

Isolation of 2,6-Dichlorophenol from the Cattle Tick *Boophilus microplus*: Receptor Cell Responses but No Evidence for a Behavioural Response

MARIEN DE BRUYNE,* PATRICK M. GUERIN*

2,6-Dichlorophenol, a compound known as a sex pheromone for several metastriate tick species, was isolated from different life-stages of the cattle tick *Boophilus microplus*. Receptor cells in two wall-pore single-walled sensilla on the tarsus I of male ticks responded to this compound in a dose-dependant manner. Using these receptors as specific detectors for compounds in the effluent of a gas chromatograph, we detected 2,6-dichlorophenol in extracts of females, males, engorged nymphs and larvae of this one-host tick, but not in an extract of eggs. No other components of the extracts elicited responses from these olfactory sensilla. However, male *B. microplus* were not arrested on a glass bead treated with 2,6-dichlorophenol and placed on a membrane in a host-simulating arena, whereas a bead treated with a female extract did evoke a strong arrestment response. In addition, no odour-conditioned anemotaxis, change in angular velocity or speed of males walking on a locomotion compensator was observed in response to this compound in a conditioned air-stream. We could therefore not establish a role for 2,6-dichlorophenol on its own as a semiochemical in males of this species.

Boophilus microplus Tick Pheromone 2,6-Dichlorophenol Walking-behaviour Arrestment

INTRODUCTION

Berger *et al.* (1971) found that one fraction of a dichloromethane extract of female ticks excited males of three species, resulting in responses typical of mating behaviour. The compound responsible for this behaviour was subsequently identified as 2,6-dichlorophenol (referred to hereunder as 2,6-DCP) from *Amblyomma americanum* (L.) (Berger, 1972), and has since been isolated from at least 14 species of metastriate ticks (Sonenshine, 1985). It has remained the only positively identified volatile sex pheromone common to the Ixodidae, though other phenols have also been suggested (Wood *et al.*, 1975). However, the precise behavioural role of 2,6-DCP has not been fully investigated in any species. The foveal glands, with terminal ducts ending in the fovea dorsalis on the tick's dorsal cuticle, are thought to be the source of 2,6-DCP production (Sonenshine *et al.*, 1981). Foveae dorsales are apparently present in all life-stages of metastriate ticks but not in prostriates such as *Ixodes ricinus* L (Schulze, 1942; Dinnik and Zumpt, 1949).

2,6-DCP causes detachment of males of different species of ticks and induces displacement towards females in experiments on the host (Sonenshine, 1985). However, in *Hyalomma dromedarii* Koch it acts only as a male attractant at relatively short distances on the host and appears not to be attractive when offered in an air-stream off the host (Khalil *et al.*, 1981). Attraction was observed in both male *Dermacentor andersoni* Stiles and *Dermacentor variabilis* (Say) to a wide range of concentrations of this product (Sonenshine *et al.*, 1976), thus providing no basis for species specific concentration dependent responses.

Sex pheromones acting over a distance, till now unidentified, have also been described in *Hyalomma asiaticum* Schulze and Schlottke (Leonovich, 1981) and three Australian reptile ticks (Bull and Andrews, 1984). In the latter three species the excitant volatile appears to be species specific. A different class of pheromones, emitted by male ticks and mediating aggregation and attachment on the host, has been described for several *Amblyomma* species (Gladney *et al.*, 1974). These pheromones generally consist of a mixture of phenols and short chain fatty acids (Schöni *et al.*, 1984; Apps *et al.*, 1988).

*Institute of Zoology, University of Neuchâtel, Chantemerle 22, CH-2007 Neuchâtel, Switzerland.

Receptor cells responding to 2,6-DCP were investigated with tungsten electrodes by Haggart and Davis (1981) in *A. americanum* (L.) and responses were thought to originate from a wall-pore single-walled (wp-sw) sensillum in the anterior pit of Haller's organ on the tarsus of the first leg pair: the d II 1 of Hess and Vlimant (1986). A study in *Rhipicephalus appendiculatus* Neumann and *A. variegatum* (Fabricius), using the tip recording technique, confirmed the presence of a similar receptor in the corresponding sensillum (Waladde, 1982). The latter also showed that responses to 2,6-DCP can be obtained from another wp-sw sensillum positioned more distally on the dorsal surface of the tarsus: the d I 1 of Hess and Vlimant.

