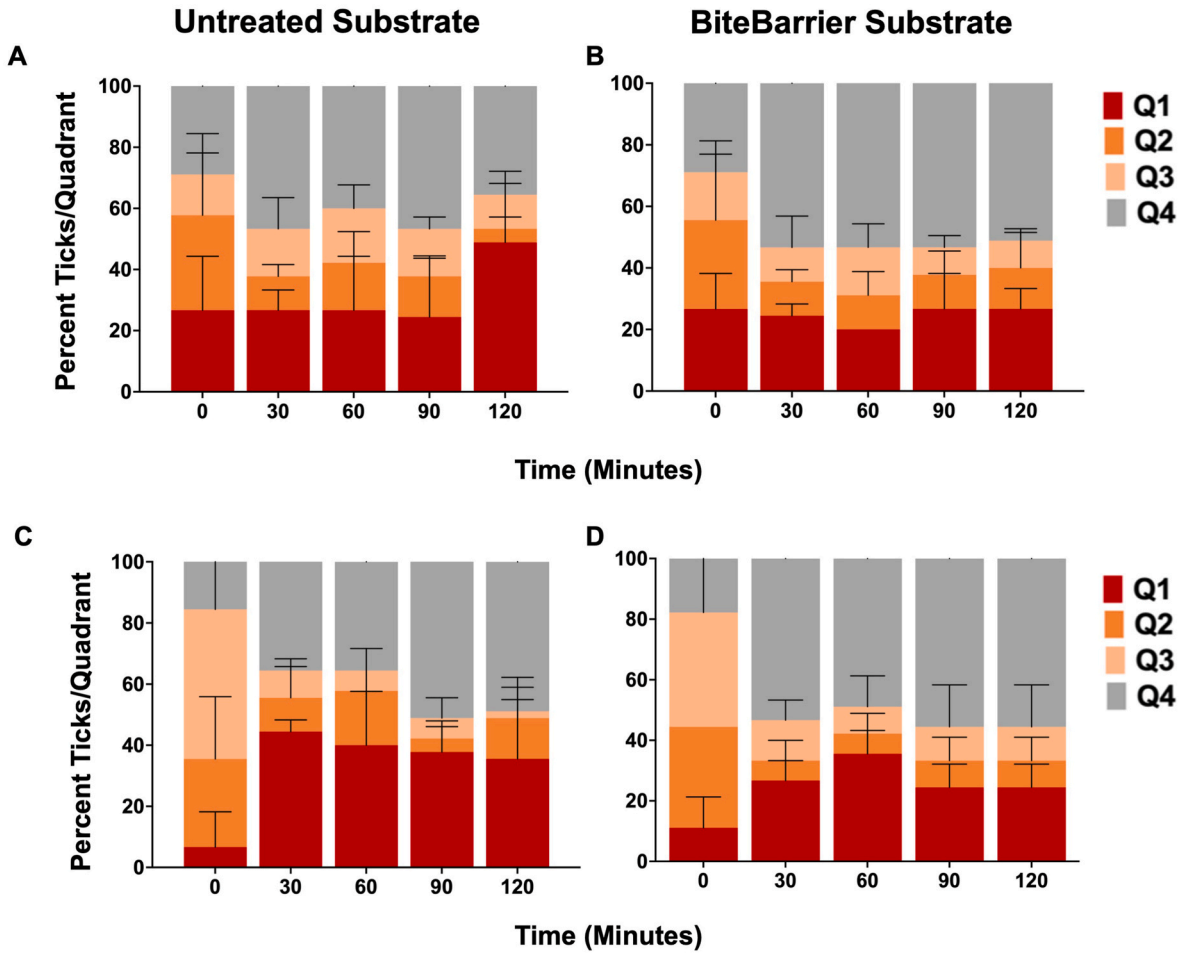

Fig. 3. Spatial efficacy and repellency of the BiteBarrier substrate (location in the test arena) to *A. americanum* and *D. variabilis* adult females in an enclosed test arena assessed using a modular assay. The bar graphs indicate the percent of ticks located in each of the four quadrants (Q1-Q4) at 30-min intervals up to 120 min during exposure of *A. americanum* (**A**, **B**) and *D. variabilis* (**C**, **D**) in the test arena. Q1 is the quadrant closest (*colored in red*) and Q4 the farthest (*colored in gray*) to the BiteBarrier substrate. Bar graph colors correspond to Q1-Q4. Error bars are cut at the y-axis maximum.

