Laboratory and field evaluation of brown dog tick behavioral responses to potential semiochemicals

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ABSTRACT

The brown dog tick, *Rhipicephalus sanguineus* sensu lato (Parasitiformis: Ixodidae), is an ectoparasite of dogs that can be found worldwide. This tick poses unique difficulties in management because it can complete its entire life cycle indoors and has demonstrated acaricide resistance. The ability to monitor for tick presence and abundance is necessary for developing effective control programs. As such, an evaluation of adult brown dog tick behavioral responses to 16 potential semiochemicals was undertaken using Y-tube and straight-tube olfactometers. Both sexes of ticks were activated by nine of the 16 semiochemicals tested, including 300 µg of 1-octen-3-ol, benzaldehyde, benzyl alcohol, hexanoic acid, nonanoic acid, methyl salicylate, o-nitrophenol, 2,6-dichlorophenol and salicylaldehyde. *Rhipicephalus sanguineus* s.l. behaviors including movement speed, direction and duration, as well as turning, were quantified following exposure to the same nine chemicals in a straight-tube olfactometer, individually at 300 µg and as mixtures. Three individual chemicals, 1-octen-3-ol, hexanoic acid, and methyl salicylate induced strong responses. These three chemicals were evaluated in a semi-field setting using a modified bed bug trap but were found to provide no significant increase in attraction compared with CO₂ alone. The results of these studies provide a foundation for future research regarding semiochemicals of *R. sanguineus* s.l.

Key words: *Rhipicephalus sanguineus*, carbon dioxide, attraction, olfactometer, pheromone, kairomone;

1. Introduction

The brown dog tick, *Rhipicephalus sanguineus* sensu lato, is an ectoparasite of veterinary importance and a vector of the pathogens causing canine ehrlichiosis (Goddard, 1987) and canine babesiosis (Boozer and Macintire, 2003). Additionally, the brown dog tick is the vector of *Rickettsia conorii*, and has been implicated as a vector of *Rickettsia rickettsii* (Demma et al., 2005; Ereemeeva et al., 2011), causative agents of Mediterranean and Rocky Mountain spotted fevers in human beings, respectively. Unlike most tick species, the brown dog tick presents a unique management challenge for homeowners and pest control companies as it can complete its life cycle indoors (Dantas-Torres, 2008; Jacobs et al., 2001; Louly et al., 2007; Dantas-Torres and Otranto, 2011). Removal of an indoor infestation is difficult due to their cryptic nature, while the use of acaricides on- and off-host is possibly complicated by the presence of acaricide resistance (Miller et al., 2001; Eiden et al., 2015). Despite the presence of this species worldwide in residential homes and dog kennels, there are no methods established for surveillance, pre- and post-treatment monitoring or mass trapping. Successful integrated pest management programs rely on monitoring pest populations for early detection and to evaluate treatment efficacy. As a single brown dog tick female can lay approximately 4,000 eggs (Koch, 1982), it is important to detect infestations at an early stage before the population establishes. One tool that can enable the early detection of tick presence is a trap with visual or olfactory attractive properties. Of particular interest are olfactory stimuli, such as kairomones, which have been investigated as trap lures for many hematophagous arthropods, including ticks.

61 However, brown dog tick chemical ecology, including response to kairomones, is not
62 well understood.

63 Adult brown dog ticks mate on the host during feeding. In order to mate or feed
64 on a host, the adult tick must first locate and get onto the host in order to attach, feed
65 and mate. In general there are two tactics that ticks use to locate hosts: ambush and
66 hunt. Tick species that use the hunt tactic, such as the brown dog tick, actively seek out
67 their host through attraction to volatiles produced by the host (Dantas-Torres, 2008;
68 2010). These volatiles, known as kairomones, guide the tick towards its host (Dantas-
69 Torres, 2010). For example, carbon dioxide (CO₂) has been demonstrated to be
70 important for brown dog tick attraction to traps (Ranju et al., 2013; Carnohan et al.,
71 2015). *Rhipicephalus sanguineus* s.l. may use kairomones other than CO₂ to determine
72 host location. However, the specific semiochemicals responsible for *R. sanguineus* s.l.
73 attraction to dogs have not been identified to date. In addition to kairomones, attraction-
74 aggregation-attachment and sex pheromones, also could contribute to a bait for use in a
75 brown dog tick trap. As brown dog ticks mate on-host, chemical communication during
76 courtship could be complex, involving a variety of chemicals at different stages
77 (attraction, mounting and mating).

78 For ticks, trapping devices have been successfully implemented in outdoor
79 settings with several *Amblyomma* species (Wilson et al., 1972; Gray, 1985; Norval et
80 al., 1989), and traps placed in dog kennels have been used with partial success in
81 attracting the brown dog tick (Ranju et al., 2013). However, no commercial tick
82 monitoring devices or traps are currently available for the brown dog tick. The purpose
83 of this study was to identify attractants that could be incorporated in a trap for the brown

dog tick. As many tick semiochemicals appear to overlap interspecifically it is feasible that chemicals previously identified for other tick species may be attractive to the brown dog tick (Allan, 2010). Hence, the specific objectives of this study were: 1) to screen a selection of chemicals, which had been previously reported as tick kairomones and pheromones, for brown dog tick activation and attraction, and 2) to evaluate selected chemicals in a semi-field setting.

2. Materials and methods

2.1. Ticks

Rhipicephalus sanguineus s.l. (hereafter *Rhipicephalus sanguineus* unless specified otherwise) were purchased from Oklahoma State University (OSU) and used within two to four weeks of receipt (i.e. six to ten weeks post-molting to the adult stage). The OSU colony was established in 1991, has wild introductions every two years and is maintained at 22.8°C and 95% relative humidity (RH) with a 15:9 (L:D) photoperiod when not feeding on sheep. When received, unfed ticks were sorted by sex and stored in ventilated 10 mL glass jars and held at 25°C and >90% RH with a 15:9 (L:D). Humidity was maintained by storing the ticks a desiccator cabinet with cups of water. The temperature and humidity was routinely measured using a data logger with an external probe sensor (U14-002, HOBO, Onset Computer Corporation, Bourne, MA). Ticks were handled using soft forceps, while wearing nitrile gloves to prevent the direct contamination of tick sensory organs by human skin volatiles, inside a plastic insect cage (BugDorm, MegaView Science Co., Taichung, Taiwan) at 25°C and 70-80% RH. Humidity was maintained in the cage with cups of water and monitored with a data

logger with an external probe sensor (U14-002, HOBO, Onset Computer Corporation, Bourne, MA).

