Medical and Veterinary Entomology

Medical and Veterinary Entomology (2020) **34**, 49–58 doi: 10.1111/mve.12405

Olfactory responses of *Amblyomma maculatum* to rumen fluid and other odourants that attract blood-seeking arthropods

K. D. GARNER[1], M. E. PAYTON[2], J. L. TALLEY[1] and B. H. NODEN[1]

[1]Department of Entomology and Plant Pathology, College of Agricultural Sciences and Natural Resources, Oklahoma State University, Stillwater, OK, U.S.A. and [2]Department of Statistics, College of Arts and Sciences, Oklahoma State University, Stillwater, OK, U.S.A.

Abstract. *Amblyomma maculatum* Koch (Ixodida: Ixodidae) has emerged as a significant vector of human and companion animal diseases in the U.S.A. When expanding in range, *A. maculatum* can be difficult to collect in the field and control on livestock. A novel method is needed to improve the field collection of *A. maculatum*, as well as to control their effects as ectoparasites of livestock and companion animals. The present study aimed to test the effects of known volatiles on the activation and selection choices of *A. maculatum* in a laboratory-based Y-tube assay and field-based assays. Although the majority of adult *A. maculatum* were activated to move by five of the seven semiochemicals tested, only rumen fluid significantly attracted ticks to make a selection in the Y-tube apparatus. Rumen fluid attracted the most *A. maculatum* in the laboratory, with 56% (84/150) making it to the rumen Y-tube arm, although the results were not replicated in semi-field experiments. These studies highlight the need for continued work to identify attractants for tick vectors that will assist field collections. These attractants could also be incorporated into management strategies that lead to prevention technologies to reduce tick burdens on cattle or in risk areas of humans.

Key words. *Amblyomma maculatum*, ammonium hydroxide, Gulf Coast tick, 2-nitrophenol, olfaction, rumen fluid.

Introduction

The incidence of tick-borne disease continues to rise among humans and companion animals in the U.S.A. (Biggs *et al.*, 2016). One area of concern is the southern region of the U.S.A. in which there have been increases in ehrlichiosis, spotted fever rickettsiosis and tularemia among human populations, as well as ehrlichiosis and American canine hepatozoonosis among canine populations (Eisen *et al.*, 2008; Allen *et al.*, 2011; Little *et al.*, 2014; Biggs *et al.*, 2016). One tick species responsible for this increased disease incidence is *Amblyomma maculatum*, which causes significant morbidity to livestock and transmits tick-borne pathogens in the region. Adult *A. maculatum* cause 'gotch ear', producing tissue and cartilage damage in the ears of a variety of livestock species (Bishopp & Trembley, 1945; Edwards, 2011). *Amblyomma maculatum* infestations can also negatively impact the weight gain and blood composition of cattle with infestations as low as 125 ticks, thereby reducing their economic value (Williams *et al.*, 1977; Stacey *et al.*, 1978; Riley *et al.*, 1995).

In addition to morbidity for livestock, *A. maculatum* has emerged as a significant vector of human and companion animal diseases in the U.S.A. (Barker *et al.*, 2004; Teel *et al.*, 2010; Paddock & Goddard 2015). Ranked as one of the top four ixodid tick species reportedly biting humans, the attachment of adult *A. maculatum* can cause tick paralysis (Paffenbarger 1951;

Correspondence: Bruce H. Noden, Department of Entomology and Plant Pathology, College of Agricultural Sciences and Natural Resources, Oklahoma State University, 127 Noble Research Center, Stillwater, OK 74078, U.S.A. Tel.: +405 744 3225; Fax: +405 744 6039; E-mail: bruce.noden@okstate.edu

Espinoza-Gomez *et al.*, 2011; Paddock & Goddard 2015), as well as the transmission of *Rickettsia parkeri*, a spotted fever group rickettsia (Paddock & Goddard 2015). *Amblyomma maculatum* is the invertebrate definitive host for *Hepatozoon americanum*, the causative agent for American canine hepatozoonosis (Mathew *et al.*, 1999; Ewing *et al.*, 2002), which infects domestic and wild canids, especially coyotes, when infected *A. maculatum* are ingested during grooming (Kocan *et al.*, 1999; Ewing *et al.*, 2000). *Amblyomma maculatum* is also a potential vector for *Ehrlichia ruminantium*, the agent of heartwater in Africa and the Caribbean (Uilenberg 1982).

When expanding in range in Central and North America (Estrada-Peña *et al.*, 2005; Teel *et al.*, 2010), *A. maculatum* can be difficult to collect in the field and control on livestock. The immature stages (larvae and nymphs) are mostly collected from birds and small mammals (Barker *et al.*, 2004; Teel *et al.*, 2010), whereas adults are mainly found, in high numbers, on the ears and head regions of large ungulates, particularly cattle. Because of their proclivity for large mammals, it is difficult to collect sizable samples of adult *A. maculatum* in the field for studies involving distribution (Mitcham *et al.*, 2017), pathogen prevalence (Paddock *et al.*, 2015) and microbiome analysis (Budachetri *et al.*, 2014). The current field-collection methods for adult *A. maculatum* are time consuming, laborious and usually result in low capture success rate (Goddard & Paddock 2005; Goddard *et al.*, 2011; Mays *et al.*, 2016; Pike 2017). Carbon dioxide traps, although highly successful for *A. americanum* (Linnaeus) (Ixodida: Ixodidae) (Koch 1987), have collected some *A. maculatum* in Oklahoma and Tennessee, although at much lower rates compared with those collected from area livestock (Semtner & Hair 1975; Mays *et al.*, 2016; Pike, 2017). A novel method is needed not only to improve the collection rates of *A. maculatum* in field settings, but also to reduce collection time and effort. Additionally, any new methods that successfully attract adults could be used to develop prevention technologies, possibly ear-tags (Kelly *et al.*, 2014), aiming to reduce or eliminate tick burdens on cattle, particularly in their ear region.