B. microplus (Canestrini) (Acari: Ixodidae) is a one-host ixodid tick. Contrary to other tick species previously investigated for sex pheromones, this tick passes through all its life-stages on the same bovine host. Widely distributed in the tropical- and sub-tropical regions, it is a vector for anaplasmosis and babesiosis and causes severe economic losses to cattle ranching. Very little is known about the mating behaviour and chemical ecology of this species. Chow *et al.* (1972) isolated a fraction from extracts of female *B. microplus* with similar chromatographic properties as that found active by Berger *et al.* (1971), but did not go so far as to identify what they called "a phenolic compound".

The olfactory wall-pore sensilla of *B. microplus*, homologous to those bearing 2,6-DCP receptors in other Ixodidae, are highly innervated: the more distal d I 1 sensillum by five neurones and the d II 1 by 15 neurones, organized in three separate bundles of 5 (Hess and Vlimant, 1986; Waladde, unpublished). Our results demonstrate responses to 2,6-DCP by sensory cells in both of these sensilla and we use these receptors as specific detectors to demonstrate the presence of 2,6-DCP in different life-stages of this species. Behavioural bioassays were then used to detect possible responses of adult males to this compound.

MATERIALS AND METHODS

Animals

Ticks were obtained from a laboratory colony at the Ciba-Geigy Agricultural Research Station, St Aubin, Switzerland and belong to the organophosphorus-resistant strain Biarra from southern Queensland, Australia. This strain has been reared on the backs of young Simmental steers for 31 generations in closed stables at 23°C and 60–70% r.h. Under these circumstances males appear on the 12th day after infestation and copulation peaks at the end of the 15th day (Falk-Vairant *et al.*, 1994).

Engorged nymphs or adult males were collected by carefully removing individuals from the host with forceps, and were transported to the laboratory in a humidified container. Engorged nymphs were kept in an incubator at 32°C and about 100% r.h. until they moulted, and adults were put on the ears of New

Zealand White rabbits enclosed in cotton bags where they readily attached. Electrophysiological experiments were made with males that had moulted in the incubator, whereas behavioural experiments were done with males collected from the host before mating. When not on the rabbits, ticks were kept at room temperature (22–28°C) over water in closed containers to assure high relative humidity.

Chemicals and extracts

Phenol, 2-nitrophenol, 4-methylphenol, 2,6-DCP and other halogenated phenols (all >98%, GC), were obtained from Supelco, USA; methyl salicylate (>99%) and benzaldehyde (>98%, GC) from Fluka, Switzerland; all solvents (analytical grade) were from Merck, U.S.A.

Ticks were extracted by submerging them for 2–6 h in small volumes (0.5–5 ml) of either dichloromethane or hexane/dichloro-methane (1:1) and sonicating for 15 min (see Table 1 for details). The extract was removed with a syringe and stored at –20°C. Air surrounding semi-engorged females on the host was collected by holding a glass cap (4.5 cm dia, 1 cm high) lightly against a steer's skin over a group of ticks. Air was sucked in over a charcoal filter via a 3 mm i.d. inlet with a portable air sampling pump (model 222-5, SKS Inc., U.S.A.) at 200 ml/min and volatiles were collected on Porapak-Q (Waters Inc., Framingham, U.S.A.) conditioned according to Byrne *et al.* (1975) and packed into a 2 ml glass cartridge. A control air collection was made, by repeating the same procedure, from the steer's skin alone after removing the females.