Tick responses to potential attractants were evaluated in Y-tube and straight-tube olfactometers. For each trial, only ticks that were active when removed from the glass jars were used, i.e. ticks that failed to move and remained quiescent were not selected. Individual ticks were evaluated with multiple chemicals but only after a period of at least two weeks of non-exposure, and they were never re-exposed to the same chemical. In between evaluations ticks were stored in jars as described above under the same conditions, i.e. 25°C and >90% RH.

2.2. Olfactometer assays

Olfactometer set-up: Glass olfactometers were purchased from Analytical Research Systems, Gainesville, FL. The Y-tube olfactometer was comprised of a 13.0 cm long stem and two 10.0 cm long arms. The tube had an inner diameter of 2.0 cm and 30 degree angle at the Y branch. The straight-tube olfactometer was 26.5 cm in total length, with an inner diameter of 2.0 cm. For both olfactometers ticks were placed in a release zone via a removable hollow glass chamber (5.0 cm length, 2.0 cm diameter) that was inserted into the downwind end of the olfactometer tube.

Both atmospheric air from the centrally located pressurized air supply (hereafter CO₂-background air) and a commercial blend of air enhanced with 5% CO₂ (v/v) (Airgas™, Tampa, FL; hereafter CO₂-enhanced air) were utilized. The latter was supplied from a pressurized tank with a similar percentage of CO₂ to the 4.5% that is emitted during human expiration (Lehane, 2005). Both airflows first passed into a two-

port humidified air delivery system (Analytical Research Systems, Gainesville, FL). The atmospheric air was cleaned to remove volatile contaminants. Prior to entering the olfactometers, the air flow passed through a flask of deionized water to produce 60-80% RH and into mixing glass chambers, which is where the chemicals were introduced. The ticks were prevented from entering the mixing chamber by metal mesh on the upwind end of the olfactometers. All airflow rates were 50 mL/min (Bissinger et al., 2011) and verified between each assay using a mass flow meter (AALBORG® GFM 171, Orangeburg, NY). Humidity in the air flow was routinely measured at the stem of the olfactometer using a data logger with an external probe sensor (U14-002, HOBO, Onset Computer Corporation, Bourne, MA). To reduce external visual stimuli and diffuse lighting, the assay arena was housed in a rectangular metal frame draped with white cotton sheeting. Glassware was hand washed with glassware detergent (Neutrad®, Decon Laboratories Inc., King of Prussia, PA) between use with different chemicals, then triple rinsed in deionized water (Yunker et al., 1992), rinsed in ethyl alcohol ($\geq$ 90%), and then heated for a minimum of one hour at 110°C until dry.

2.2.1. Experimental procedure: Assays were conducted in the light between 08:00-20:00 hr at 25°C and 60-80% RH. The experimental unit consisted of five adult ticks of the same sex. Ticks were inserted into the removable hollow chamber, and then the chamber was placed with the open end down on a smooth surface for 1 to 2 min. This technique allowed for tick acclimatization, resulting in quiescence.

Diluted chemical solutions (30 μ L) were applied to 1.5 cm diameter filter paper circles (Whatman® no. 1; Maidstone, England) placed on flat Teflon coated disks (US Plastics Corp., Lima, Ohio). The solvent was allowed to evaporate for 30 s and then

disks were placed in the mixing chambers of the olfactometer. A listing of all chemicals, including purity and manufacturer information, and chemical combinations evaluated in the two olfactometers and the semi-field evaluation are provided in Table 1. These chemicals were all previously identified to be semiochemicals of other tick species (Allan, 2010).

The hollow chamber containing quiescent ticks was attached to the downwind end of the olfactometer, which placed the ticks in the release zone. Ticks were left undisturbed for 15 min to allow for movement in response to the chemical.

2.2.2. Y-tube olfactometer assays: Y-tube olfactometer tests were used to conduct a preliminary screening of tick activation caused by potential attractant semiochemicals. Two Y-tubes were set up simultaneously, at each time interval a chemical was tested in the presence of CO₂-background air and CO₂-enhanced air, with the same air going into both arms of the Y-tube. Chemicals were tested in an incomplete block design so that one replicate of a set of four chemicals at each amount (i.e. 3, 30 and 300 µg) was completed each day. The order of chemicals tested each day and sex of ticks used were randomized. Tests were conducted with a chemical in one arm and a hexane control in the other arm. An external control with hexane in both arms (with CO₂-background air and CO₂-enhanced air), also was completed (hereafter control). Each treatment was replicated 10 times, resulting in the exposure of 50 ticks to each chemical/amount/air combination. Tick activation was defined as the proportion of ticks that left the release zone.

2.2.3. Straight-tube olfactometer assays: Chemicals eliciting the strongest activation ($p < 0.001$ compared to controls) were further evaluated for searching behavior in straight-tube olfactometers (Table 1). Four straight-tubes were set up simultaneously, at each time interval two chemicals were tested with one tube using CO₂-background air and the other using CO₂-enhanced air. The nine individual chemicals, and selected blends were compared to a control. Each treatment was replicated three times, resulting in the exposure of 15 ticks to each chemical/air combination. Tick responses in the olfactometer were videotaped from directly above using a charge-coupled device (CCD) camera (Philips® LTC 0500/60), an automatic iris lens (1/3 in, 5-55 mm, F 1.4, Rainbow, Irvine, CA), and a real-time mpeg encoder (Canopus, Kobe, Japan). Each video was later manually scored for specific tick behavioral events using a behavioral analysis software package (The Observer® XT 8.0, Noldus, Wageningen, The Netherlands).