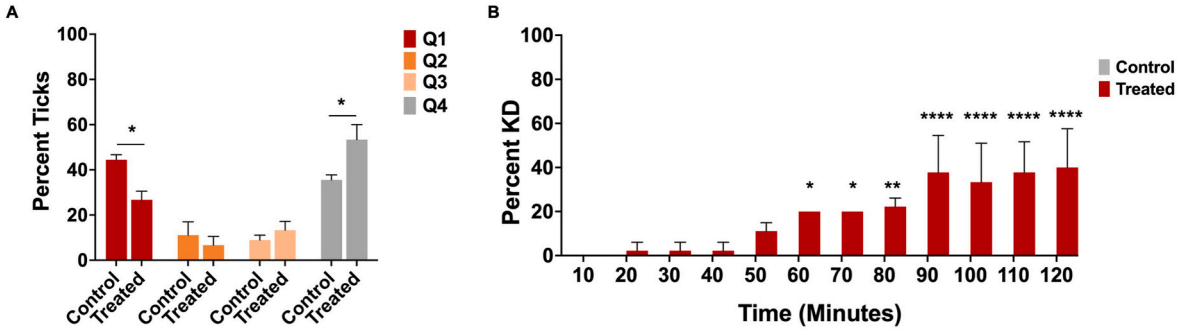

Fig. 4. Spatial efficacy of the BiteBarrier substrate (location and knockdown in test arena) to *D. variabilis* and *A. americanum* adult females in an enclosed chamber assessed using a modular assay. **A** Bar graph indicating the percent of control and treated *D. variabilis* females located in each of the four quadrants of the test arena at 30 min exposure. **B** Bar graph indicating the percent knockdown (KD) of *A. americanum* females at 10-min intervals up to 120 min exposure in the test arena. Q1 is the quadrant closest and Q4 the farthest to the BiteBarrier substrate. The results represent $n = 3$ independent biological replicates, with $n = 3$ technical replicates per biological replicate. Two-way ANOVA with Šídák's multiple comparison test ($P < 0.05$) was used to compare the two groups (*$P < 0.05$; **$P < 0.005$; ****$P < 0.0001$).

untreated and treated locations (Supplementary Table S4). In comparison, there was no statistically significant difference in the distribution of *I. scapularis* ticks between treated and untreated halves of the arena at any time point during the experiment (Supplementary Table S5).

The "hot foot" effect described by Eisen et al. (2017) was observed in all three tick species following contact with the BiteBarrier treated substrate. This phenotype, characterized by extreme irritation and avoidance behavior, was more pronounced in *I. scapularis* and *A. americanum* adults, as compared to *D. variabilis* adults (data not shown). During the 5-min test period, a small number of each species contacted and crawled on the volunteer. Approximately 9% of *I. scapularis* adults (4/45 ticks) contacted and crawled on the volunteer,

