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Responses of the tropical bont tick, *Amblyomma variegatum* (Fabricius), to its aggregation-attachment pheromone presented in an air stream on a servosphere

C. McMahon · P. M. Guerin

Abstract Male *Amblyomma variegatum* ticks feeding on a host release a mixture of *o*-nitrophenol and methyl salicylate which serves to attract conspecifics. The behavioural responses of *A. variegatum* on a servosphere to these volatiles presented in an air stream are detailed here. In still air, ticks walked on all eight legs, but with long halts. In contrast, the air stream caused continuous walking and induced a reaching response where the forelegs actively sampled the air. Such reaching increased the angular velocity and reduced walking speed, effects that were amplified in the presence of vapours from *o*-nitrophenol and methyl salicylate in the air flowing over the ticks. Vapour from a 1:1 mixture of *o*-nitrophenol and methyl salicylate was attractive over a 10^4-fold concentration range providing an increase in upwind displacement of 20–40%, significantly higher than the natural ratio where *o*-nitrophenol vapour predominates. Although the responses to *o*-nitrophenol vapour were variable when presented alone, this chemical was consistently attractive when delivered with steer hair odour – unattractive on its own. Moreover, the upwind walk to this combination did not cause a change in speed or angular velocity. This supports the hypothesis that the response to the pheromone is enhanced by host odour.

Key words *Amblyomma* · Tick · Pheromone · Behaviour · Servosphere

Abbreviations *AAP* aggregation-attachment pheromone · *MS* methyl salicylate · *ONP* *o*-nitrophenol · *RH* relative humidity

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Introduction

Amblyomma variegatum Fabricius (Acari, Ixodidae), the tropical bont tick, is an obligate haematophagous ectoparasite which causes extensive economic damage to bovids both in the Caribbean and in sub-Saharan Africa (where it is native). It is a typical three-host tick species with each stage (larva, nymph and adult) parasitising different mammals for several days. Being a metastriate tick species, *A. variegatum* mates on its host and feeding adult males secrete an aggregation-attachment-pheromone (AAP), which is perceived in the Haller's organ on the forelegs, the principal olfactory organ of ticks. The secretion of an AAP is fundamental to the life cycle of this tick, for females on the host will only attach and feed near a source of pheromone (Norval and Rechav 1979; Barré et al. 1998). The AAP also promotes the aggregation and attachment of other males (Norval and Rechav 1979). Field tests with *A. hebraeum*, a sister species of *A. variegatum* with a similar AAP composition (Diehl et al. 1991), indicate that the semiochemical helps to mediate long-range attraction of conspecifics of both sexes to the host: an extract of feeding male *A. hebraeum* applied to cattle preferentially attracted wild conspecifics to the host and even to the area of application (Rechav and Whitehead 1978). Furthermore, Norval et al. (1989) demonstrated that cattle infested with *A. hebraeum* were more attractive to conspecifics than uninfested cattle, and postulated that the AAP may aid in marking a suitable host for further infestation. *A. hebraeum* adults were also preferentially attracted to rabbits on which ticks were feeding (Yunker et al. 1990). Although such 'host baiting' as suggested by Norval et al. (1989) by feeding males still remains to be proven for *A. variegatum* (Barré et al. 1998), extracts of feeding males of this species proved very attractive in the field at a distance of over 10 m when delivered with CO_2, whereas the latter was a poor attractant on its own (Norval et al. 1992).

The principal volatile components of the AAP of *A. variegatum* have been identified as *o*-nitrophenol (ONP) and methyl salicylate (MS), both released at an average rate of 15 μg day^{-1} and 4 μg day^{-1} or, respectively, ca. 0.05% and 0.01% of body weight day^{-1} by males from their dermal glands type 2 after feeding for 3–4 days (Diehl et al. 1991). Olfactory receptors have been located in Haller's organ for both ONP and MS (Schöni 1987), but behavioural studies on the responses of *A. variegatum* to these compounds have yielded mixed results. ONP has been shown to induce aggregation in still air (Schöni et al. 1984), and to attract the tick in the field (Hess and De Castro 1986), probably via odour-mediated anemotaxis. Hess and De Castro (loc. cit.) did not find MS to be attractive in the field, although Norval et al. (1991) reported attraction to this compound in the presence of CO_2. Yunker et al. (1992) reported strong attraction by *A. variegatum* to MS vapour in a two-choice olfactometer, but not to the vapour of ONP.

The above studies did not attempt to test different doses of the semiochemicals or to systematically investigate possible interactions between them. The detailed knowledge of the ultrastructure of Haller's organ (Hess and Vlimant 1982) and characterisation of several of the chemoreceptors contained there (Steullet and Guerin 1994 a) make *A. variegatum* an excellent model for a behavioural study of an acarine pheromone. We set out to investigate the responses of *A. variegatum* adults to the two main components of its AAP under controlled conditions in an air stream on a servosphere. Evidence is presented to show that *A. variegatum* adults are attracted upwind to the components of its AAP and we describe the modification of the basic walking behaviour of this acarine in response to the pheromone.