Four behavioral response variables were analyzed: movement, activation, end touch, and direction change. Movement (secs) was the total time that *R. sanguineus* spent moving. Activation (secs) was the time until a tick moved out of the release zone. End touch was the frequency that a tick contacted the mesh boundary at the upwind end of the olfactometer. Direction change was the number of times a tick changed direction of movement.

Further analysis of the recorded videos was completed to investigate changes in the speed of behavior in ticks exposed to several of the chemicals. The tracks were manually digitized using motion analysis software (Peak Motus v. 8, Vico, Centennial, CO) and the resulting tracks were analyzed using EthoVision v. 7.0 (Noldus, Asheville, NC). The treatments subjected to further analysis were the control (hexane), and those

that resulted in strong tick activation and attraction, which were 300 µg 1-octen-3-ol, 30 and 300 µg kairomone blends, and the 30 and 300 µg pheromone blends. The variables assessed included velocity (cm/sec), and angular velocity (degrees/sec). Angular velocity is defined as the change in direction of movement between two consecutive samples, calculated per unit time, or turning rate. The average was calculated for each variable for each experimental unit (group of five ticks).

2.3. Semi-field assays

2.3.1. Assay design: Selected chemicals were evaluated for their efficacy using the commercially-available bed bug trap, ClimbUp[®] (Susan McKnight Inc., Memphis, TN) in two specially designed assay rooms or mesocosms described previously by Carnohan et al. (2015). In previous studies, the ClimbUp[®], when baited with carbon dioxide, was the most efficacious of those tested for capture of brown dog ticks (Carnohan et al., 2015). In the mesocosms, the average temperature was $24.54 \pm 0.27^{\circ}\text{C}$ and $26.19 \pm 0.22^{\circ}\text{C}$ in the morning (6:00-12:00 hr) and afternoon (13:00 -19:00 hr) evaluation periods, respectively.

2.3.2. Treatments: Eight treatments were evaluated as potential tick attractants, including three chemicals that were selected for use based on the straight-tube assay results. 1-Octen-3-ol and methyl salicylate had the shortest activation, and hexanoic acid induced the greatest movement. Preliminary studies verified that 500 µL of undiluted chemicals lasted for at least six hours. Each 500 µL aliquot was placed into a glass vial with a hole in the lid (5mm diameter), and a piece of pipe cleaner (8 cm) was folded in half and inserted into the lid of the vial to facilitate diffusion of volatiles. The vial

was placed on the top of the CO₂-emitting thermos. Each chemical was used individually with and without CO₂. CO₂ was provided in the form of dry ice pellets (~1.8 kg), which were placed in a blue and white insulated thermos (1.89 Liter Igloo Legend, Igloo Products Corp.) with a 1.2-cm orifice, and produced 992.5 ml/min CO₂. Similar thermoses, to the one used in this trap, provided no attractive stimulus to the ticks in the absence of CO₂ in a previous study (Carnohan et al., 2015). In the treatments without CO₂, an empty thermos was placed in the center of the trap. Additionally, two controls were established, a ClimbUp[®] trap with a heat source and without chemicals or dry ice, and a ClimbUp[®] trap with dry ice without heat or chemicals. The heat source was an air-activated hand warmer (Hot Hands, HeatMax Inc., Dalton, GA), which produced a temperature of 44.5 ± 0.5°C and was placed in the center of the trap. All treatments except the heat treatment contained a thermos.

2.3.3. Trap evaluation: Ten ticks of mixed sex were released in each of the four corners of the mesocosm, at least 15 cm from the walls, for a total of 40 ticks. Following a 30 min acclimatization period, a trap baited with one of eight attractant combinations (treatments) was placed in the center of each mesocosm approximately 1 m from the walls. Nitrile gloves and shoe guards (Trimaco, Morrisville, NC) were worn when entering the rooms. The doors were closed and the rooms were left undisturbed for six hours. At the end of this period, the positions of the ticks within the rooms and on traps were recorded. The number of ticks captured, attracted, and activated were recorded for each room. Ticks recovered in the pitfall of the trap were considered captured. Ticks counted both on the trap and in the pitfall of the trap were classified as attracted. It was noted in a previous study (Carnohan et al., 2015) that ~90% of the ticks were on the

walls after the 30 min acclimatization period. Therefore, any ticks that were on the trap, in the pitfall of the trap, and on the floor of the room were considered to be activated.

After each assay, all ticks and traps were removed from the two rooms and a high powered exhaust fan was run for 30 min. Thereafter, a new group of ticks were released into each room for the next assay.

Each of the eight treatments (Table 1) were tested seven times with 40 ticks per test. Traps were randomized by room and time of day (morning or afternoon) ensuring that all treatments were equally represented by using a randomized Latin-square design.

2.4. Statistical analyses

2.4.1. Y-tube olfactometer assays: The response variable activation (definition as previously described) was evaluated for normality with no transformation necessary. A linear mixed model consisting of three factors of chemical (see Table 1), amount (3, 30 and 300 μ g) and air (CO₂-background and CO₂-enhanced) and all two- and three-way interactions was fitted. Chemical group was considered a random blocking factor, while the treatment arm (arm that received the chemical) and sex served as fixed blocking factors. All models were fitted using restricted maximum likelihood (REML) as implemented in PROC MIXED with SAS v. 9.2 (SAS, 2008) with a significance level of 5%. Least significant difference (LSD) tests were used to compare means of tick responses, particularly of each chemical against the control, and to compare mean tick responses under CO₂-background air to CO₂-enhanced air within a given chemical.

2.4.2. Straight-tube olfactometer assays: The six response variables analyzed were duration of movement, time to activation, number of end touches and direction changes, velocity and angular velocity, with definitions as previously described. All data were tested for normality. Activation and angular velocity did not follow a normal distribution, therefore, these variables were log transformed prior to statistical analysis and the means were back-transformed for presentation. All six response variables, were analyzed by fitting a linear mixed model using PROC MIXED with SAS 9.2 (SAS, 2008) that included the factors of chemical (see Table 1), air (CO₂-background air and CO₂-enhanced air), and their interaction. Significant differences ($\alpha = 0.05$) between means for each of the variables analyzed were evaluated using LSD tests.