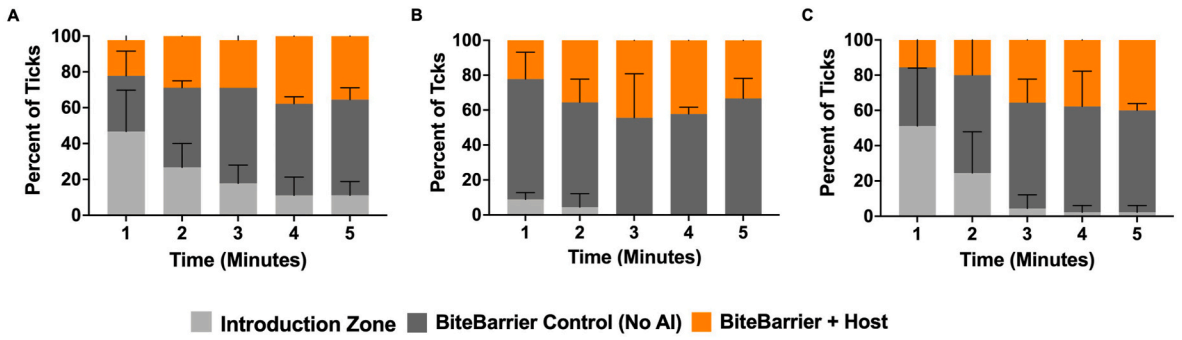

Fig. 5. Impact of the BiteBarrier substrate on the location of adult *I. scapularis*, *A. americanum* and *D. variabilis* in the test arena during forced exposure in the horizontal behavioral choice assay. Bar graphs show the percent of adult females of *I. scapularis* (**A**), *A. americanum* (**B**), and *D. variabilis* (**C**) in the different locations of the test arena (Introduction Zone, BiteBarrier substrate + Host Zone, BiteBarrier substrate Control (no AI) Zone) at 1-min intervals up to 5 min. Results represent $n = 3$ independent biological replicates (15 ticks/biological replicate). Error bars are cut at the y-axis maximum. See Supplementary Tables S3–S5 for statistical analyses.

with an average time to first contact of 100 s and time on the volunteer of 14 s. In the negative control group, approximately 26% (12/45) *I. scapularis* females crawled on the volunteer over a 5-min test period, with an average time to first contact of 36 s and time on the volunteer of 22 s (data not shown). Approximately 20% (6/30) of *A. americanum* adults contacted the volunteer with an average time on the subject of 12 s, and average time to first contact with the host of 80 s. Thirteen percent (6/45) of *D. variabilis* adults contacted the volunteer; the average time on the volunteer was 26 s and average time to first contact was 133 s.

3.3. BiteBarrier perimeter assay

The efficacy of the BiteBarrier prototype perimeter against *I. scapularis* adult females was evaluated under forced exposure and controlled conditions in a Peet Grady-style chamber. Ticks were placed in the lower left corner, at 1 cm equidistant from the edge of the BiteBarrier prototype (containing untreated or transfluthrin-treated substrate). As shown in Fig. 6, 50% of control ticks were located in the outer area, followed by the perimeter (30%) and inner area (20%) at the end of the 2-h forced exposure period, although these differences were not statistically significant. In comparison 57% of treated ticks were located inside the perimeter, with 16% and 27% located on the perimeter inner and outer areas, respectively by conclusion of the 2-h forced exposure. For treated ticks, there was a statistically significance difference in tick location between the perimeter and inner area.

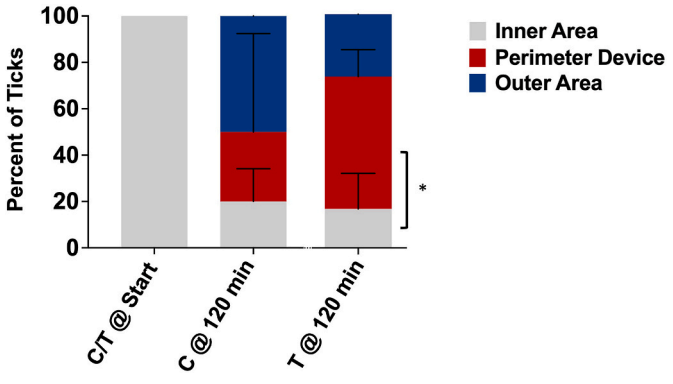

Fig. 6. Impact of the BiteBarrier perimeter on the location of adult *I. scapularis* females at the end of the forced exposure period in a Peet Grady-style Chamber. The bar graph shows the percent of ticks in the different areas of the test arena (inner area, perimeter device, outer area) at the start and at the end of the 120-min exposure. Results represent $n = 3$ independent biological replicates (10 ticks/biological replicate). Error bars are cut at the y-axis maximum. Two-way ANOVA with Tukey's multiple comparison test ($P < 0.05$) was used to compare the location of *I. scapularis* females in the test arena (inner area, perimeter device, outer area) at 120 min for control and treated groups (*$P < 0.05$). *Abbreviations*: C, control; T, treated (BiteBarrier-exposed).