Various volatile compounds have been identified as attractive to ticks in laboratory and field studies. First, pheromone-mediated behaviour, most notably by the attraction-aggregation-attachment pheromone (AAAP), has been reported for a number of ixodid tick species. AAAP is a pheromone excreted by feeding adult male ticks to attract the same species of non-feeding males and females to the specific feeding site, forming clusters on cattle and other large ruminants (Sonenshine, 1985). Female *A. maculatum* are significantly more attracted to cattle with actively feeding males (Sleeba *et al.*, 2010), whereas free-living nymphs and adults of *Amblyomma hebraeum* Koch (Ixodida: Ixodidae) in Zimbabwe were attracted to traps pairing AAAP and CO_2 if placed in suitable habitats with known populations of the target species (Bryson *et al.*, 2000). Although, *Amblyomma variegatum* (Fabricius) (Ixodida: Ixodidae), an important tick vector in southern African and the Caribbean, was not found to be attracted to traps with CO_2 and AAAP (Maranga *et al.*, 2003), tail and collar tags impregnated with deltamethrin and AAAP were reported to reduce *A. variegatum* infestations on cattle (Kelly *et al.*, 2014). Other compounds that have attractant potential for ixodid ticks include 2,6-dichlorophenol (Kellum & Berger 1977; Sonenshine 1985; Norval *et al.*, 1991; Barré *et al.*, 1997; Allan *et al.*, 1998), 1-octen-3-ol (Osterkamp *et al.*, 1999; Ranju *et al.*, 2012; Carr *et al.*, 2013), ammonium hydroxide (Haggart & Davis 1980; Carr *et al.*, 2013), squalene (Yoder *et al.*, 1998, 1999), ear exudate (Wanzala *et al.*, 2004) and rumen fluid (Donzé *et al.*, 2004), Currently, only 2,6-dichlorophenol extracted from fed ticks (Kim 2004) and CO_2 have been identified as marginally attractive to *A. maculatum*. A lack of attractive chemicals, the low number of field-collected *A. maculatum* compared with other tick species and the difficulty of collecting ticks off host all demonstrate the need to identify putative chemical attractants for enhancing field collection rates. The present study aimed to test the effects of known volatiles on the activation and selection choices of *A. maculatum* in a laboratory-based Y-tube assay and also test the effect of any significant attractant in field-based assays.

Materials and methods

Ticks

Adult *A. maculatum* and adult *A. americanum*, both approximately 2 months old, were obtained from the Tick Rearing Facility, Oklahoma State University, Stillwater, Oklahoma. After receipt, ticks were held in a humidity chamber, using potassium sulphate to maintain humidity at the saturation point. The chamber was kept at room temperature under am LD 15 : 9 h light/dark photocycle (Thangamani & Bente, 2014). Ticks were acclimated to the laboratory environment for at least 48 h after acquisition and prior to use in bioassays. All ticks were unfed for the entirety of the study.

Y-tube olfactometer assay design

All tests took place within a fume hood equipped with fluorescent lights at room temperature. Two-choice selection assays were conducted using a glass Y-tube olfactometer (Glassworks, Bartlesville, OK, U.S.A.) adapted from methods described previously (Carr *et al.*, 2013; Pike, 2017). Filtered air was introduced into the Y-tube arms via the fume-hood installed air delivery system with an exception for CO_2 delivery (described below). Air from the fume hood ports was directed through filters using activated charcoal then further filtered using fine glass wool. After filtration, air flow rates were regulated using 150-mm correlated flowmeters (Cole-Parmer, Vernon Hills, IL, U.S.A.). Flow rates per arm were adjusted symmetrically. Air from the flowmeters was directed into glass vacuum traps (Wilmad-LabGlass, Vineland, NJ, U.S.A.), used as volatile holding chambers, which then allowed air to flow into the ports of the arms of the olfactometer. When required, CO_2 (3% in breathing quality air) was introduced into the system using a compressed gas tank (Stillwater Steel, Stillwater, OK, U.S.A.) and was not subjected to filtration. A designated 150-mm correlated flowmeter was used for CO_2 to reduce risk of contamination. Carbon dioxide was then introduced into the Y-tube through the glass vacuum trap and into

a port of one Y-tube arm, as described above. To avoid positional bias, odourants were alternated between the two ports of the olfactometer. A vacuum integrated into the fume hood was used to remove gasses at the downwind end of the Y-tube equal to the rate at which air flowed into the system. Using gloves to avoid contamination, everything was washed with Alconox® (Alconox, Inc., White Plains, NY, U.S.A.) detergent and hot water, with all glassware being dried in an oven at 100 °C between use, whereas any equipment incapable of being heat dried was rinsed with 95% ethanol and air-dried for at least 8 h.