2.4.3. Trap evaluation: The three response variables analyzed to evaluate trap efficacy were captured, attracted, and activated (definitions as previously described). All response variables were evaluated for normality and no transformations were deemed necessary. All three responses were analyzed by fitting a linear mixed model using PROC MIXED with SAS 9.2 (SAS, 2008) that included the blocking factors of mesocosm and time (morning and afternoon), together with the main factor of attractant (8 treatments) and the random effect of trapping event. Inclusion of environmental covariates of average temperature and average RH also were evaluated due to noticeable differences in morning and afternoon trap catches, and if significant ($\alpha = 0.05$) were kept in the final model. Significant differences ($\alpha = 0.05$) between pairs of attractants were evaluated using LSD tests.

3. Results

3.1. Y-tube olfactometer assays

There was a significant difference between the responses of males and females when comparing activation ($F_{1,946} = 4.24$, $p = 0.0397$), however, these differences were slight and likely to be without biological significance. For example, proportion of activation for female and male ticks was 0.33 ± 0.03 and 0.30 ± 0.03 , respectively. Therefore, the data for male and female ticks were pooled and sex was considered to be a random factor in the analysis.

All the model factors and interactions were significant ($p < 0.05$), with the exception of treatment arm, which confirms the lack of directional bias in the olfactometer. Exposure of adult *R. sanguineus* to 300 μg of γ -valerolactone, 1-octen-3-ol, benzaldehyde, benzyl alcohol, hexanal, hexanoic acid, butanoic acid, methyl salicylate, o-nitrophenol, 2,6-dichlorophenol or salicylaldehyde, resulted in activation, that was significantly greater than ticks exposed to the control ($p < 0.001$; Table 2).

Chemicals that caused greater than 60% activation of ticks at 300 μg included o-nitrophenol, 1-octen-3-ol, 2,6-dichlorophenol, benzaldehyde, benzyl alcohol, butanoic acid, methyl salicylate and salicylaldehyde ($p < 0.05$). The activation in response to chemicals at the lowest amount tested, 3 μg , was not significantly different from the control (data not shown). Regardless of air type, ticks exposed to 30 μg of methyl salicylate, salicylaldehyde, o-nitrophenol, butanoic acid and 2,6-dichlorophenol demonstrated more activation than ticks exposed to the hexane control (data not shown; $p < 0.05$). The presence of CO_2 -enhanced air resulted in significant increases

for activation ($F_{1,946} = 5.02$, $p = 0.0253$). Chemicals at 300 μg with an increase in tick response in the presence of CO_2 -enhanced air were benzyl alcohol, hexanal, and hexanoic acid (Table 2; $p < 0.05$).

3.2. Straight-tube olfactometer assays

Analysis of the videotaped behavioral assays revealed that after leaving the release zone, adult *R. sanguineus* actively moved in the direction of the airflow and against the airflow in the straight-tube olfactometer when exposed to the various chemicals tested. There were significant differences in movement depending on exposure to different chemicals and mixtures of chemicals ($F_{15,61} = 36.89$, $p < 0.0001$) and a significant interaction ($F_{15,61} = 2.24$, $p = 0.0140$) was found between chemical and air (CO_2 -background or CO_2 -enhanced). Movement was significantly increased during exposure to seven individual chemicals and four blends (Table 3). Ticks spent the most time moving in the presence of benzyl alcohol ($2,444 \pm 139$ s), the 300 μg kairomone blend ($2,285 \pm 170$ s), and hexanoic acid ($2,142 \pm 139$ s), all in the presence of CO_2 -enhanced air. However, tick movement did not significantly increase with the addition of CO_2 . On the contrary, the presence of CO_2 -enhanced air significantly lowered movement in the presence of butanoic acid ($p = 0.003$).

Time to activation in CO_2 -enhanced air (149 ± 1.0 s), was significantly shorter than for CO_2 -background air (228 ± 1.0 s) irrespective of the chemical treatment ($F_{1,61} = 4.72$, $p = 0.0337$). There were significant differences ($F_{15,61} = 13.6$, $p < 0.0001$) between chemical treatments in time to activation (Table 4). In the control treatment, time to activation was 3,501 s, significantly longer than for all chemicals, except for

benzaldehyde ($1,018 \pm 1.7$ s). Time to activation was shortest when ticks were exposed to 1-octen-3-ol (15 ± 1.5 s) and the 300 μ g all-chemical blend (25 ± 1.5 s). Ticks responded rapidly to several other treatments, including methyl salicylate (50 ± 1.5 s), the 300 μ g kairomone blend (50 ± 1.5 s), the 300 μ g pheromone blend (64 ± 1.5 s), and butanoic acid (76 ± 1.5 s).

There was a significant difference ($F_{1,61} = 6.29$, $p = 0.0148$) between the number of end touches for CO₂-background air (7.44 ± 0.37) and CO₂-enhanced air (6.14 ± 0.36), irrespective of the chemical treatment. Significant differences ($F_{15,61} = 13.6$, $p < 0.0001$) between chemical treatments in number of end touches are displayed in Table 4. The groups of five ticks exposed to the 300 μ g kairomone blend, 1-octen-3-ol and the all-chemical blend touched the upwind end of the straight-tube 13.33 ± 1.11 , 12.83 ± 0.99 and 9.17 ± 0.99 times within the test period, respectively; which was significantly more than all other treatments evaluated (range: 1.83 to 8.83), including the control (3.00 ± 0.99) (Table 4).