Materials and methods

Ticks

A. variegatum adults from Africa used in this study have been reared in captivity at Novartis Animal Health S.A. (St. Aubin, Switzerland) since 1981 for 20 generations. A second strain, originating from a wild population collected in the Ivory Coast in 1997, was reared at the same site and used in this study solely for comparison with the other strain. All stages (immature and adult) are fed on the tails of Simmental calves at 22–24°C and kept under constant darkness during moult at 28°C/80–90% relative humidity (RH). After moult, unfed adult males and females forseen for these tests were maintained in an environmental cabinet at 10 h light and 10 h darkness with 2-h ramps of dawn and dusk at between 18°C and 23°C and 60–90% RH for up to 9 months. *A. variegatum* adults placed in a radar actograph (actometer model RA-12B Syntech, The Netherlands), sensitive enough to even record foreleg raising, were sessile when held undisturbed under these conditions. In this state the ticks maintain their legs folded underneath the abdomen.

Before an experiment, a small piece (ca. 4 mm^2) of an adhesive reflector (3 M, No. 7610, Switzerland) was placed over 2 mm posterior to the capitulum on the back of each tick before the start of an experiment. The tick was then placed on the servosphere, and where necessary, was activated to walk by gently nudging its body with forceps. Unless otherwise specified, ticks were allowed at least 3 min to acclimatise in the air stream on the sphere so as to record walks at a stable speed. All tests were carried out in the dark under filtered incandescent light (filter cut-off, 780 nm) as these animals are acutely sensitive to visual stimuli. Each tick was only tested once.

Stimulus and stimulus delivery for behavioural recordings

ONP (50 mg, Merck) and MS (50 μl, Fluka) were each dissolved in 2 ml solvent (dichloromethane, Merck, analytical grade), hereafter referred to as 25 mg ml^{-1} stock solutions. Both chemicals were >98% pure as indicated by gas chromatography linked mass spectrometry. Solutions of ONP, MS and ONP plus MS were made up from stock solutions to obtain the concentration desired. To test the responses of ticks to chemicals, 0.4 ml from the ONP, MS and ONP plus MS solutions were placed on filter paper (7 cm diameter) and left to evaporate until dry. The filter paper was then placed upright at the bottom of a 500-ml gas-wash bottle. The same procedure was followed for the control using 0.4 ml of solvent. Gas-wash bottles were then left to equilibrate for at least 5 min before presenting the evaporated vapours to the ticks. Filter papers for both the control and test gas-wash bottles were replaced after every two tests. Ticks did not show a response to residual dichloromethane vapour from control gas-wash bottles.

Vapours from the gas-wash bottles were introduced upwind via Teflon tubing (2 mm i.d. × 4 mm o.d.) attached to a metal tube blocked at one end (3.8 cm long, 1.7 mm i.d. × 2 mm o.d.) with four 0.5-mm diameter holes along one side of its length. The tube was inserted horizontally, with its holes positioned against the airflow, 26 cm upwind of the apex of the sphere through a rubber septum in the wall of the stainless-steel tube conducting the main air stream to the sphere. Computer-controlled solenoid valves switched the charcoal-filtered air flow (150 ml min^{-1}) between control and test gas-wash bottles for consecutive 1-min control, test and end-control periods, thus voiding 30% of the volume of the gas-wash bottle in 1 min. To determine if the consecutive presentation of the control followed by test treatment might bias results, 15 ticks were presented with charcoal-filtered air (150 ml min^{-1}) for two consecutive 1-min recording periods. In another experiment, 24 ticks were presented with air (150 ml min^{-1}) passing through a control gas-wash bottle for 1 min (i.e. one containing filter paper from which solvent had been evaporated) followed immediately for another minute by air flowing through a second control gas-wash bottle, as in the normal test procedure. No difference between the parameters of the ticks' walk (see below) in the 1st and 2nd min was apparent in either experiment.

Estimation of release rates of chemicals in behavioural tests

To estimate the release rate of ONP and MS from the gas-wash bottles, 100 μg of each, individually and together, were dosed on filter paper and the solvent (dichloromethane) left to evaporate as for behavioural tests. This filter paper was then introduced into a 50-ml flask and left to equilibrate for 15 min. A Porapak Q column (0.2 g, 50–80 mesh; Millipore, USA), 3 cm long and 2 mm in diameter was attached at the exit of the flask through which pressurised charcoal-filtered air (20 ml min^{-1}) passed for 1 min. The same procedure was carried out for the control, using only solvent. Chemicals collected on Porapak were eluted with dichloromethane and the recovery of volatiles in the eluate was quantified using gas chromatography linked to flame ionisation detection by comparing peak height and retention times with known quantities of standards. The efficiency of the recovery of chemicals from the polymer was estimated by introducing 10 μg of both ONP and MS to the top of the Porapak column (above) and drying it by attaching it to the air stream at 20 ml min^{-1} as above for 1 min. The chemical load on the trap was estimated by extracting and quantifying as above. The percentage recovery of the known amounts applied to the polymer was used as a correction factor in estimating the true quantity recovered from the headspace. All tests and controls were run in duplicate.

Responses to ONP in the presence of host odour

As sex pheromones of metastriate ticks are released in the context of host odour, the response of *A. variegatum* to ONP vapour (10-ng source dose) in the presence of steer hair odour was tested on three different days. Animals were presented for three consecutive 1-min periods with (1) air passing through a control gas-wash bottle (150 ml min^{-1}), (2) air passing through both a control gas-wash bottle and a bottle containing steer hair (25 g) at 75 ml min^{-1}, and (3) air passing through both a gas-wash bottle containing 10 ng of ONP (75 ml min^{-1}) and the one with steer hair (75 ml min^{-1}) passing to the main air stream. The CO_2 concentration within the gas-wash bottle containing the steer hair was estimated by sealing the gas-wash bottle and inverting it for 15 min before sampling the air at the open end with a CO_2 indicator tube sensitive in the range 100–3000 ppm (accuracy: ±10–15%; Drägerwerk, Germany).