Olfactometer assay with Amblyomma americanum

Preliminary behavioural assays were conducted to establish that this Y-tube olfactometer system was an appropriate way of measuring tick responses to odourants. Carbon dioxide is a known attractant for *A. americanum* (Koch, 1987). Previously, mixed-sex adult *A. americanum* were shown to be significantly attracted to CO_2 (3% in breathing quality air) in a Y-tube olfactometer assay using an air flow rate of 100 mL/min and a trial time of 5 min (Carr *et al.*, 2013).

Additionally, tick responses to being marked with fluorescent powder (DayGlo ECO®; DayGlo Color Corp., Cleveland, OH, U.S.A.) were evaluated. As a result of the time involved in testing the effects of odourants on individual ticks and the need to generate sufficient data for comparisons, it was necessary to use different colours of fluorescent powder so that ticks could be evaluated in groups instead of individually. Ticks were marked with powder at least 24 h before assays were conducted. All ticks were acclimated to the test setting at least 30 min prior to use. Each trial was conducted with five unfed, previously untested, mixed-sex adults. Twelve replications were conducted for a total of 60 ticks tested per assay. After placement at the starting point into the olfactometer (2.5 cm past the exhaust outlet), each trial ran for five minutes and positive responses were recorded along with the time it took to make a selection using a stop watch with multiple stop functions. Movement of 4 cm or more into one the arms of the olfactometer was recorded as a positive response. When two different arm treatments were used, the test substance and control were alternated between the arms to reduce positional bias.

To ensure the system was responding 'normally' in accordance with previously reported results (Carr *et al.*, 2013), *A. americanum* were tested in specific assays: marked and unmarked ticks (60 per assay) to air only at a flow rate of 100 mL/min/port, as well as marked and unmarked ticks (60 per assay) to air and CO_2 at a flow rate of 100 mL/min/port. The air-only assay consisted of air only flowing into both arms of the Y-tube olfactometer. The air-only assay allowed behavioural observations to occur between the two treatment groups, ticks marked with fluorescent powder and ticks not marked with powder. The air and CO_2 assay consisted of air flowing through one arm of the Y-tube when CO_2 flowed through the opposing arm. This air and CO_2 experiment evaluated the behaviours between the marked and unmarked ticks and also evaluated the attractiveness of CO_2.

Olfactometer assay with A. maculatum

Each assay trial was conducted using five mixed-sex adult *A. maculatum*, with six trial replicates being conducted (total ticks = 30), each time with previously untested ticks. Ticks were only handled when wearing gloves. On the day of testing, ticks were acclimated to the experimental setting for at least 30 min prior to placement into the Y-tube olfactometer. Ticks were placed into the olfactometer at a starting point 2.5 cm past the exhaust opening. Each trial ran for 10 min (time increased by 5 min as a result of differences in activation to movement compared with *A. americanum*) and selection times were recorded for the first positive response from individual ticks within the Y-tube apparatus as described by Carnohan *et al.* (2017). Tick species respond differently to varying odourants in Y-tube systems, with *A. americanum* responding quickly and selecting arms of the Y-tube containing odourants, whereas others, including *Rhipicephalus sanguineus* (Carnohan *et al.*, 2017) and *A. maculatum* (Kim, 2004), take more time to respond. Because of the low activity of *A. maculatum* ticks, positive responses were recorded at two points: (a) 'Tick activation' occurred when the tick moved 2 cm into the Y-tube apparatus and (b) odourant selection was recorded when a tick moved 4 cm into one of the arms of the olfactometer.

A standard air flow rate to be used for all trials was established. Using the previously described protocols, trials were conducted using filtered air in both arms at 0 mL/min, 24.8 mL/min, 47.8 mL/min, 76.7 mL/min, 97.2 mL/min, 122.1 mL/min and 138.3 mL/min. The flowmeters were correlated, scaled units were chosen for simplicity of adjustment. Additionally, responses to CO_2 and filtered air introduced into opposite arms were evaluated at the same rates described above. The volatile holding chambers remained empty for the flow rate determination trials.

Because trials were conducted with five ticks at a time, differentiation of individuals was necessary. Ticks were marked with one of five different DayGlo ECO® pigments. Marking occurred at least 24 h prior to testing. Using the assay methods described, trials using no air, air in both arms, and air and CO_2 ensured marking with fluorescent powder did not alter behaviour. A flow rate of 47.8 mL/min/port was chosen as a result of responses observed in initial air and CO_2 flow rate assays. To ensure that marking did not impact *A. maculatum* behaviour, a no-air assay using 30 marked ticks and a no-air assay using 30 unmarked ticks were conducted and the responses timed and recorded. An assay with only air entering through both arms of the Y-tube was similarly conducted, with 30 marked ticks and 30 unmarked ticks. Finally, behavioural responses to CO_2 and air alternated between ports with 30 marked ticks and 30 unmarked ticks were also investigated. Much like the air and CO_2 assay conducted with *A. americanum*, this was performed with *A. maculatum* to evaluate the effect of marking on tick behaviour, as well as the effect that CO_2 has on their behaviour.