Signs of rapid onset of KD and toxicity were observed in treated ticks. Immediately on placement adjacent the treated BiteBarrier prototype perimeter, ticks exhibited signs of irritation and crawled on or beneath the perimeter. A series of sublethal behaviors were observed during exposure to the treated BiteBarrier prototype, ranging from irritation (including the "hot foot" effect, a behavior previously described by Eisen et al. (2017) which defines the response of *I. scapularis* nymphs upon contact with permethrin-treated fabric) to uncoordinated movement, with ticks that exhibited the latter phenotype, frequently progressing to a KD phenotype (defined as ticks being incapable of movement in a horizontal or vertical plane, or on their scutum and unable to correct to standing or exhibiting spastic movement of the legs) and death at 48 h PE. Control ticks exhibited active movement in the test arena over the 2-h test period, and alternated between crawling and resting, with occasional waving of the forelegs, indicative of questing behavior. We observed 90% KD of treated ticks at 1 and 2 h (Fig. 7A), and 90% mortality at 48 h PE (Fig. 7B).

4. Discussion

4.1. Spatial efficacy of the BiteBarrier treated substrate against *A. americanum* and *D. variabilis* in the modular spatial assay

The study of Murgia et al. (2022) showed greatest contact efficacy of the BiteBarrier treated substrate to *I. scapularis* (> 90% KD at 1 h and >90% mortality at 48 h PE after a minimum of 10 s contact time) followed by *A. americanum* (> 90% KD at 1 h PE after a minimum of 10 s contact time). For *D. variabilis*, minor contact efficacy was observed in nymphs only, which exhibited 90% KD at 1 h PE after a minimum of 180 s contact time. The study also revealed modest spatial efficacy of the BiteBarrier treated substrate in the modular assay at a short-range (5–28 cm) to *I. scapularis* adult females as demonstrated by 80% KD at 2 h exposure and 100% KD by 40 min PE, and no evidence of repellency (Murgia et al., 2022). In the present study, we extended the evaluation of the spatial efficacy of the BiteBarrier-treated substrate to *A. americanum* and *D. variabilis* adults using the modular assay described in Murgia et al. (2022). Our results revealed differences in BiteBarrier efficacy between these species, both during and post-exposure to the treated substrate. Specifically, there was no evidence of repellency to *A. americanum* during 2 h exposure to the BiteBarrier treated substrate under the conditions used for this assay. However, KD and sublethal phenotypes such as reduced activity or inactivity (characterized by ticks remaining in the same spatial location without evidence of body/leg tremors or spastic movement of the legs) were observed during exposure, indicating a toxic

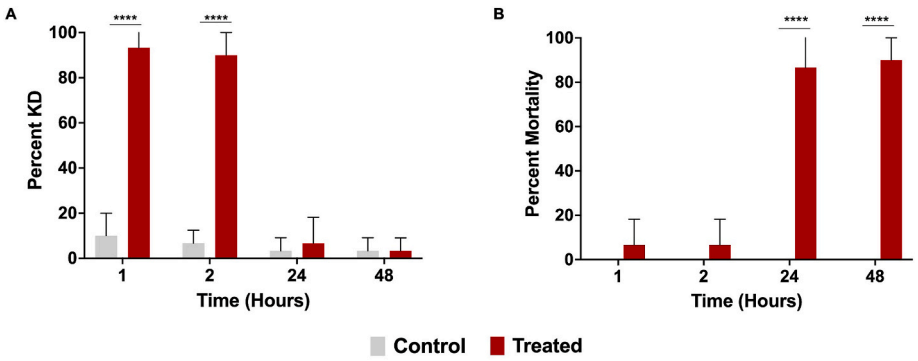

Fig. 7. Spatial efficacy of the BiteBarrier perimeter to *I. scapularis* adult females in a forced exposure assay in a Peet Grady-style chamber. Bar graphs show percent KD (**A**) and percent mortality (**B**) of ticks at 1, 2, 24, and 48 h post-exposure for control and treated groups. Error bars are cut at the y-axis maximum. The results represent $n = 3$ independent biological replicates with $n = 3$ technical replicates per biological replicate. Two-way ANOVA with Šídák's multiple comparison test ($P < 0.05$) was used to compare the two groups (*$P < 0.05$; **$P < 0.005$; ****$P < 0.0001$).