Servosphere and recording of behaviour

A servosphere (Kramer 1976) was used to record the responses of *A. variegatum* adults to chemical stimuli. This apparatus functions in such a way as to keep the arthropod at the same position in space, i.e. at the apex of a Perspex sphere (50 cm diameter) to which a stimulus delivery tube is directed. The detail of the equipment and procedure followed was as given by Taneja and Guerin (1995) with certain modifications. The feedback and the reaction time of the servosphere motors were adjusted to allow the ticks to walk unimpeded in all directions within a circle (2 cm diameter) with the photosensor placed 25 cm directly above the animal. A metal dome (28 cm diameter and 4 cm high) coated with a non-reflecting black paint and with a hole (5.5 cm diameter) at its centre to permit passage of light from the sensor, covered the sphere apex to minimise interference from air-currents and residual light in the room. The air stream (3.5 l min^{-1}) flowing over the ticks exited from a rectangular orifice (4 cm long, 2.5 cm high) positioned 6 cm from the apex of the sphere and was maintained at a speed of 0.18 m s^{-1} (23–25°C; 70% RH) as measured by an anemometer (Thermo-air, Schiltknecht Messtechnik, Switzerland; response time < 1 s). This wind velocity is more than five times greater than the maximum walking velocity of *A. variegatum* adults in these conditions. Temperature and humidity were measured with a thermo-electric hygrometer (Hygro-Air II, Schiltknecht Messtechnik, Switzerland; response time ca. 1 s). Two black-and-white infrared-sensitive video cameras (Canon Ci 20PR, Japan and PCO Computer Optics 77 CE), one positioned at a 70° angle over the orifice, another looking tangentially to the apex of the sphere from a downwind position, were employed to view the behaviour of the ticks. Both views were observed simultaneously by entering the CCD camera signals to a screen splitter (Panasonic production mixer, WJ-MX12, Japan), to a video recorder (Panasonic VCR NV-180, Japan) and a video monitor (Panasonic, WV-5360, Japan).

Data analysis

The co-ordinates of the sphere's displacement while compensating for the displacement of the tick were collected by computer and the vectors obtained were used to reconstruct the tracks described by the tick (Taneja and Guerin 1995). It was established from video and track analysis of several ticks' walks at different speeds that the walking behaviour was best described by choosing a sampling rate of 0.2 s per vector and by applying a running mean over five successive vectors. Movements of less than 0.1 mm (the lowest increment sampled by the servosphere) were excluded. In this manner, some noise was removed from the track records to give a realistic picture of the turning angles described. Any remaining high angles described when the tick pivoted were deleted manually. These modifications typically removed < 4% of all vectors. As the speed of individual ticks fell in the range 10–30 mm s^{-1}, the chosen sampling rate provided an average resolution of 40–120% of the body length of the tick per vector. These vectors were used to calculate speed, angular velocity (° s^{-1}), stop time, mean direction ϕ relative to the air flow and path straightness r (derived from the variance of ϕ; circular statistics, after Batschelet 1981). An analysis (Spearman test, two-tailed) undertaken to investigate any interrelationships between these parameters showed that they were poorly correlated (correlation coefficient < 0.15, $n > 1600$).

Apart from speed, parameters calculated from the vectors in control and test periods were not normally distributed and all comparisons were undertaken using non-parametric statistical tests (two-tailed). Differences between walks in test and control periods were analysed pair-wise using the Wilcoxon signed rank test. The significance of differences between treatments was determined using the Wilcoxon-Mann-Whitney test (MWU) by comparing differences pairwise between the test and control for different parameters.

The increase in time spent walking in the cone (60° either side of due upwind) over the relative time spent walking there in the preceding control period of equal duration was chosen as a means of measuring attraction and, when significant, termed an upwind or attractive response. A 'target vector' was also calculated by multiplying the cosine of the mean direction ϕ by the path straightness r (after Jones 1977). This provides an estimate of the efficiency with which the mean direction was followed and is independent of the arbitrarily chosen upwind cone. The target vector was in close agreement with the relative time spent walking upwind (Spearman correlation coefficient 0.93, $P < 0.001$, two-tailed, $n > 1600$). The paired differences in the time spent walking in the upwind cone between test and control were also closely correlated with corresponding paired differences in target vector (Spearman correlation coefficient 0.91, $P < 0.001$, two-tailed, $n > 1600$).