Semiochemical testing

The chemicals (Sigma-Aldrich, St Louis, MO, U.S.A.) tested were: 2-nitrophenol (98%), 1-octen-3-ol (98%),

2,6-dichlorophenol (99%), squalene (≥98%, liquid) and ammonium hydroxide (28–30% NH_3 basis, ACS). At two different times, 50 mL of rumen fluid was collected from a single fistulated donor cow by veterinary staff or technicians under the supervision of the Oklahoma State University Center for Veterinary Health Sciences (OSU-CVHS). Although only rumen fluid has been reportedly used (Donzé *et al.*, 2004), we chose to test rumen fluid at three stages of freshness. Fresh rumen fluid was used within 1 h of acquisition from the cow donor. Aged rumen fluid was created by storing fresh rumen fluid in a closed container in a refrigerator for 2 and 6 months. Ear exudate was obtained via rubbing the ear surfaces of cattle with a clean flannel cloth (5 × 5 cm) with gloved hands. Flannel ear swabs were collected on three different occasions, with two swabs per collection being obtained for a total of six cloths. Ear swabs were placed in glass vials and immediately transported to the laboratory and tested for bioactivity within 1 h of collection. All interactions with cattle in the present study follow the protocols outlined in the protocol (AG-15-11) approved by the OSU Animal Care and Use committee.

Potential attractants were tested using the Y-tube olfactometer methods described previously. An air flow rate of 47.8 mL/min/port was selected based off the air flow and CO_2 flow rate assays. Five different chemical dilutions (10%, 5%, 2.5%, 1% and 0.1%) were made using methanol as the diluting solution with 2-nitrophenol; methanol was used as the control. Five different dilutions (10%, 5%, 2.5%, 1% and 0.1%) were made using hexane as the diluting solution for 1-octen-3-ol, squalene and 2,6-dichlorphenol; hexane was used as the control for these chemicals. Five different dilutions (25%, 10%, 5%, 1% and 0.1%) were made using water as the diluting solution for ammonium hydroxide with water acting as the control during testing. Additionally, 2-month-old rumen fluid was tested at volumes of 50 µL and 100 µL with air as the test control. This aimed to determine whether a greater volume increased activity as a result of the potentially higher microbial activity observed for this age of rumen fluid at 25 µL compared with fresh and 6-month-old fluid. In accordance with similar protocols described by Wanzala *et al.* (2004), individual cattle ear swabs were placed into one odourant chamber and a clean flannel piece was used as a control in the opposing chamber. Odour chambers containing flannel swabs were also submerged in a water bath at 38.6 °C, equivalent to the average body temperature of a cow. This aimed to facilitate release of any odours collected from the ears on the flannel swabs. All dilutions were made when wearing gloves, using a serial dilution method in 10-mL volumetric flasks immediately before testing. With the exception of ear swabs, each putative attractant (25 µL) was placed onto cellulose filter paper (2.5 cm circles) and immediately transferred into an odourant chamber with forceps. The appropriate control (25 µL) was also placed onto cellulose filter paper (2.5 cm circles) and transferred into the opposite odourant chamber with new forceps to avoid contamination. Unaltered cellulose filter paper acted as the control for rumen fluid and was placed into the opposite odourant chamber via clean forceps. Rotation of controls and treatments were carried out between the two ports of the Y-tube arms to prevent positional bias. After placement of ticks into the olfactometer, a stopwatch with multiple stop capability was started and ran for 10 min.

Mark–release–recapture field bioassay

The principle chemicals eliciting attraction in the laboratory assays were then field tested. Rumen fluid, aged 2 months, was tested against the controls: water and CO_2 (produced by dry ice). Field trials used a mark–release–recapture method to study efficacy of the traps. Laboratory-reared mixed sex adult *A. maculatum* were marked using the same methods described for use in the laboratory choice selection assays. Field trials were conducted at the Oklahoma State University North Range Research Station in Stillwater, OK, U.S.A. Three trials were conducted in May and June 2016 with ambient temperatures ranging from 24 °C to 27 °C and relative humidity ranging from 37% to 47%. The field site was a pasture interspersed with Eastern red cedars and oak trees with cattle present before and during the time period of testing. Field trials were conducted between 10.00 and 14.00 hours and lasted 2 h. Testing was not carried out if the wind speed was greater than 15 mph or if the ground cover was damp or wet, aiming to maintain consistency for comparisons between trials.

Rumen fluid and water were dispersed by placing 20 mL of liquid into small glass dishes (60 × 15 mm) (PYREX®; Corning Glass Works, Corning, NY, U.S.A.) (Pike, 2017). Rumen fluid was tested in both heated and unheated states; the water control was also heated. A CO_2 control (dry ice) was also added. Samples were heated in an attempt to induce more volatility for better air dispersal. Air-activated single use heat packets (HotHands® Warmers; Walmart, Bentonville, AR, U.S.A.) were used as the heat source. Heat packets were activated when removed from the protective packaging and exposed to air 20 min prior to use to allow for optimal temperature (100 °F to 180 °F) to be reached, in accordance with the manufacturer's instructions. All test and control chemicals were placed into individual plastic storage containers with holes cut into the lower portions to direct released gases outwards into the test sites. These containers were then placed individually onto plywood boards (1 × 1 m). Folded masking tape was placed on all four edges of the board, with the tape's sticky side facing the environment. The taped boards and containers were then placed at the test locations.

In total, four boards with test or control chemicals was placed at the field site per trial (Pike, 2017). The boards were placed 10 m apart, in a linear fashion. Twenty ticks were released per trap, each set of ticks marked with a different colour fluorescent powder. Ten ticks were released two meters to either side of the trap. Two hours after ticks were released, traps were examined for presence of recaptured ticks or wild caught *A. maculatum*.