effect of the BiteBarrier to *A. americanum*. In comparison, modest repellency was observed in *D. variabilis* with 55% of treated ticks detected in Q4, the quadrant located farthest from the BiteBarrier treated substrate, at the conclusion of the 2-h forced exposure test period. While there was no evidence of KD, sublethal phenotypes (reduced activity or inactivity) were observed during exposure to the BiteBarrier treated substrate in this species. It is known that pyrethroids can cause repellency at sublethal concentration and toxicity at lethal concentration (Bibbs and Xue, 2016). The modest efficacy of the BiteBarrier to *D. variabilis* was expected based on the previous study of Murgia et al. (2022). The repellency observed in *D. variabilis* could reflect the accumulation of TF in the test arena in vapor phase or on the chamber surface, to a level sufficient to elicit repellency but insufficient to cause toxicity. Furthermore, effects did not persist post-exposure, with less than 20% KD observed at 1 h PE and no mortality in either species. These results indicate minimal spatial efficacy of the BiteBarrier treated substrate against *A. americanum* and *D. variabilis* in the modular assay. The results may reflect challenges to the delivery of a lethal dose of TF chemistry *via* volatile actives (i.e. in vapor phase) and the need to rely on contact modalities to achieve control of both *A. americanum* and *D. variabilis*. For this reason, subsequent spatial studies were limited to *I. scapularis*.

There is need to better understand the mode of action of volatile SPs to ticks, including the mechanisms that underpin repellency, KD, and mortality. The Haller's organ, a specialized sensory structure in the forelegs of ticks, has been implicated in olfaction and the detection of heat and humidity, among other functions (Mulenga, 2014). Its structure, including the identification of olfactory sensilla and olfactory receptor neurons (ORNs), has been studied (reviewed in Gebremedhin et al., 2023). Laboratory and field assays have been employed to identify attractive pheromones and kairomones to be utilized in lure and kill traps for tick control (Carr and Roe, 2016). In addition, electrophysiology studies employing single-sensillum recording have been employed to detect odors that elicited a response on individual ORNs and, when coupled with gas chromatography, to identify individual odor components (Lingeman et al., 2024). The Haller's organ has been implicated in the repellency of DEET to *D. variabilis* (Carr et al., 2017). It was also shown that low concentrations of DEET interfered with thermosensation but not with olfactory functions in *A. americanum* (Carr and Salgado, 2019). Olfactory receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs), together with olfactory binding proteins (OBPs) have been implicated in the olfactory and gustatory processes of insects (reviewed in Suh et al., 2014). Genes coding for ionotropic glutamate receptors (iGluRs) and GRs, but not for ORs or OBPs, have been identified in the genomic sequences of seven tick species belonging to five genera, *Ixodes*, *Haemaphysalis*, *Dermacentor*, *Hyalomma* and *Rhipicephalus*, within the family Ixodidae (Gulia-Nuss et al., 2016; Jia et al., 2020; De et al., 2023; Nuss et al., 2023), suggesting potential fundamental differences between the olfactory and gustatory processes of ticks and insects. In a transcriptomic study by Carr et al. (2017), the authors proposed a G-protein coupled receptor (GPCR)-mediated olfactory pathway based on the identification of Haller's organ-specific GPCR transcripts, two putative adenylate/guanylate cyclase, several putative odorant degrading enzymes, and the absence of transcripts corresponding to IRs or GRs. However, in a subsequent transcriptomic study of *I. scapularis* forelegs and hind legs, Josek et al. (2018) identified two IRs and two GRs as preferentially expressed in the forelegs but did not find transcripts corresponding to the GPCRs of Carr et al. (2017) and suggested a possible role of the former receptors in olfaction, instead. In addition, candidate odorant-binding proteins have been identified in different tick species using transcriptomics, proteomics, and genomics approaches; however, functional studies are needed to confirm their involvement in olfaction (reviewed in Gebremedhin et al., 2023). Further research is needed to further elucidate the molecular mechanism of olfaction and better support the development of repellents and HVSEs.

Pyrethroids are known to act at the voltage-gated sodium channel (VGSC) in the insect nervous system causing KD and mortality. The VGSC consists of an alpha subunit composed of four domains (I to IV), each with six transmembrane segments (S1 to S6) connected by loops forming a pore. Additionally, auxiliary transmembrane proteins, TipE or TipE-homologous, were found necessary to express the insect VGSC and to increase its functionality *in vitro* (Dong et al., 2014). In insects, binding of pyrethroids stabilizes the VGSC in the open state, disrupting the nerve function by precluding the channel from regaining the resting state (Dong et al., 2014).