Results

Walk of *A. variegatum* on the servosphere in still air

In still air, *A. variegatum* adults walked on the servosphere in brief bouts interrupted by longer (< 3 min) pauses. While walking, the tick maintained its rostrum parallel to the surface of the sphere and proceeded at constant speed with shifts to the left and right of the path (Fig. 1). These shifts consisted of 30 half-turns min^{-1},

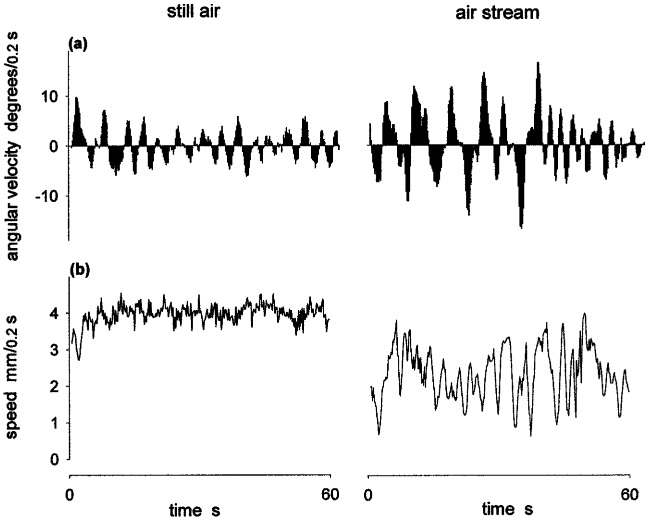

Fig. 1 The influence of an air stream (0.18 m s^{-1}) on the angular velocity (**a**) and speed (**b**) of an *Amblyomma variegatum* adult walking on a servosphere as recorded using 0.2-s successive vectors. A running mean over five vectors was applied once to speed and twice to angular velocity for graphical representation. Note the reduced less stable speed and increased angular velocity of the walk in the presence of wind

with each half-turn between 20° and 40° for males and 20–30° for females. The ticks tended to follow a given direction for at least 1 min before switching to another direction, yet the overall trajectory was circular.

Walk of *A. variegatum* on the servosphere in an air stream

In an air stream, *A. variegatum* usually walked continuously, often for some hours. Circular trajectories were uncommon (< 10%). The gait of *A. variegatum* walking in the air flow alternated between two distinct categories, an eight-legged walk as in still air and a six-legged walk. The six-legged walk consisted of the animal lifting its forelegs above the ground to sample the air vigorously. To achieve this type of walk, the alloscutum was fixed as vertically as possible using the hind legs as a buttress. This laboured walking was always undertaken before the tick made sharp angles or pivoted, i.e. turns > 150° s^{-1}, and was associated with a lower speed (ca. 20%) and higher angular velocity (ca. 30%) compared to the eight-legged walk (Fig. 1). Generally, males exhibited this behaviour more than females, and this reaching behaviour was only seen in the presence of the air stream. This pattern of locomotion induced by the air stream was also observed for other tick species running on the sphere (*A. hebraeum*, *Rhipicephalus sanguineus*, *Ixodes ricinus*, *Argas persicae* and *Ornithodorus moubata*). Straighter avoidance walks for *A. variegatum* were recorded when the ticks were presented with stimuli such as a light source (ca. 400 lux) and high air-stream speeds (0.5 m s^{-1}) of low RH (< 10%).

To investigate the effects of the air stream, five ticks were left walk in still air followed by the air flow (0.18 m s^{-1}), and this order was reversed for five other ticks. Tracks were recorded after 2 min acclimatisation to either condition. The order of the experiments had no discernible effect on the subsequent walking, so the data for the two lots of ticks were pooled for analysis. The air stream affected tick walking behaviour: the median speed was significantly ($P < 0.001$, Wilcoxon signed rank test) reduced from 16 mm s^{-1} to 11 mm s^{-1} and there was a tendency towards an increased turning angle (Fig. 1), due in large part, to the induction of the six-legged walk. No difference in walking behaviour was evident at air speeds in the range 0.1–0.5 m s^{-1}. The air stream did not cause anemotaxis, but ticks were acutely sensitive to small variations in the air flow (< 0.02 m s^{-1}) passing over the sphere. Although all tests were carried out in an air stream at 70% RH, a comparison with the walk of 20 ticks at 95% RH yielded no significant differences.

One-minute walks of over 1600 ticks walking in an air stream at 0.18 m s^{-1} preceding test periods for chemicals (below) were analysed. There was no preference for upwind walks. The ticks spent a median relative time of 22.7% walking in the upwind cone (60° either side of due upwind) and the mean direction (ϕ) was 177° relative to upwind (0°) with a median path straightness (r) of 0.26, and a median target vector of −0.21. Ticks usually walked continuously at a median speed of 19.3 mm s^{-1} and at a median angular velocity of 24.7° s^{-1}, yet walked slower in the upwind cone (median 16.9 mm s^{-1}, $P < 0.001$, Wilcoxon sign rank test) and at a lower angular velocity (median 22.4° s^{-1}, $P < 0.001$).

A comparison between male and female ticks showed that females ($n = 478$) walked slightly faster (median 21.3 mm s^{-1}) than males (median 18.9 mm s^{-1}, $P < 0.001$, MWU, $n = 1450$). However, females spent a similar time (median 22.6%) walking in the upwind cone to males (median 22.7%), and both genders did not differ greatly in angular velocity (median 23.8° s^{-1} and 24.2° s^{-1} for females and males, respectively).

The influence of the age of adult ticks (1–13 months after moult) on the way they walked was also analysed. Only speed was significantly correlated (Spearman correlation coefficient 0.4, $P < 0.001$, two-tailed, $n > 1928$) as ticks walked slower with increasing age. Over 1 year, the average speed of ticks decreased by 30%.