Statistical analysis

All data from the olfactometer studies were analysed using SAS, version 9.4 (SAS Institute Inc., Cary, NC, U.S.A.).

Table 1. Screening of various odourants on *Amblyomma maculatum* activation and choice in a Y-tube olfactometer after 10 min of exposure to a particular odourant: five replicates of 30 ticks each (males and females tested together).

	Movement‡	Odour arm		Control arm	
	% (*n*)	Choice % (*n*)	Group mean ± SE	Choice % (*n*)	Group mean ± SE
Air	55.3 (83/150)^{cd}	13.3 (20/150)	0.67 ± 0.15	10.0 (15/150)	0.5 ± 0.11
CO_2	68.6 (103/150)^{ab}	22.6 (34/150)	1.13 ± 0.13†	13.3 (20/150)	0.67 ± 0.13
Rumen fluid	79.3 (119/150)^{a}	56.0 (84/150)*	1.5 ± 0.21†	19.3 (29/150)	0.7 ± 0.16
Fresh rumen	76.6 (23/30)	23.3 (7/30)		10.0 (3/30)	
2 months	82.2 (74/90)	37.7 (34/90)		15.5 (14/90)	
6 months	73.3 (22/30)	23.3 (7/30)		3.3 (1/30)	
2-nitrophenol	76.0 (114/150)^{a}	17.3 (26/150)	0.87 ± 0.16	12.6 (19/150)	0.63 ± 0.14
Squalene	78.0 (117/150)^{a}	17.3 (26/150)	0.87 ± 0.16	13.3 (20/150)	0.67 ± 0.1
Ammonium hydroxide	63.3 (95/150)^{bc}	8.6 (13/150)	0.43 ± 0.12	14.0 (21/150)	0.7 ± 0.16
1-octen-3-ol	72.6 (109/150)^{ab}	8.6 (13/150)	0.43 ± 0.11	12.6 (19/150)	0.63 ± 0.12
2,6-dichlorophenol	50.6 (76/150)^{d}	5.3 (8/150)	0.27 ± 0.1	10.0 (15/150)	0.5 ± 0.15
Ear exudate	26.7 (8/30)^{d}	3.3 (1/30)	0.17 ± 0.17	6.7 (2/30)	0.33 ± 0.21

Data are presented as the percentage of ticks activated, as well as the percentage of ticks that chose the odourant or the control arm in the Y-tube and the mean ± SE of replicate groups for each odourant tested.

*Comparison between arms ($P = 0.0001$).

†Comparison between arms ($P < 0.05$).

‡Chi-squared analysis comparing activation by air vs. odourants. Different lowercase letters indicate significant differences (chi-squared compared with responses to air only controls).

All of the odourants had some effect on the ticks within the Y-tube system. Chi-squared was used to compare the choices of *A. americanum* in the Y-tube, as well as the activation of adult *A. maculatum*, comparing movement towards air vs. each odourant. Because there was no significant difference between activation of adult *A. maculatum* in air at 47.8 mL/min/port vs. the other air flow speeds ($P = 0.8695$), CO_2 at 47.8 mL/min/port vs. the other CO_2 flow concentrations ($P = 0.8603$) or between the different types of rumen fluids ($P = 0.5361$), it was possible to combine all tests together (air, CO_2 and rumen fluid) for the chi-squared analysis for movement in the Y-tube. Because ticks were tested in groups of five, mean ± SE values were determined and analysed using a *t*-test to demonstrate whether choice trends existed at the group level as well. Standard contingency table analyses using PROC FREQ tested for relationships between marked vs. unmarked, direction in the Y-tube, relationships between Y-tube choices for no air and air vs. CO_2, and choices for particular concentrations of the odourants tested. If insufficient ticks in the samples reacted to odourants, Fisher's exact test and gamma statistics were used to test for overall relationships in the assay and for potential concentration gradients, respectively. The multiplicity of hypothesis testing for each *P*-value was accounted for by the use of a false discovery rate adjustment in PROC MULTTEST. Fisher's exact test was used to analyse the results from the field study.

Results

Response of A. americanum *in Y-tube olfactometer assay*

Amblyomma americanum were not affected in assays by alternating the air and CO_2 between the arms of the olfactometer ($P = 0.7910$) or by marking using florescent dye in the air-only assays ($P = 0.8695$). Additionally, *A. americanum* were significantly more attracted to CO_2 in the Y-tube olfactometer assay with more adult *A. americanum* choosing CO_2 ($n = 48$) than air ($n = 23$) ($P = 0.0255$).

Response of A. maculatum *in Y-tube olfactometer assay*

Air flow rate assays. Air flow rate did not affect *A. maculatum* response (air vs. air) ($\chi^2 = 2.045$, d.f. = 5, $P = 0.8695$), indicating that tick movements observed in the Y-tube olfactometer were in response to various chemicals. CO_2 caused significant activation of *A. maculatum* to move into the apparatus, although individual *A. maculatum* adults did not choose the CO_2 arm of the Y-tube more than the arm with air ($\chi^2 = 3.019$, d.f. = 5, $P = 0.8200$) (Table 1). However, there was a significant choice of CO_2 when compared at the group level ($t = 2.19$, d.f. = 29, $P = 0.037$) (Table 1). The air flow rate of 47.8 mL/min was chosen for subsequent tests as a result of the higher percentage of ticks making selections for CO_2 and air treatments compared with other rates, although differences in activation of adult ticks were not significant between the different air flow rates ($P = 0.8695$).