VGSC and/or ORs have been implicated in the repellency effect of volatile pyrethroids to mosquitoes. Valbon et al. (2022) showed that activation of both ORs and the VGSC is involved in the repellent effects of bioallethrin in *Aedes aegypti*. On the other hand, Andreazza et al. (2021) demonstrated that TF (> 98% pure) elicited repellency in *Aedes aegypti* preferentially *via* VGSC activation, and not *via* olfactory receptors and the mechanism of TF repellency in mosquitoes is as yet, uncharacterized. In the modular assay of Murgia et al. (2022), ticks exhibited irritation at the beginning of the test period and variable KD during and post-exposure, presumably due to an increase in TF concentration in the test arena over time (including potential accumulation of TF on the surface of the test arena). This observation aligns with the toxicological response reported for mosquitoes exposed to increasing doses of TF in vapor phase (Andreazza et al., 2021). It is possible that TF repellency and toxicity to ticks are elicited *via* functionally different subsets of VGSCs (to be identified) possibly produced by alternative splicing, as no ORs have been identified in the Haller's organs. Although no information on post-transcriptional mechanisms is available for ticks,

alternative splicing sites on the VGSC gene have been identified in the varroa mite (*Varroa destructor*) (Wang et al., 2003). In insects, it has been shown that alternative splicing and RNA editing produce functionally different VGSC in terms of expression level and channel gating properties (reviewed in Dong et al., 2014). As hypothesized by Andreazza et al. (2021) for mosquitoes, the repellency effect observed in ticks could be due to a low dose of TF acting at a subset of hypersensitive VGSCs (to be identified). Higher doses of TF could act broadly on VGSCs, causing a systemic effect and producing a progression of behavioral responses from the "hot foot" effect to the KD phenotype, culminating in death. Future *in vitro* and *in vivo* studies to better understand the neurophysiological response, including the role of the Haller's organ, in tick olfactory and gustatory processes could help elucidate the MoAs of volatile pyrethroids against these pests.

It is unknown whether respiration plays a role in the intake of volatile chemistries in ticks. Fielded et al. (1994) investigated the dynamics of gas exchange in unfed *Amblyomma hebraeum* adults and showed a difference between actively moving *vs* resting ticks. Actively moving ticks had "elevated but erratic CO_2 emission rates", while resting ticks had two types of CO_2 emission patterns: a "regular discontinuous burst of CO_2" and an "extended discontinuous ventilation" characterized by long period without CO_2 emission during which the spiracles were likely closed (Fielded et al., 1994). In the modular assay, ticks exposed to the BiteBarrier treated substrate at distance showed irritation which manifested as an increase in crawling activity in the test arena, and could have favored an increase intake of TF. However, after ~30 min exposure, ticks exhibited a marked reduction in activity, which could have limited gas exchange and decrease TF intake. This phenomenon might explain the limited effect of the BiteBarrier treated substrate in terms of percent tick KD and mortality post-exposure in the modular assay.

Another open question remains the potential re-crystallization of the AI on the surfaces of the test arena as well as on the tick integument, and the contribution of TF absorbed *via* the tick cuticle (including *via* the tarsi, the major route of absorption in mosquitoes and presumably in ticks), to tick repellency, KD and mortality. As discussed in Murgia et al. (2022), the terrestrial nature of hard ticks and the modular assay design complicate the assessment of repellency resulting from TF in vapor phase *versus* contact resulting from TF crystallization on surfaces. Future studies involving assays designed to tease apart these contributions will be of value to understanding the mode of action of TF and other volatile actives and HVSEs to ticks.