Release rate of test chemicals in air over the servosphere

Analysis of the headspace from a 50 ml flask containing ONP and MS showed no difference in the rate of volatilisation between these two compounds of similar vapour pressure when added either together or separately (coefficient of variation within the most differing pair was 25%). An interval experiment where the release rates during the first and last 30 s of the 1-min test period at the specified flow rate were compared, showed only a slight reduction (ca. 10%) in the recovery of volatiles in the 2nd half minute compared to the 1st. Some 2–3% of the 100-μg source was recovered from the headspace of the 50 ml flask in 1 min. Extrapolating from this figure to the experimental conditions on the sphere, a 1-μg source dose would release an estimated 4×10^{10} molecules ml^{-1} of either ONP or MS in air flowing over the tick at the apex of the sphere, taking into account the dilution of the gas-wash bottle effluent in the main air stream.

Attraction of *A. variegatum* to pheromone components

Vapours from the two main components of the aggregation-attachment pheromone of *A. variegatum* were tested individually and together. The index of attraction chosen was the percentage difference in time spent walking in the upwind cone in the test period over the control (see Materials and methods). ONP, the predominant AAP constituent, was found attractive at source doses from 1 pg to 1 μg, but the upwind response was variable at any of these doses. By contrast, MS was always attractive in a range from 100 pg to 1 µg (lower doses not tested). Moreover, the response to MS did not differ significantly over this range. A binary 1:1 mixture of equal source doses of MS and ONP was significantly more attractive than the natural ratio (3:1) of these compounds present over feeding males, but not more attractive than MS presented on its own at this dose (Fig. 2). This 1:1 binary mix was attractive in four tests spanning a 10,000-fold concentration range (Fig. 3). Furthermore, the degree of attraction between the 10-ng and the 10-µg source doses was not significantly different. However, there was a significant trend towards reduced attraction as the source dose of the 1:1 binary mix was increased ($P < 0.01$, Jonckheere test; after Siegel and Castellan 1988). *A. variegatum* was less attracted to the 100-µg source dose of the 1:1 mixture, and the 1-mg source dose was overdosed, for although ticks turned upwind towards the source this did not lead to upwind walks.

No significant difference was found in either the upwind response or in the parameters of the ticks' walk between pheromone vapour delivered at a constant rate (above) from a gas-wash bottle flask containing 1 µg of the binary 1:1 mix or in gradual increments over 1 min (0–150 ml min^{-1}). Also, the responses of ticks to the pheromone presented at different air stream humidities (70% and 95%) did not differ. Neither did gender, time of day, age (between 1 month and 9 months) or the number of generations the colony was reared in captivity influence the behaviour. Moreover, the strain of ticks reared in captivity since 1981 and the more recent (1997) colony originating from a wild tick population from the Ivory Coast did not differ in their responses to this attractant.

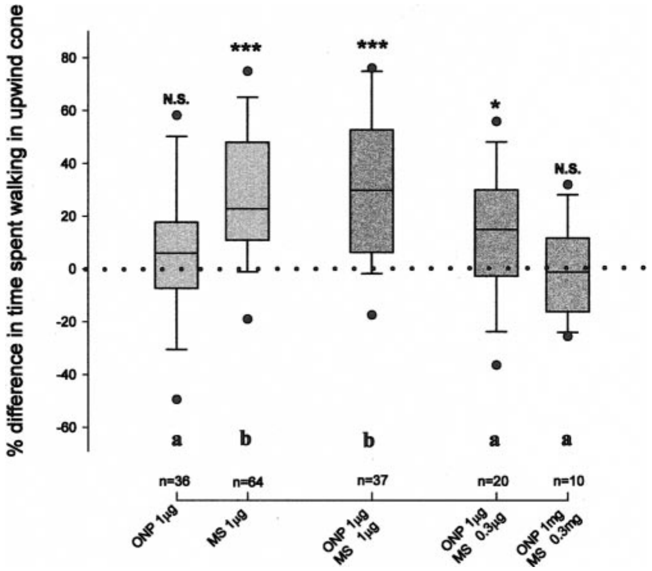

Fig. 2 Box plots of the difference from the control in time spent walking on the servosphere in the upwind cone (60° either side of due upwind) as a percentage of the total for the period by *A. variegatum* adults in response to delivery of vapours of the aggregation-attachment pheromone components *o*-nitrophenol (ONP) and methyl salicylate (MS) on their own or as mixtures in the air stream. Source doses are indicated under box plots. The *line within a box* marks the median, the lower and upper boundaries of a box indicate the 25th and 75th percentiles, error bars below and above a box indicate the 10th and 90th percentiles, and the 5th and 95th percentiles are shown as *circles*. *Asterisks* above a treatment indicate the significance of the response (*$P < 0.05$; ***$P < 0.001$; Wilcoxon signed rank test). Treatments denoted by the same letter (*a*, *b*) are not significantly different ($P < 0.05$, Wilcoxon-Mann-Whitney test)

Responses of the ticks to the binary mixtures and components in terms of angular velocity and walk in the upwind cone were also calculated using distance based sampling. This analysis of 40 tests (10–20 ticks for each test) provided no increase in resolution between test and control for angular velocity (°/0.4 mm) compared to time based sampling (°/0.2 s). Further, the relative displacement in the upwind cone was found to be a less efficient index of attraction than the relative time spent walking in the upwind cone as ticks frequently slowed down in the presence of the attractant.