Tick marking effect assays. Marking ticks did not significantly influence the behaviour of *A. maculatum* in the olfactometer assays. Equal numbers of marked and unmarked ticks selected both arms in the no air trial (30%) ($\chi^2 = 0.6007$, d.f. = 1, $P = 0.7910$) and in the air-CO_2 trial (26.7%) ($\chi^2 = 0.3409$, d.f. = 1, $P = 0.7910$).

Responses to putative attractants. Five odourants (CO_2, 2-nitrophenol, squalene, 1-octen-3-ol and rumen fluid) significantly activated the movement of *A. maculatum* compared

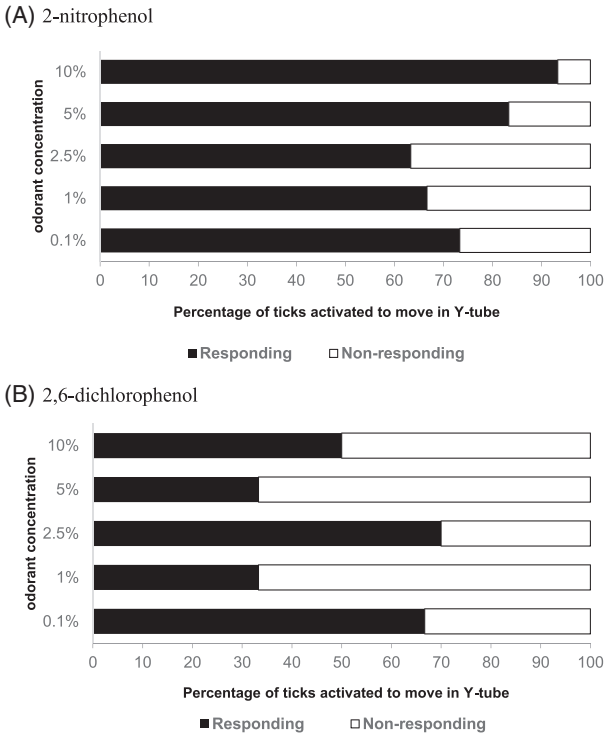

Fig. 1. Percentage of adult *Amblyomma maculatum* responding to varying concentrations of (A) 2-nitrophenol and (B) 2,6-dichlorophenol in a laboratory Y-tube olfactometer. Each concentration of odourant was tested with 30 ticks.

with movement using air only, whereas ammonium hydroxide, 2,6-dichlorophenol and ear exudate did not activate movement in the Y-tubes (Table 1). Only rumen fluid significantly attracted *A. maculatum* to make a choice at the end of the Y-tube apparatus.

Although most odourants did not induce a choice in the Y-tube, varying concentrations of only 2-nitrophenol and 2,6-dichlorophenol elicited significant activation responses in adult *A. maculatum* ticks. For 2-nitrophenol, there was no significant relationship between movement of *A. maculatum* in the Y-tube (Fisher's exact, $P = 0.0871$), although there was a significant relationship because of increasing concentrations (gamma = −0.3056, $P = 0.0284$) (Fig. 1A). Between concentrations of 2.5% and 10%, there was an increased movement of 30% in adult *A. maculatum*. For 2,6-dichlorophenol, there was a significant relationship between movement of *A. maculatum* in the Y-tube (Fisher's exact test, $P = 0.0255$), although the effect was not a result of an increasing concentration (gamma = 0.1302, $P = 0.4910$) (Fig. 1B). Although most ticks responded to the various concentrations of 2,6-dichlorophenol positively, more ticks were attracted to the control arm at two concentrations (1% and 5%), which could not be explained because the concentration of 10% demonstrated results similar to those for concentrations of 2.5% and 0.1%.

Response to ear exudate in olfactometer assays. Amblyomma maculatum was not activated by or attracted to ear exudate of cattle (Table 1). Of the 30 ticks evaluated, only one selected for the arm with ear volatiles, whereas two selected for the control arm.

Response to rumen fluid in olfactometer assays. In general, adult *A. maculatum* were more responsive to rumen fluid than to any other odourant tested. Rumen fluid caused the activation of 79% (119/150) of the ticks to move in the Y-tube and 56% (84/150) made it to the rumen fluid arm (Table 1). The activation of ticks by rumen fluid, however, was not affected by the age of rumen fluid ($\chi^2 = 1.2470$, d.f. = 2, $P = 0.7910$). Of the 113 ticks that chose a particular arm in the Y-tube, adult *A. maculatum* were significantly more attracted to rumen fluid than air controls ($P = 0.0020$), with 56% (84/1150) of responding ticks moving into the rumen fluid arm of the Y-tube vs. 19% (29/150) moving into the air control arm (Table 1). This also occurred at the group level of analysis ($t = 2.69$, d.f. = 29, $P = 0.012$) (Table 1). Choice of the rumen arm ($\chi^2 = 1.0045$, d.f. = 2, $P = 0.7910$) was not affected by the age of rumen fluid. However, when comparing responses of ticks moving into the rumen arm of the Y-tube by each age of rumen fluid compared with those moving into the air controls, adult *A. maculatum* were significantly attracted to 2-month old (38%, 34/90 ticks vs. air controls: 16%; 14/90) ($P = 0.0100$) but not 6-month old (23%; 7/30 ticks vs. air controls: 3%; 1/30) ($P = 0.0563$) rumen fluid or fresh rumen fluid (23%; 7/30 ticks vs. air controls: 10%; 3/30) ($P = 0.2289$) (Table 1).