There were significant differences in the number of direction changes depending on exposure to individual chemicals or the chemical mixtures ($F_{15,61} = 27.8$, $p < 0.0001$; Table 3). There also was a significant interaction of chemical and air (CO₂-background or CO₂-enhanced) with this variable ($F_{15,61} = 2.43$, $p = 0.0076$). In treatments without CO₂ enhancement, introduction of 1-octen-3-ol (69.67 ± 5.05), hexanoic acid (61.67 ± 5.05) or the 300 μ g kairomone blend (48.33 ± 5.05) resulted in significantly more direction changes than exposure to any of the other treatments (range: 9.33 to 36.33), including the control (9.33 ± 5.05). In CO₂-enhanced air, treatments containing the 300 μ g kairomone blend (81.50 ± 6.19) and hexanoic acid (74.00 ± 5.05) caused

significantly more direction changes than the remaining chemical treatments (range: 6.67 to 58.00), including the control (6.67 ± 5.05). Ticks exposed to CO₂-enhanced air and 300 µg kairomone blend changed direction 59.3% more frequently ($p < 0.05$) than ticks exposed to the same blend under CO₂-background air conditions.

Chemical treatments also affected speed of behavioral responses with a significant effect of chemical treatment on velocity ($F_{5,22} = 15.11$, $p < 0.0001$) and angular velocity ($F_{5,22} = 6.00$, $p = 0.0012$) with no other factors or interactions being significant (Table 5). The velocities of ticks exposed to the 300 µg kairomone blend (2.72 cm/sec) and 300 µg 1-octen-3-ol (2.64 cm/sec) were significantly greater than all other treatments and more than twice as fast compared to the control (1.04 cm/sec). The angular velocity, was significantly greater in the control (79.09 degrees/sec) compared to all other chemicals analyzed.

3.3. *Trap evaluation*

There was a significant effect of the mean temperature ($F_{1,23.3} = 28.74$, $p < 0.0001$) on the number of ticks captured; a 1°C increase in temperature translated into 0.75 more ticks captured. Using the model with the covariate of average temperature for the variable captured, the mean number of ticks captured in the trap was significantly different for the treatments evaluated ($F_{7,40.9} = 7.65$, $p < 0.0001$). Ticks captured following exposure to the eight treatments separated into two distinct groups (Table 6). Treatments that included CO₂ resulted in significantly greater numbers of ticks captured than treatments that did not include CO₂.

There were no significant effects on the numbers of ticks attracted and activated due to temperature ($F_{1,45} = 3.17$, $p = 0.0817$ and $F_{1,45} = 2.4$, $p = 0.1282$, respectively). Using the model without the covariate of average temperature, there were significant differences in the number of ticks activated ($F_{7,46} = 3.19$, $p = 0.0077$) dependent upon the treatment presented in the trap. Trends were similar for activation as they were for capture. All CO₂-containing treatments generated the greatest activation, ranging from 14.94 (± 1.74) to 19.69 (± 1.74) and were statistically equivalent ($\alpha = 0.05$; Table 6). There were no significant differences between the treatments for the number of ticks attracted ($F_{7,46} = 1.98$, $p = 0.0782$). The mean number of ticks attracted to the different treatments ranged from 7.89 to 13.81 (19.72% to 34.53%; data not shown).

4. Discussion

Understanding of the chemical ecology of the brown dog tick is limited. However, it is known that many tick semiochemicals, both kairomones and pheromones, overlap in function between species (Allan, 2010). Therefore, the aim of this study was to screen a selection of chemicals, previously reported as tick-associated pheromones and kairomones, for brown dog tick activation and attraction. The Y-tube olfactometer served as a tick activation screening device enabling the evaluation of 16 chemicals previously identified from the literature as semiochemicals. The nine chemicals that resulted in strong activation (i.e. 1-octen-3-ol, benzaldehyde, benzyl alcohol, butanoic acid, hexanoic acid, methyl salicylate, o-nitrophenol, 2,6-dichlorophenol and salicylaldehyde), were further tested in the straight-tube olfactometer to provide insight into the searching behavior of ticks exposed to these chemicals. Based on these

studies, three chemicals, 1-octen-3-ol, hexanoic acid, and methyl salicylate were selected for semi-field trap testing. All traps with CO₂ captured similar numbers of ticks, and the addition of one of the three chemicals had no additive effect on trap catch demonstrating the overriding importance of the kairomone CO₂ for host location in brown dog ticks.

In the straight-tube olfactometer, ticks demonstrated odor-conditioned anemotaxis, directional movement in response to air currents carrying stimulating volatiles (Kennedy, 1977), by approaching and contacting the upwind end of the olfactometer closest to the odor source. In addition, chemokinesis, non-directional responses to odors, were recorded, such as reduced turning rate (angular velocity) in the presence of certain chemicals. In the presence of the control, ticks had a turning rate three-fold higher than in the presence of potential semiochemicals. For example, in the presence of 1-octen-3-ol, ticks turned 10.12 ± 3.49 degrees/sec compared to 79.09 ± 23.63 degrees/sec in the presence of the control (Table 5). The increased random turning of the ticks exposed to the control is likely the result of the absence of volatile cues, which would stimulate directional movement.

Of the 16 chemicals tested in this study, four had been previously implicated in brown dog tick chemical ecology: 2,6-dichlorophenol, methyl salicylate, squalene and benzaldehyde (Yoder et al., 2008; Ranju et al., 2013; Borges et al. 2015). As in the current study, 2,6-dichlorophenol and methyl salicylate have been demonstrated previously to attract brown dog ticks (Yoder et al., 2008; Ranju et al., 2013). Squalene, a skin associated compound, elicited aggregation rather than attraction in brown dog ticks (Yoder et al., 2008), which is possibly why this chemical failed to induce behavioral

responses in the current study. Benzaldehyde has been shown to be repellent to brown dog ticks when in conjunction with 2-hexanone but not when alone (Borges et al., 2015). When tested alone in the current study, benzaldehyde resulted in increased activation in the Y-tube olfactometer and increased movement in the straight-tube olfactometer, which could be indicative of a repellent action of this compound. In addition to 2,6-dichlorophenol, methyl salicylate and benzaldehyde, six chemicals were demonstrated to induce changes in the behavior of the brown dog tick for the first time. These were 1-octen-3-ol, butanoic acid, hexanoic acid, benzyl alcohol, salicylaldehyde and o-nitrophenol (Rechav et al., 1977; Apps et al., 1988; Lusby et al., 1991; Yunker et al., 1992; Steullet and Guerin, 1994a; Osterkamp et al., 1999).