Generalities on the responses of *A. variegatum* to test chemicals on the servosphere

In the presence of any attractant the time taken by ticks to actually turn upwind was variable, happening within the first 30 s of the 1-min test period. The kinesis responses of ticks to the binary mixtures (whether attractive or not) and components presented alone were always the same, usually occurring within the 1st 10 s (Fig. 4). These responses were characterised by an increase in angular velocity and a reduction in speed, effects also associated with the six-legged walk and concurrent reaching in the air with the forelegs. Such increases in angular velocity and decreases in speed were not always significant for a given treatment yet were closely correlated (Spearman correlation coefficient 0.44, $P < 0.001$, two-tailed, $n > 1600$) and contributed to a lower overall displacement by the tick (Fig. 5a–d). These kinesis responses were independent of the increased upwind walk (Spearman correlation coefficient 0.01; $P > 0.2$, $n = 357$, two-tailed) and an analysis of the change in angular velocity and speed over a range of concentrations (Fig. 3) indicated no dependency on dose (Jonckheere test, $P < 0.2$).

Ticks varied in the manner they approached the source: a small majority maintained an upwind approach from one side, or very rarely, walked straight upwind. Other ticks, however, crossed the axis of the air stream, and while veering downwind often made abrupt turns to re-orientate towards the source. This behaviour never occurred in the controls. The upwind walk to an attractant usually (ca. 80% of ticks) persisted long into the end control. In the other cases (ca. 20%), ticks responded to the loss of chemical either by describing small circles, abruptly turning downwind, or both, behaviours that were also absent in controls. If a tick happened to be walking consistently downwind at the

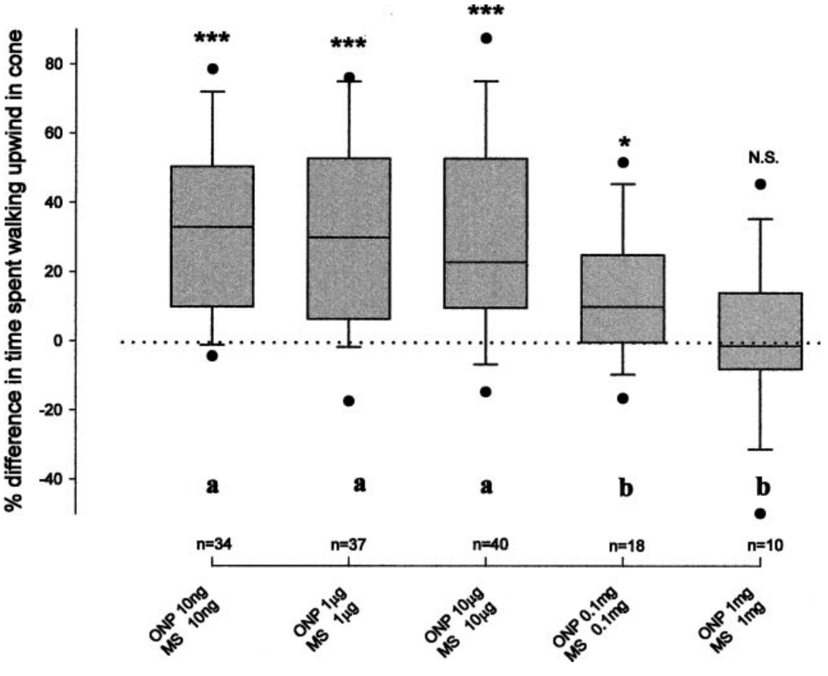

Fig. 3 Box plots of the difference from the control in time spent walking on the servosphere in the upwind cone by *A. variegatum* adults in the presence of vapours from increasing source doses of a 1:1 mixture of ONP and MS. *Asterisks* above a treatment indicate the significance of the response ($*P < 0.05$; $***P < 0.001$; Wilcoxon signed rank test). Treatments denoted by the same letter (*a*, *b*) are not significantly different ($P < 0.05$, Wilcoxon-Mann-Whitney test). Source doses are indicated under box plots. Note the significant ($P < 0.01$, Jonckheere test) trend towards reduced attraction at increasing source doses of the binary mix. The 1:1 mixture was clearly overdosed at 1 mg

end of the test period, it too usually maintained this direction in the end control, turning upwind after loss of the chemical only in a minority of cases (ca. 20%). Ticks could not be induced to respond to presentation of pheromone components in the air when in the resting pose with legs folded beneath the abdomen on the servosphere.

Responses to ONP in the presence of host odour

Although steer hair odour itself was not attractive ($P < 0.6$, $n = 33$), the addition of ONP (source dose 10 ng) to steer hair (source dose 25 g) was attractive in each of three tests ($P < 0.001$, $n = 33$, Figs. 5e, 6). In contrast, the response to ONP presented alone was

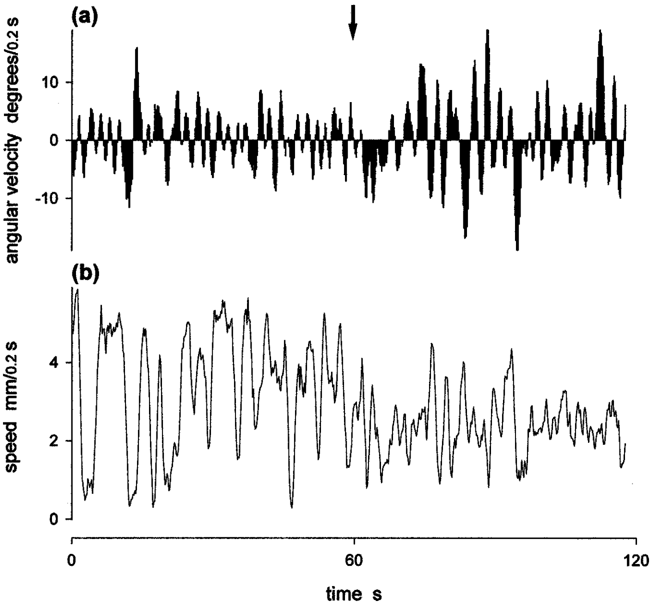

Fig. 4 The effect of vapours from the binary mixture of the aggregation-attachment pheromone components ONP and MS each at a 1-µg source dose on the angular velocity (**a**) and speed (**b**) of an *A. variegatum* adult. Running means were applied as detailed in the legend to Fig. 1. Speed dropped and angular velocity increased significantly upon adding the ONP and MS odours (*arrow at 60 s*)