Field collection of A. maculatum

Two-month old rumen fluid was used in the field setting because of the success of the laboratory olfactometer assays. In total, 240 mixed-sex adult *A. maculatum* were marked and released during three different field attraction trials. Eleven marked ticks were recaptured during the assays and no wild *A. maculatum* were collected (Table 2). In the field, rumen fluid (heated and unheated) was tested in field trials against water and CO_2. Three trials were conducted, although one trial failed to recapture ticks. In the remaining trials, dry-ice baited CO_2 traps returned 10 more ticks than water or unheated rumen fluid. One marked tick was collected on a trap using heated rumen fluid, whereas the CO_2 trap in the same trial recaptured three marked ticks. Over the entirety of the two successful field studies conducted, CO_2 traps returned nine more marked ticks than heated rumen fluid traps (Table 2). Traps baited with water or unheated rumen fluid recaptured zero ticks in each of the three field trials.

Discussion

Amblyomma maculatum impact the well-being of cattle, as well as transmit human and animal pathogens, although little is known regarding how they find their host and what odourants attract them. We have demonstrated that a variety of odourants, including those known to attract other species of ticks, activated

Table 2. Results from field assays∗testing effect of rumen fluid on the mark–release–recapture of laboratory-reared *Amblyomma maculatum*.

	Number of ticks released	Number (%) of ticks recaptured
Dry ice (CO_2)	40	10 (25%)^
Hot rumen	40	1 (2.5%)
Cold rumen	40	0
Water	40	0

^ Fisher's exact test, $P < 0.0001$ with CO_2 significantly differing from the other three categories.
∗One round was not presented as a result of no ticks being recaptured.

a majority (51–79% depending on the compound) of adult *A. maculatum* to move into a Y-tube olfactometer. Although most odourants activated adult ticks, only rumen fluid attracted a significant number to select the odourant arm over the control.

Although unique for *A. maculatum* in the present study, rumen fluid has also been shown in laboratory studies to be attractive to other ixodid tick species [*A. variegatum*, *A. hebraeum*, *Ixodes ricinus* (Linnaeus) (Ixodida: Ixodidae), *Ixodes persulcatus* (Schulze) (Ixodida: Ixodidae) and *Ixodes scapularis* Say (Ixodida: Ixodidae)] (Donzé *et al.*, 2004). Fermentation in the rumen, facilitated by microflora, produces short-chain fatty acids that include butanoic acid, isobutanoic acid, 3-methylindole, 4-methylphenol, acetic acid, propanoic acid and methane (Erwin *et al.*, 1961; Donzé *et al.*, 2004; Ranju *et al.*, 2014). When four rumen-associated volatiles (butanoic acid, isobutanoic acid, 4-methylphenol and 3-methylindole) were combined at a 100 : 10 : 1 : 1 dilution, both *A. variegatum* and *I. scapularis* were attracted at half the rate of whole rumen fluid, indicating that the proportion of volatiles is important to ticks (Donzé *et al.*, 2004). This selection response among ticks, however, is not always genus-specific (Ranju *et al.*, 2014).

Although aged rumen fluid was attractive to *A. maculatum* in the laboratory, it did not produce similar results under field conditions, with CO_2 eliciting more effect than the aged rumen fluid (Table 2). This may have been a result of the simplified hand warmer, which may have not heated the rumen samples to a sufficiently high temperature to release the attractive volatiles. Under natural conditions, cattle eruct gases from their rumen fluids every 2–3 min, with most of the contents of the rumen being converted into breath every 1 h (Donzé *et al.*, 2004). These bovine eructions would be paired with host skin odours and physical cues, such as vibrations and heat, and could trigger more aggressive host seeking in the field. Carbon dioxide acts alone or synergistically with chemical lures for blood-feeding insects (Carr 2011; Carnohan *et al.*, 2017) and may have the same effect with other host-associated volatiles.

Although more work needs to be carried out to identify specific volatiles in the rumen fluid, it is possible that rumen fluid might actually be involved in the attraction process of *A. maculatum* to cattle where they would be likely to mate and find a blood meal. *Amblyomma maculatum* are normally associated with pasture settings and are not as likely to be found in woodlands or the ecotone between the woodlands and pastures as are other tick species (Barker *et al.*, 2004; Teel *et al.*, 2010). As a tick species that is not known to move towards specific stimuli (ambush strategy) (Dr T. Dubie, unpublished data; Teel *et al.*, 2010), the volatiles within the frequent eructions of cattle in a pasture might cue an adult *A. maculatum* that a potential host is in proximity. During the rumination process, cattle produce copious amounts of methane and other gases (20–30 L/h) (Bowen 2017). Because methane originates from the fermentation of feed in the digestive track and CO_2 comes from respiratory activity (Pinares-Patiño *et al.*, 2007), a combination of both volatiles from rumen fluid with CO_2 could potentially attract more *A. maculatum*. One of the primary determining factors for methane in ruminants is dry matter intake (DMI) (Rischewski *et al.*, 2017) and both DMI and methane increase from early to mid-lactation (Bielak *et al.*, 2016). The increase of methane associated with cows in the early to mid-lactation periods coincides with adult *A. maculatum* infestations on cattle in Oklahoma (J. L. Talley, personal communication). Consequently, it is possible that the ticks use the volatiles from the rumen fluid as a directional cue to orient them to a nearby cow (Dr Pete Teel, personal communication; Donzé *et al.*, 2004). Once on the cow, the female tick uses pheromones and other compounds in the attraction–aggregation–attachment response to orient to the ears of the cattle (Gladney, 1971; Kim, 2004; Sleeba *et al.*, 2010).