2,6-Dichlorophenol is a female-produced sex pheromone for many tick species, reportedly present in extracts of partially-fed female brown dog ticks (Chow et al., 1975). However, the presence of the chemical does not confirm its role as a sex pheromone. In fact, current literature supports the lack of a role for this chemical in brown dog tick mating behavior (de Bruyne and Guerin, 1994; Borges et al., 2006; Louly et al., 2008). Methyl salicylate is a component of the male-produced attraction-aggregation-attachment pheromone attractive to immatures and adults of several *Amblyomma* species (Yunker et al., 1992), but is not known from brown dog ticks. Attraction of unfed *R. sanguineus* to 2,6-dichlorophenol and methyl salicylate was demonstrated previously in two-choice, short-range olfactory tests (Yoder et al., 2008). The role of 2,6-dichlorophenol and methyl salicylate remains unclear and responses to these compounds may possibly be related to generalized responses to phenols in the context of host finding.

Several host-derived chemicals were demonstrated for the first time to induce changes in brown dog tick behavior. 1-octen-3-ol is a kairomone used for host location by many hematophagous arthropods, including ticks (Osterkamp et al., 1999; Carr et al., 2013). Interestingly, *R. sanguineus* larvae were not attracted to 1-octen-3-ol in the Petri dish bioassays performed by Ranju et al. (2012). In the current study, 1-octen-3-ol emerged as one of the chemicals that induced the greatest degree of tick behavioral response, including activation and attraction, in the straight-tube assays. Along with 1-octen-3-ol, hexanoic acid and o-nitrophenol also elicited behavioral responses in the brown dog tick. All three chemicals have been isolated from bovine skin extracts and found to cause attraction of *Rhipicephalus (Boophilus) microplus*, (Canestrini) (Osterkamp et al., 1999). The potential for these two chemicals to be important in *R. sanguineus* attraction was confirmed in the present study.

The other two host-derived chemicals that influenced brown dog tick behavior in the current study, butanoic acid and benzaldehyde, have been isolated from volatile odor collections from bovines and rabbits (Steullet and Guerin, 1994a, 1994b; Donzé et al., 2004). These host-derived chemicals were shown to excite olfactory receptors of the tropical bont tick, *Amblyomma variegatum* Fabricius (Steullet and Guerin, 1994a, 1994b). Butanoic acid, when presented to *A. variegatum* resulted in significantly greater attraction and locomotion compared to ticks presented with a control (Donzé et al., 2004).

In addition to kairomones, brown dog ticks responded to previously identified tick pheromone components for other tick species. These included benzyl alcohol, benzaldehyde, o-nitrophenol and salicylaldehyde, the first three of which are

components of the male-produced attraction-aggregation-attachment pheromones produced by many tick species (Yunker et al., 1992). Salicylaldehyde is considered a female-produced sex pheromone with previously demonstrated low attraction of unfed males (Wood et al., 1975; Yunker et al., 1992). While it is currently unknown if brown dog ticks produce any of these compounds, they were still included as potential attractants due to the known overlap in function between species (Allan, 2010). In our study, benzyl alcohol produced more movement in the straight-tube olfactometer than any of the other chemicals or mixtures tested. Brown dog ticks also responded to benzaldehyde, salicylaldehyde and o-nitrophenol in both olfactometers.

The addition of carbon dioxide did not significantly alter activation behavior of *R. sanguineus* in the olfactometer experiments for most of the chemicals. This was an unexpected observation, as CO₂ is an important activator, attractant and/or synergist for many hematophagous arthropods (Vale and Hall, 1985; Takken and Kline, 1989; Steullet and Guerin, 1992; Barré et al., 1997; Osterkamp et al., 1999; McMahon and Guerin, 2002; Allan et al., 2010; Carr et al., 2013). Carr et al. (2013) demonstrated that *Amblyomma americanum* (L.) and *Dermacentor variabilis* (Say) responded differently to air containing 3% CO₂, depending on the flow rate. For example, *D. variabilis* responded at flow rates of 50 and 75 mL/min, but not at 25 or 100 mL/min (Carr et al., 2013). In regard to the current study, it is possible that *R. sanguineus* would respond to CO₂ if a flow rate other than 50 mL/min was used. Additionally, responses to several of the most activating chemicals were so strong in CO₂-background air that the data did not allow for an increase in activation when evaluated with CO₂-enhanced air. For

example, when 300 µg 1-octen-3-ol was tested with CO₂-background air, 100% of the ticks were activated (Table 2).

Tick attractants often are complex blends of chemicals consisting of optimal mixtures and ratios. Although Donzé et al. (2004) observed *A. variegatum* attraction to individual components of bovine rumen, attraction was only observed when the ratio of chemicals was adjusted to reflect those present in the rumen fluid. In the current study, no strong conclusions about the synergistic effects of mixing chemicals could be drawn. In each case, where a blend was found to activate ticks, at least one chemical that was included in the blend had a statistically similar effect on the ticks, thus indicating that the individual chemical may have been solely responsible for the tick response.

Traps baited with CO₂ consistently lured brown dog ticks. The importance of CO₂ in tick activation and attraction has been demonstrated many times, either as a standalone attractant (Wilson et al., 1972; Gray, 1985; Carnohan et al., 2015), or in combination with other chemicals (Norval et al., 1989; Maranga et al., 2006; Nchu et al., 2010; Ranju et al., 2013). In the current study, *R. sanguineus* were both significantly more activated and captured in the traps that produced CO₂. These findings indicate that CO₂ is an important component for brown dog tick attraction and trapping. In the current study, 1-octen-3-ol, hexanoic acid, or methyl salicylate at the doses and ratios tested did not significantly increase the number of ticks activated, attracted or captured by the trap above CO₂ alone. Ratios and concentrations of these compounds should be optimized to enhance attraction, however, currently CO₂ alone is sufficient as a bait for brown dog tick traps.