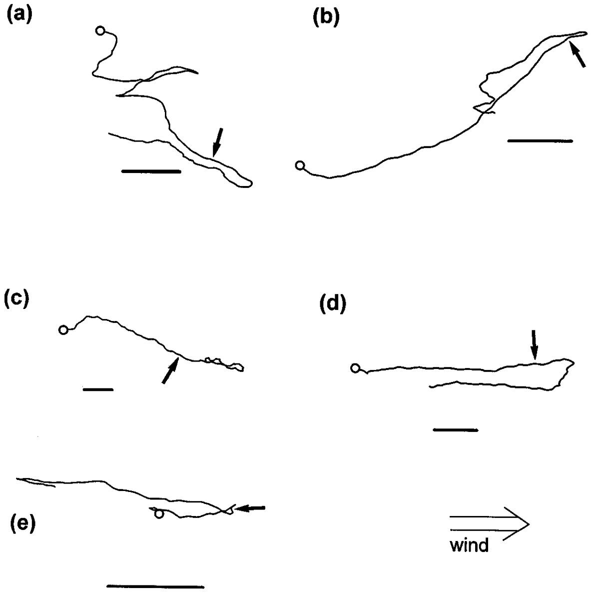

Fig. 5 Tracks described by *A. variegatum* adults on the servosphere in response to vapours from a binary mixture of ONP and MS each at a 1-µg source dose (**a–d**), and to vapours from a 10-ng source dose of ONP in the presence of steer hair odour (**e**; see text). The tracks started (o) with the ticks walking downwind (*open arrow*) in the control period. The *bold arrows* on the tracks indicate odour on. *Bars* beneath each track represent a displacement of 20 cm

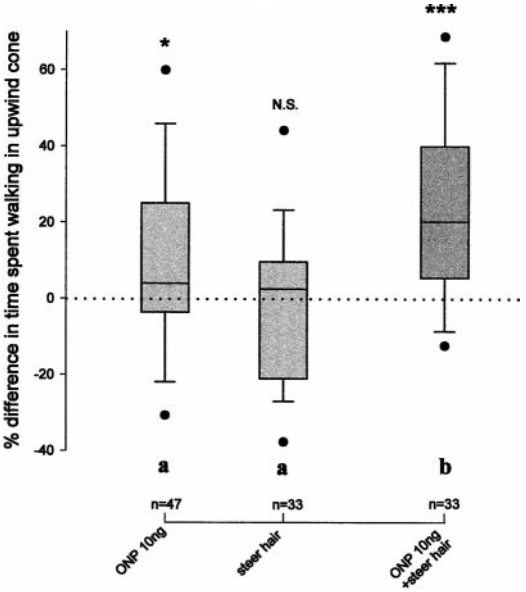

Fig. 6 Box plots of the difference from the control in time spent walking in the upwind cone by *A. variegatum* adults in the presence of vapours of ONP , steer hair, and ONP plus steer hair. *Asterisks* above a treatment indicate the significance of the response (*$P < 0.05$; ***$P < 0.001$; Wilcoxon signed rank test). Treatments denoted by the same letter (*a*, *b*) are not significantly different ($P < 0.05$, Wilcoxon-Mann-Whitney test). Source doses are indicated under box plots

variable: at this source dose (10 ng) ticks were attracted to this chemical in only one of three other tests. The nature of the responses to ONP in the presence of steer hair odour was also markedly different to that seen for either of the pheromone components and their binary mixtures, in that the ticks did not show any consistent or significant change in speed or turning angle. Ticks responded to ONP plus steer hair odour with a significantly ($P < 0.05$) greater increase in the walk in the upwind cone (median 20.1%, $n = 33$) compared to ticks presented ONP alone (median 3.9%; $n = 47$; Fig. 6). The CO_2 concentration in the gas-wash bottle containing steer hair was not sufficient to cause the CO_2 indicator tube to respond.

Discussion

In common with some other arthropods, *A. variegatum* described circular paths on the servosphere in the absence of other sensory cues (Bell and Kramer 1979; Heinzel and Böhm 1989; Lönnendonker and Scharstein 1991). The air stream, however, modified this walk by inducing vigorous vertical movements of the forelegs, a reaching behaviour also seen for other species of tick running on the servosphere during this study. Raising of the forelegs and body axis has been reported to serve in the aerial dispersal of the two-spotted spider mite (Smitley and Kennedy 1985), occurring only in the presence of wind, so the reaching observed here might be a general response of acarines to this stimulus.