The present study also demonstrated that five different odourants activating other tick species, including those in the genus *Amblyomma*, also activated the majority of *A. maculatum* to move in the Y-tube olfactometer. Once activated, however, tick species do not all react the same way in a Y-tube olfactometer. *Amblyomma americanum* become active within the tube and make their selection within five minutes (Carr *et al.*, 2013) (a hunter host-seeking behaviour). Others, however, such as *R. sanguineus* (Carnohan *et al.*, 2017) and *A. maculatum*, and possibly *Dermacentor variabilis* (Carr *et al.*, 2013), are slow to get started and thus need longer time to make choices (ambush host-seeking behaviour). This possibly explains the low capture rates for *A. maculatum* and *D. variabilis* in field collection traps involving dry ice (CO_2) (Kim, 2004). In the case of *A. maculatum*, they appeared to be either disoriented in the tube or clumped together, needing time to separate when the testing started. Because of this slowness to move into the area of the tube where a choice can be made (unlike other species and lifestages that readily move into the tube structure) (Carr *et al.*, 2013; Van Duijvendijk *et al.*, 2017) and the inability to test for vertical movement of 'ambush' tick species, Y-tube olfactometers have been used as 'tick activation screening devices' for the preliminary screening of potential semiochemicals that may be attractive for tick species (Carnohan *et al.*, 2017). The need for this distinction was apparent in the present study because significant movement in the horizontally-positioned Y-tube was observed for 2-nitrophenol and 2,6-dichlorophenol, although activity varied considerably as a result of small differences in odourant concentration. These odourants may have activated the ticks to move, although only 15–36% of the ticks made it to the selection area in the Y-tube. In general, the numbers of ticks tested by the odourants were too low to measure significant differences, thus demonstrating the difficulties with respect to interpreting the results from Y-tube olfactometers. To overcome this, Carnohan *et al.* (2017) screened activating odourants using a Y-tube olfactometer and then tested the

activating compounds using a straight-tube olfactometer that measured anemotaxis (movement toward the source of the particular odour) and chemokinesis (non-directional responses such as turning around). Although this next level of testing was not performed in the present study, the activation effect of the semiochemicals tested on the majority of *A. maculatum* indicated that *A. maculatum* are activated by many of the same odourants reported by in other studies and have the potential for use after further testing.

All studies have limitations that need to be taken into consideration. Other studies have paired odourants with CO_2 aiming to enhance the effect on a particular species (Barré *et al.*, 1997; Carnohan *et al.*, 2017). Because 2-nitrophenol is a component of AAAP, pairing with other components (such as CO_2) could have made it more attractive (Barré *et al.*, 1997). It was also interesting that 2,6-dichlorophenol did not significantly activate the ticks in the Y-tube, although a very small proportion (10%) were significantly attracted to the control arm as opposed to the odourant arm. 2,6-dichlorophenol normally attracts fed male *Amblyomma* ticks and has no effect on unfed males or females (Sonenshine, 1985; Kim, 2004), which were used in the present study. This is probably the reason for the lack of response to 2,6-dichlorophenol, although the choice of the control arm by more ticks may have been by chance or a repellant effect of the odourant used. Finally, although working with five ticks at a time aided in the collection of greater numbers of ticks in a shorter amount of time, the clumping that occurred when *A. maculatum* placed in the Y-tube olfactometer suggests that using an individual tick in the olfactometer per trial may have reduced the time the ticks spent not moving or clumping together and could have resulted in increased overall selection rates.

In conclusion, the present study demonstrates that there are odourants that activate and attract *A. maculatum*. Given the increasing importance of this tick species as a vector for human and animal pathogens, as well as its continued impact on cattle production, there is a need for continued development of odourant-based prevention strategies. These strategies would improve the collection of *A. maculatum* in field settings where hundreds of *A. maculatum* can be collected on cattle but almost none are collected using traditional methods in the field (T. Dubie and J. L. Talley, unpublished data). Moreover, their incorporation into developing prevention technologies, possibly ear- or tail-tags (Kelly *et al.*, 2014), could reduce or eliminate tick burdens on cattle and assist producers in the regions where this species is a nuisance.

Acknowledgements

We thank Ms Lisa Coburn and the staff at the Oklahoma State University Tick Rearing Facility for their assistance in providing the ticks for this project, as well as in the procurement of the ear odour samples; Dr Ann Carr for helpful advice for setting up the Y-tube assays; Dr Jack Dillwith for assistance with the chemicals used in the study; Dr Trisha Dubie for assistance with the IACUC permit and the field component; and Dr John Gilliam and the technicians at the OSU Center for Veterinary Health Sciences for providing the rumen fluid. This project was funded by NIFA/USDA Hatch Grant funds through the Oklahoma Agricultural Experiment Station (OKL-02909) and the Oklahoma State University Tick Rearing Facility (OKL-0272). The authors declare that they have no conflicts of interest.

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Accepted 10 August 2019
First published online 21 August 2019