During trap evaluations, the temperature during the morning ($24.54 \pm 0.27^{\circ}\text{C}$) was lower than during the afternoon ($26.19 \pm 0.22^{\circ}\text{C}$). Covariate analysis revealed that temperature significantly influenced the number of ticks captured and more ticks were caught in traps in the afternoon evaluations, when tick testing rooms were warmer, than in the morning evaluations. Gray (1985) demonstrated that higher temperatures coincided with greater numbers of captured *Ixodes ricinus* L. in the field using a CO₂-baited trap. Our findings suggest that slight differences in temperature impact *R. sanguineus* behavior with tick activity increasing at higher temperatures and this should be considered for residential deployment of a trapping or monitoring device.

In conclusion, this study provided preliminary insight into adult *R. sanguineus* s.l. activation and attraction in response to several semiochemicals. Some of these chemicals eventually may serve as attractants for use in *R. sanguineus* s.l. management efforts. However, there remain many questions that need to be addressed given the multitude of doses, combinations and ratios in which these and other chemicals may be applied and mixed to improve tick responses. Nevertheless, identifying ideal attractants can be a very complex endeavor, and this study has established a base from which to expand our understanding of *R. sanguineus* s.l. chemical ecology.

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679 **Table 1**

680 Chemicals tested in olfactometer assays and included in blends.

Test Chemical (% purity)	Y-tube ^a	Straight -tube ^b	All chemical blend ^c	Kairomone blend ^c	Pheromone blend ^c
Acetone (≥ 99.9) ^e	X				
Benzaldehyde (≥ 99) ^e	X	X	X		X
Benzoic acid (≥ 99.5) ^d	X				
Benzyl alcohol (≥ 99) ^e	X	X	X		X
Butanoic acid (≥ 99) ^e	X	X	X	X	
2,6-Dichlorophenol (99) ^e	X	X	X		X
Hexanal (96) ^d	X				
Hexanoic acid (99) ^d	X	X	X	X	
Methyl salicylate (≥ 99) ^e	X	X	X		X
o-Nitrophenol (98) ^e	X	X	X	X	X
Nonanoic acid (≥ 97) ^e	X				
1-Octen-3-ol (98) ^d	X	X	X	X	
3-Pentanone (98) ^d	X				
Salicylaldehyde (98) ^e	X	X	X		X
Squalene (≥ 98) ^e	X				
γ -Valerolactone (≥ 99) ^e	X				
Control (Hexane, ≥ 99.7) ^e	X	X			

681 ^a Y-tube chemicals were tested at 3, 30 and 300 μg 682 ^b Straight-tube individual chemicals were tested at 300 μg

683 ^c Blends were tested at a total amount of 300 and 30 µg; within these blends the
684 amounts varied depending on the number of chemicals. In the 30 and 300 µg all
685 chemical blend each chemical was at 3.3 and 33.3 µg, respectively. In the 30 and 300
686 µg kairomone blend each chemical was at 75.0 and 7.5 µg, respectively. In the 30 and
687 300 µg pheromone blend each chemical was at 50.0 and 5.0 µg, respectively.

688 ^dAcros Organics, Thermo Fisher Scientific, Fair Lawn, NJ

689 ^eSigmaAldrich, St. Louis, MO

Table 2

Preliminary screening of chemicals on adult *Rhipicephalus sanguineus* sensu lato activation in a Y-tube olfactometer. Responses recorded after 15 min exposure to a chemical (300 µg). Ten replicates with 5 ticks per replicate (males and females tested separately but data were pooled). Data are presented as predicted means; the standard errors of the mean were 0.07 for all treatments except the control, which was 0.04

Chemical	Proportion activated ^a	
	CO ₂ -background Air ^b	CO ₂ -enhanced Air ^b
Acetone	0.093	0.153
Benzaldehyde	0.933 ***	0.813 ***
Benzoic acid	0.230	0.130
Benzyl alcohol	0.613 ***b	0.873 ***a
Butanoic acid	0.890 ***	0.930 ***
2,6-Dichlorophenol	0.816 ***	0.896 ***
Hexanal	0.036 b	0.316 *a
Hexanoic acid	0.390 **b	0.910 ***a
Methyl salicylate	1.021 ***	0.981 ***
o-Nitrophenol	0.921 ***	1.021 ***
Nonanoic acid	0.070	0.030
1-Octen-3-ol	1.013 ***	1.033 ***
3-Pentanone	0.161	0.101

Salicylaldehyde	0.956 ***	0.956 ***
Squalene	0.056	0.076
γ -Valerolactone	0.281	0.341 **
Control	0.155	0.130

^a Within a column, predicted means were compared to control using Least Significant Difference (LSD) tests (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

^b Differing letters indicate differences between chemicals tested in CO₂-background versus CO₂-enhanced air. For each chemical p-values were calculated using LSD tests.

Table 3

Movement (sec) and number of direction changes of adult *Rhipicephalus sanguineus* sensu lato exposed to potential semiochemicals in a straight-tube olfactometer. Responses recorded after 15 min exposure to a chemical (300 µg unless stated otherwise). Each chemical consisted of three replicates of each air type with five mixed-sex ticks per replicate.

Chemical	Movement ^{a,b,e}		Direction Change ^{a,b,f}	
	CO ₂ -	CO ₂ -enhanced	CO ₂ -	CO ₂ -enhanced Air
Benzyl alcohol	1,824 abc	2,444 a	36.33 cd	50.33 bc
300 µg Kairomone blend ^c	1,979 ab	2,285 a	48.33 bc*	81.50 a*
Hexanoic acid	2,032 a	2,142 a	61.67 ab	74.00 a
Methyl salicylate	1,481 cd	1,743 b	36.00 cde	30.00 de
1-Octen-3-ol	1,844 abc	1,640 b	69.67 a	58.00 b
300 µg All chemical blend	1,613 bcd	1,521 bc	29.67 def	39.00 cd
o-Nitrophenol	1,323 de	1,232 cd	23.33 defgh	21.67 efgh
Benzaldehyde	1,371 cde	1,132 cde	23.00 defgh	27.33 def
Butanoic acid	1,695 abcd	1,093 de*	32.00 de	25.33 defg
30 µg Pheromone blend ^d	677 fg	936 def	15.00 gh	22.67 efgh
300 µg Pheromone blend ^d	818 ef	772 efg	17.67 fgh	18.33 efghi
2,6-Dichlorophenol	769 fg	614 fg	15.33 gh	15.00 fghi
30 µg All chemical blend	865 ef	597 fg	22.00 efgh	10.67 hi

Salicylaldehyde	721 fg	518 gh	15.00 gh	15.00 fghi
30 µg Kairomone blend ^c	751 fg	432 gh	24.67 defg	12.33 ghi
Control (Hexane)	391 g	201 h	9.33 h	6.67 i

^a Letters a-h following means within a column indicate significant differences ($p < 0.05$) calculated using Least Significant Difference tests.