Electrophysiology

A male tick (2–14 days old) was fixed with adhesive tape on a glass plate with an anterior tarsus extended in such a way that its sensilla were visible in the transmitted beam of light on the stage of an inverse microscope (Nikon, Diaphot-TMD at 600×, working distance: 15 mm). Two olfactory wall-pore single-walled sensilla on the tarsus of the forelegs were studied (Fig. 1), the d II 1 in the anterior pit of Haller's organ (20 µm long, 5 µm dia at base) and the d I 1 on the knoll distal from the Haller's organ (36 µm long, 5 µm dia at base) (Hess and Vlimant, 1986). To facilitate electrical contact, the tip of the sensillum was cut using the tip of an oscillating glass stylet (Gödde, 1989), and a glass electrode filled with 0.05% polyvinylpyrrolidone K90 (Fluka) in 0.15 M KCl was immediately placed over it with the aid of a micro-manipulator. The reference electrode filled with 0.15 M NaCl was inserted into the coxa of the same leg and put to earth. The recording electrode was connected via a chlorinated silver electrode to a high impedance preamplifier, mounted on the micro-manipulator, and to a universal a.c./d.c. amplifier (UN-03, Syntech, The Netherlands) and signals amplified 1000×. a.c. and d.c. Signals were recorded separately on two channels of a DAT recorder (DTR-1200, Biologic, France) and the

a.c. channel was played back via a DAS 16 analogue-digital card (Metrabyte Corp., U.S.A.) into an IBM compatible PC equipped with the spike analysis programme SAPID (Smith *et al.*, 1990).

Known amounts of chemicals dissolved in CH_2Cl_2 were pipetted onto filter paper strips (45 mm^2) and, after evaporation of solvent, inserted into 5 ml plastic syringes serving as stimulus cartridges. Charcoal-filtered humidified air at $26\text{--}28^\circ\text{C}$ and $85\text{--}95\%$ r.h. was blown over the tick preparation at 60 cm/s from a 6 mm i.d. glass tube whose orifice was at 2 mm from the preparation. A second air-stream (1 ml/s) from a blank cartridge was added to the main air-stream at 45 mm from the preparation and solenoid valves were used to switch for 1 s to the cartridge containing the stimulus.

Since it was not possible to consistently associate responses to stimuli with distinct olfactory units in the sensilla studied here, increase in action potential frequency was calculated from the total number of spikes counted in the second after stimulus arrival minus the number in the second before stimulus delivery. Mainly due to travel time in the glass air delivery tube, a delay of 100 ms existed between solenoid valve activation and arrival of the stimulus at the sensillum as determined from the response to $1\text{ }\mu\text{g}$ of synthetic 2,6-DCP on filter paper; the relatively high dose was used to produce a sharp rise in spike activity. Only recordings where signal to noise ratio was at least 2:1 were analysed.

GC, GC coupled electrophysiology and GC-MS

Separation of extracts and comparison of peaks with known amounts of synthetic standards was done with cold on-column injection on a 30 m high resolution fused silica capillary column (DB-wax, J&W Scientific, U.S.A., $0.25\text{ }\mu\text{m}$ film thickness, 0.25 mm i.d.) in a Carlo Erba HRGC 5160 gas chromatograph (GC) with H_2 as carrier gas at 1.5 ml/min (0.5 m/s) temperature programmed from 60°C after 1 min to 200°C at 25°C/min , 200 to 230°C at 5°C/min and held at 230°C for at least 5 min . ECD (Ni^{63}) and FID detectors were installed in series. Quantification was by peak area integration using a Spectra-physics SP-4270 integrator and by comparing with known amounts of standards injected in the same session.

A splitter was installed allowing 60% of the capillary column effluent to pass to the detectors and the remainder was led through a heated transfer-line in the oven wall (at 250°C) into the conditioned airflow mentioned above at 30 cm from the preparation. A level discriminator incorporated in the amplifier allowed us to sort impulses from noise in the a.c. signal recorded from the sensillum and impulse frequency was converted into a d.c. voltage with a frequency to voltage converter (time constant 1 s). This voltage and the d.c. potential drop recorded from the sensillum upon stimulation were used as indicators of biological activity of eluting products and printed simultaneously with ECD and FID responses on a chart recorder. A water-jacketed glass tube

TABLE 1. Gas chromatography linked electron capture and olfactory sensillum detection, and gas chromatography linked mass selective detection of 2,6-dichlorophenol in different life stages of *B. microplus* (amounts of 2,6-DCP detected are rounded off to one significant digit)