We detected modest repellency of *D. variabilis* during exposure to the BiteBarrier treated substrate at a distance, and no evidence of repellency in *A. americanum* ticks. These findings agree with the study of Siegel et al. (2022), who reported a higher repellency effect of TF on *D. variabilis*. However, this and our previous study (Murgia et al., 2022) disagree with Siegel et al. (2022), as we did not identify a repellency effect to *A. americanum* or *I. scapularis*. However, exposed ticks exhibited KD, which agreed with the report of a "drunken-like state" in ticks exposed to TF. Siegel et al. (2022) proposed a definition of repellency based on the ability of the tick to climb onto a vertical stick. "Climbing deterrence" defined as ticks unable to reach the top of the stick and remain there over the 10-min testing period, was identified as a key parameter and used to score ticks as repelled by the AI. Using this definition of repellency, the authors reported greater repellency of metofluthrin (MT) > TF to *D. variabilis* > *A. americanum* > *I. scapularis* adults. In the present study, repellency was defined as "tick movement away from the source of the AI, with or without physical contact of the tick with the treated surface". Differences in assay design, definition of repellency, and testing parameters likely account for the discrepancy between the two studies.

Other studies have tested the efficacy of the volatile pyrethroids metofluthrin and allenthrin against ticks. Bibbs and Xue (2016) assessed the efficacy of two commercial products, Therma CELL and OFF!Clip-On against *A. americanum* nymphs and showed short-range repellency and toxicity post-exposure against this species. In the present study, there was no evidence of spatial activity for *A. americanum* exposed to the BiteBarrier treated substrate. Differences in the AI tested, AI concentration in the test arena, and AI volatilization due to the use of active (former products) *vs* passive (BiteBarrier) devices could explain this discrepancy.

Differences between the methodology and parameters used to assess the efficacy of AIs and products across multiple studies present challenges to comparative analyses. Studies of spatial activity and repellency in ticks are hampered by the lack of standardized assays and defined, universally accepted parameters to assess the efficacy of AIs, formulations, and active/passive devices against ticks. This issue has also been discussed by other investigators who point to impediments to AI discovery and product development (Bissinger et al., 2011).

4.2. Tick behaviors following contact with BiteBarrier treated substrate in the presence of a human stimulus

Murgia et al. (2022) reported stronger contact efficacy of the BiteBarrier treated substrate against *I. scapularis* > *A. americanum* > *D. variabilis*, performed in the absence of host cues. In this study, we developed an assay to test the capability of treated substrate to control ticks in the presence of a human host and explore the impact of the BiteBarrier treated substrate on a range of tick behaviors that precede tick bite (orientation and movement toward the host, host contact, sustained attachment and vertical movement on the host). The dual-choice behavioral assay revealed the impact of the BiteBarrier treated substrate on multiple tick behaviors, including those behaviors typically associated with host location, attachment and feeding. Almost universally, exposure induced the "hot foot" effect indicative of tick irritation resulting from TF exposure. This represents an important finding as the irritation response is likely to impair questing, attachment, and movement on the host. Despite presence of the BiteBarrier treated substrate, a small percentage of ticks (20%, 13%, and 9% of *A. americanum*, *D. variabilis*, and *I. scapularis*, respectively) located and crawled on the host at some point during the 5-min test period. On average, ticks crawled in the test arena for over a minute before locating and crawling on the host, which may reflect an overall confusion and irritation. In addition, *I. scapularis* and *A. americanum* ticks crawled on the finger of the volunteer for less than 15 s and *D. variabilis* for less than 26 s before crawling off. These data indicate the need for further behavioral studies to investigate the impact of the BiteBarrier on tick-host location and attachment in the presence of a host. The longer contact time with the human volunteer observed for *D. variabilis* agrees with the results of Murgia et al. (2022) which revealed limited evidence of mortality in this species after 3 min of contact with the BiteBarrier treated substrate, presumably reflecting the greater biomass and detoxification capacity in this species.

While the BiteBarrier treated substrate did not prevent ticks from contacting and crossing the treated substrate (Fig. 5) or eliminate tick contact with the host, ticks that contacted the treated substrate exhibited rapid contact irritancy ("hot foot" effect), leading to avoidance (crawling on the wall of the Petri dish) and protective (crawling beneath untreated/treated substrates) behaviors. These behaviors were also reported by Murgia et al. (2022) and other studies of tick exposure to pyrethroids such as permethrin (Eisen et al., 2017). Eisen et al. (2017) tested *I. scapularis* nymphs in a double-choice assay using a test arena comprising equal areas of treated and untreated substrate and a human finger placed on each half. Ticks were found to preferentially contact the finger located in the untreated area, but 9% of ticks also contacted the finger placed on the permethrin-treated area. Our results agree with the findings of Eisen et al. (2017) both in terms of tick preference for the untreated zone and proportion of ticks contacting the human finger placed in the treated zone. It is known that *I. scapularis* has an "ambush-style" questing behavior in which the tick exploits direct physical contact and electrostatic force, among other factors to gain host attachment, as opposed to positive movement over distance toward the