^b Within a row, * indicates a significant difference ($p < 0.05$) calculated using LSD tests comparing each chemical tested with CO₂-background and CO₂-enhanced air for each behavioral parameter.

^c Kairomone blend included: 1-octen-3-ol, o-nitrophenol, butanoic acid, and hexanoic acid

^d Pheromone blend included: methyl salicylate, salicylaldehyde, 2,6-dichlorophenol, benzaldehyde, benzyl alcohol, and o-nitrophenol

^e For movement the standard errors of the mean were 139 for all treatments except for benzaldehyde, which was 240

^f For direction change the standard errors of the mean were 5.05 for all treatments except benzaldehyde, which was 8.76

Table 4

Time to activation (sec) and number of end touches of adult *Rhipicephalus sanguineus* sensu lato during exposure to potential semiochemicals in a straight-tube olfactometer. Responses recorded after 15 min exposure to a chemical (300 µg unless stated otherwise). Replicates of both air types were combined giving a total of six replicates for each chemical with five mixed-sex ticks per replicate. Values in parenthesis correspond to standard errors of the mean.

Chemical	Activation (sec) ^a	End touch ^a
1-Octen-3-ol	15 (1.5) a	12.83 (0.99) f
300 µg All chemical blend	25 (1.5) ab	9.17 (0.99) f
Methyl salicylate	50 (1.5) bc	8.67 (0.99) de
300 µg Kairomone ^b	50 (1.5) bc	13.33 (1.11) f
300 µg Pheromone ^c	64 (1.5) bcd	5.50 (0.99) bc
Butanoic acid	76 (1.5) cd	7.83 (0.99) d
o-Nitrophenol	168 (1.5) de	7.00 (0.99) cd
2,6-Dichlorophenol	182 (1.5) de	4.50 (0.99) ab
Salicylaldehyde	233 (1.5) ef	6.17 (0.99) bc
Benzyl alcohol	283 (1.5) efg	6.67 (0.99) bcd
30 µg All chemical blend	336 (1.5) efg	4.17 (0.99) ab
Hexanoic acid	426 (1.5) efg	8.83 (0.99) e
30 µg Pheromone blend ^c	567 (1.5) fg	5.33 (0.99) b
30 µg Kairomone blend ^b	660 (1.5) fg	3.83 (0.99) ab
Benzaldehyde	1,018 (1.7) gh	1.83 (1.40) a
Control (Hexane)	3,501 (1.5) h	3.00 (0.99) ab

^a Letters a-h following means within a column indicate significant differences ($p < 0.05$)

calculated using Least Significant Difference tests.

^bKairomone blend included: 1-octen-3-ol, o-nitrophenol, butanoic acid, and hexanoic acid

^cPheromone blend included: methyl salicylate, salicylaldehyde, 2,6-dichlorophenol, benzaldehyde, benzyl alcohol, and o-nitrophenol

Table 5

Velocity (cm/sec) and angular velocity (degrees/sec) of movement of adult

Rhipicephalus sanguineus sensu lato exposed to potential semiochemicals in a straight-tube olfactometer. Responses recorded after 15 min exposure to a chemical. Replicates of both air types were combined giving a total of six replicates for each chemical with five ticks of mixed sex per replicate. Values in parenthesis correspond to standard errors of the mean.

Chemical	Velocity ^a	Angular Velocity ^a
300 µg Kairomone blend ^b	2.72 (0.21) a	10.37 (3.27) a
300 µg 1-Octen-3-ol	2.64 (0.21) a	10.12 (3.49) a
300 µg Pheromone blend ^c	1.45 (0.21) b	16.95 (5.74) a
30 µg Pheromone blend ^c	1.21 (0.21) b	25.24 (8.79) a
Control (Hexane)	1.04 (0.21) b	79.09 (23.63) b
30 µg Kairomone blend ^b	0.88 (0.21) b	23.64 (7.23) a

^a Letters a-b following means within a column indicate significant differences ($p < 0.05$) calculated using Least Significant Difference tests.

^b Kairomone blend included: 1-octen-3-ol, o-nitrophenol, butanoic acid, and hexanoic acid

^c Pheromone blend included: methyl salicylate, salicylaldehyde, 2,6-dichlorophenol, benzaldehyde, benzyl alcohol, and o-nitrophenol

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Table 6

Captured and activated adult *Rhipicephalus sanguineus* sensu lato following release into a room containing a trap with different treatments. Numbers out of 40 mixed-sex ticks captured or activated with a total of seven replicates. Values in parenthesis correspond to standard errors of the mean.

Treatment	Captured^{a,b}	Activated^b
1-Octen-3-ol + CO ₂	5.97 (0.88) a	16.99 (1.78) abc
CO ₂	5.22 (0.87) a	19.69 (1.74) a
Methyl salicylate + CO ₂	4.87 (0.86) a	18.52 (1.72) ab
Hexanoic acid + CO ₂	4.74 (0.87) a	14.94 (1.74) abcd
Methyl salicylate	2.10 (0.86) b	12.09 (1.72) cd
Hexanoic acid	0.64 (0.86) b	10.84 (1.72) d
Heat	0.60 (0.87) b	14.23 (1.74) bcd
1-Octen-3-ol	0.42 (0.86) b	13.98 (1.72) bcd

^a Response variable captured was adjusted by the covariate average temperature

^b Letters a-d following means within a column indicate significant differences ($p < 0.05$) calculated using Least Significant Difference (LSD) tests.