Sample (number extracted in parentheses)	Extraction method*	2,6-DCP pg/tick (ECD peak area)	Also detected by	
			Olfactory sensillum	MS
<i>Immature Stages</i>				
Eggs 17 days old (10,000)	CH_2Cl_2 6 h	0	n.t.	n.t.
Larvae 16 weeks old (1500)	CH_2Cl_2 6 h	2	d I I	n.t.
Engorged nymphs (50)	CH_2Cl_2 6 h	30	d I I	n.t.
<i>Adult Females</i>				
Pharate (45)	CH_2Cl_2 /hex 5 h	20	d II I	n.t.
Newly moulted <i>in vitro</i> (80)	CH_2Cl_2 6 h	300	d I I	n.t.
1 day old (100)	CH_2Cl_2 /hex 3 h	600	d II I + d I I	n.t.
2 days old (200)	CH_2Cl_2 /hex 5 h	500	d I I	++
3 days old (20)	CH_2Cl_2 /hex 5 h	300	n.t.	n.t.
Fertilized 5 days old (67)	CH_2Cl_2 /hex 2 h	100	n.t.	+
Unfertilized 5 days old (50)	CH_2Cl_2 /hex 2 h	400	n.t.	++
<i>Adult Males</i>				
Pharate (215)	CH_2Cl_2 /hex 5 h	10	n.t.	n.t.
Newly moulted <i>in vitro</i> (76)	CH_2Cl_2 6 h	200	d II I + d I I	n.t.
1 day old (200)	CH_2Cl_2 /hex 3 h	300	d II I	n.t.
2 days old (247)	CH_2Cl_2 /hex 5 h	200	d I I	n.t.
<i>Air Sample</i>				
31 air over 25 host-attached Unfertilized females in 20 min On Porapak®	CH_2Cl_2 /hex	10	n.t.	n.t.

*All extractions terminated with 15 min sonication.

n.t., Not tested; hex., hexane; ECD, electron capture detector; MS, mass selective detector.