host, as is typical of some hard tick species (e.g. *Amblyomma* spp.). The dual-choice assay is designed to exploit this questing phenomenon and enable meaningful assessment of product effects in the presence of a host. Overall, the data suggest the potential of the BiteBarrier treated substrate to interfere with tick questing by triggering irritation and avoidance behaviors that could reduce bite risk. The findings reveal that a small percentage of BiteBarrier-exposed ticks (9–20%, depending on species) are capable of host location when exposed, but that these ticks experience minimal time on the host (14–26 s), take on average 80–133 s to locate the host, and frequently crawl off the host during the 5-min test period, suggesting potential of the BiteBarrier to minimize host location, attachment and tick bite.

4.3. BiteBarrier prototype perimeter device is effective against Ixodes scapularis female ticks at near range in the perimeter assay

In a series of laboratory contact and modular assays, Murgia et al. (2022) demonstrated contact efficacy and short-range spatial activity of the BiteBarrier treated substrate against *I. scapularis* adult females. Those data strongly supported a perimeter device as the ideal configuration for tick control. Therefore, we developed an assay to test the efficacy of a BiteBarrier prototype in a perimeter configuration under controlled conditions against *I. scapularis* adults. The perimeter assay presented here enables assessment of the efficacy of novel tick-bite prevention technologies under laboratory and semi-field or full clinical field trial conditions. The assay is specifically designed to test products against terrestrial pests such as ticks and enables assessment of the phenotypic response of unrestrained ticks in order to mimic application in the field as would be typical in military or civilian use cases.

Our results indicate the potential of the BiteBarrier prototype perimeter to protect from ticks, possibly *via* delivery of TF dose sufficient to disrupt tick activity and to control ticks located inside the perimeter *via* KD and mortality. Furthermore, we obtained $>$ 90% KD at 1 h PE and $>$ 90% mortality at 48 h PE, indicating potential of the BiteBarrier perimeter prototype to meet the EPA targets for product registration. The BiteBarrier device is a novel one-of-a-kind tick control technology with potential multiple applications as a personal protection device to protect an area from ticks. The device could be used to protect the deployed warfighter from tick bites *via* placement under an open-floor tent in sleeping/dining areas, and as a tick protection tool for the general public. In this study, we addressed the ability of the BiteBarrier to control ticks in an area internal to the perimeter device. Open questions remain about the impact of the perimeter device on the area outside the perimeter. The BiteBarrier presumably has an effect in the outer area as well, and future work will address these questions by evaluating the area protected by the device.

Ongoing studies aim to determine the dimensions of the area protected by the BiteBarrier perimeter and the effect of sublethal responses to TF on pre-attachment behaviors. In addition, the device is under evaluation under semi-field conditions to determine applicability for use in limited outdoor settings. Environmental factors such as air movement, temperature, and humidity are expected to affect TF volatilization and dispersal, reducing efficacy (KD/mortality) under semi-field conditions, and requiring further assay optimization. Future studies will also investigate the efficacy of the BiteBarrier prototype perimeter against additional tick species, including *A. americanum* and *D. variabilis*.

5. Conclusions

We present an initial assessment of the BiteBarrier prototype configured as a perimeter device for area-wide protection against *I. scapularis* adult females and tick-bite prevention. Our data demonstrate the potential of the BiteBarrier prototype as a barrier device for protection of an area such as a sleeping/work area from ticks and containment of ticks within the BiteBarrier perimeter. Behavioral data revealed possible interference of the BiteBarrier treated substrate with tick behaviors that precede attachment and feeding and highlight the need for further studies to assess its bite prevention capability.

CRediT authorship contribution statement

Maria V. Murgia: Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Laurie Widder:** Conceptualization, Resources, Writing – review & editing. **Catherine A. Hill:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Ethical approval

This study does not require ethical approval.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2025.100246.

Data availability

The data supporting the conclusions of this article are included within the article and its supplementary files.

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