The air stream velocity had no discernible effect on the walk of *A. variegatum*. This contrasts with studies by Bell and Kramer (1979) and Heinzel and Böhm (1989) who noted that although cockroaches and carrion beetles, respectively, described circles in still air, these were modified to ever decreasing arcs as wind speed increased. For *A. variegatum*, however, the air stream seemed to act as a simple switch that initiated reaching behaviour, and consequently angular velocity increased and walking speed decreased. This ortho- and klinokinesis induced by moving air was further enhanced by the presentation of ONP (where attractive), MS, and ONP plus MS. As such it may represent a search strategy that enables the tick to sample the air stream vigorously while still maintaining an upwind course towards the source. An increased angular velocity might also aid the tick to stay in contact with the odour plume. Wolf and Heisenberg (1991) working with tethered *Drosophila* found that when the flies were stimulated with host odour in a open-loop design, the increase in angular velocity recorded could explain the animal staying three times longer in the odour field.

A 1:1 binary mixture of ONP and MS was a robust attractant at source doses of less than 1 mg. Based on the known release rates of ONP from feeding adult males (Diehl et al. 1991), the 1-mg source dose released an equivalent amount to the tick on the servosphere as the quantity released from >1000 feeding males in 1 min. As the average population of *A. variegatum* adults on cattle rarely exceeds 200 per host (Pegram et al. 1986), it might be that the presence of one quarter of this population as males only at a predilection site would deter further males from arriving there and account for the lack of progress upwind to 1-mg source doses. High doses of AAP components have been shown to attract in the field when delivered with CO_2: Norval et al. (1991) reported that a mixture of 20 mg ONP and 200 μl MS attracted more than 40% of all ticks released, and Barré et al. (1997) found a synthetic pheromone mix equivalent to the extracts from 100 feeding males to be attractive, and suggested that the dose could be increased. In the open-loop design of experiments done here on the sphere, the tick was required to respond in a confined system, whereas the concentration will vary widely in the field. Another possible reason for the discrepancy between results obtained in our laboratory study and the field may have been the manner in which ticks were activated. On the servosphere, it was the air stream that stimulated the tick to keep walking, whereas in the field experiments, either breath (Hess and De Castro 1986) or CO_2 (Norval et al. 1991; Barré et al. 1997) was required.

In this study, female and male *A. variegatum* adults responded with an upwind walk to very low concentrations of ONP and MS. At the estimated 7–8% release of chemical from the filter paper into the gas-wash bottle head space of a 1-pg source dose of ONP, the lowest attractive dose tested, the chemical was present at ca. 4×10^4 molecules ml^{-1} of air at the apex of the sphere, a value comparable with the sensitivity of moths to their sex pheromones (Kaissling 1990). This is well below the levels (1×10^7 molecules ml^{-1}) of 2,6 dichlorophenol (Waladde 1982) and ONP (Steullet and Guerin 1994a) shown to stimulate specific chemoreceptors in the Haller's organ of this species.

It was unexpected that a naturally occurring 3:1 ratio of ONP to MS would be less attractive than a 1:1 binary mix of both compounds at the same dose. This demonstrates that ratios of attractant components are critical in evoking an upwind response in this tick and raises the possibility that a natural ratio of ONP to MS might prove more attractive if delivered to ticks as part of a more complete pheromone blend. Extracts from fed males have provided several candidate compounds which may be integral to the AAP of *A. variegatum* such as nonanoic acid (Schöni et al. 1984; Pavis and Barré 1993) and benzaldehyde (Lusby et al. 1991). Further, it is known that feeding *A. hebraeum* males release several short-chain fatty acids, especially isobutyric acid (Apps et al. 1988). Interestingly many of these substances are, like ONP, host compounds for which *A. variegatum* has chemoreceptors (Steullet and Guerin 1994a). Therefore, what constitutes a host odour or an AAP component may be somewhat arbitrary since some may occur in both the host odour and AAP blends, modulating the responses to ONP and MS.

The attraction to ONP in the presence of steer hair odour (the latter unattractive) was not associated with a change in turning angle or speed. This may suggest that ticks do not need to reach extensively when orienting to the source of a more complete blend of what constitutes an adequate attractant. This phenomenon of a straighter upwind track to the source has also been demonstrated for the flight of the European grape berry moth (*Lobesia botrana*) males to calling females as compared to a synthetic mix (Witzgall and Arn 1990). Moreover, the constancy of the attraction to ONP plus steer hair odour compared to the variability of the upwind response to ONP presented alone is consistent with release of this principal pheromone component of *A. variegatum* in the context of host odours. Olfactory receptor cells in Haller's organ sensilla have been characterised as responding to a wide range of host chemicals such as CO_2, H_2S, mercaptans, NH_3, branched and unbranched short-chain fatty acids, saturated and unsaturated aliphatic aldehydes, lactones, furfural, benzaldehyde and related compounds, and nitrophenols such as ONP (Steullet and Guerin 1992a, b; 1994a, b). Steer hair may contain some or all of these products. Steullet and Guerin (1994a) postulated that components of the AAP, especially ONP, have been selected in the evolution of this pheromone system for the purpose of amplifying specific host chemical cues to which this tick is particularly sensitive (Steullet and Guerin 1994a). This would suggests a virtuous circle for host seeking by this ectoparasite between host odour and the AAP. Our findings that the ratios of the attractive components are important in the attraction of *A. variegatum* adults, and that responses to the main pheromone component are both enhanced and modified in the presence of host odour, are consistent with their hypothesis.

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