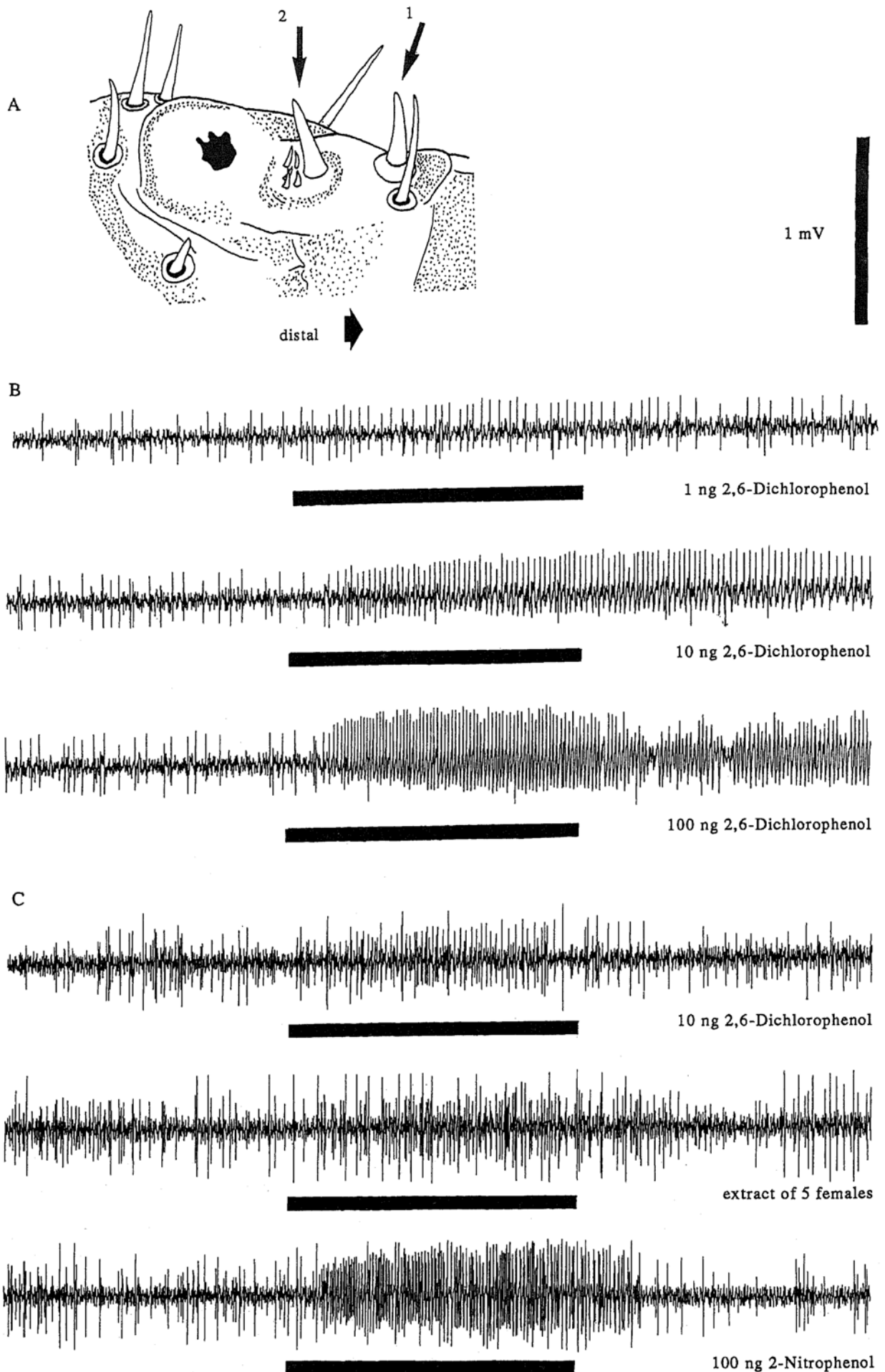


FIGURE 1. Characterization of olfactory receptors in two wall-pore single-walled sensilla on the dorsal side of tarsus I of male *B. microplus* ticks. (A) Dorsolateral abaxial view of Haller's organ and surrounding sensilla, showing the d I 1 (1) and d II 1 (2) sensilla. (B) and (C) Electrical signals obtained by tip recordings of these two sensilla, the d I 1 (B) and the d II 1 (C) upon stimulation with different volatiles. Stimulus dose is in ng of substance on filter paper in an odour cartridge from which air was displaced at 1 ml/s into a humidified air stream of 60 cm/s flowing over the preparation. Horizontal bar represents 1 s stimulus period. In (C) the frequency of the largest amplitude spike is irregular but was not modified by any of the volatiles tested.

(8 mm i.d.), circulating water from a bath (28°C), served to ensure constant conditions of the airflow (26–28°C, 80–90% r.h.) right up to the preparation. Any decline in the activity of the preparation was monitored with a 100 ng dose of 2,6-DCP as stimulus which was added to the air-stream as described above, before and after each GC run and the responses calibrated.

Gas chromatography–mass spectrometry (GC-MS) analyses were conducted with an HP-5971A mass selective detector (ionization energy 70 eV, temperature 180°C) linked to a HP-5890 series II GC equipped with the DB-wax column described above and programmed from 60°C after 5 min to 230°C at 8°C/min and held at 230°C for 15 min with helium as carrier gas at a flow rate of 1.2 ml/min. Mass spectra of unknowns were analysed and compared with standards held in a library using the HP Chemstation program on an HP IBM compatible computer.

Behavioural bioassay 1: dummy female on a membranous substrate

A 0.1–0.2 g glass bead (5 mm dia), roughened with a wet-stone and flattened on one side to inhibit rolling (3 mm high), was placed in the centre of a round arena (40 mm dia) consisting of a Baudruche® membrane (Joseph Long Inc., U.S.A.) stretched over a 0.9% NaCl solution held at $36 \pm 2^\circ\text{C}$ on a warm plate. A 40 mm high plastic tube placed around this arena and the permeability of the membrane assured a constant r.h. of >95% (Kröber, unpublished). Two such arenas were used simultaneously on the same warm plate, one bearing a bead with a tick extract or 2,6-DCP applied with a micro-syringe, the second treated with just solvent alone as control. When 2,6-DCP was applied, both beads were treated afresh for each tick having been washed in solvent and heated to 42°C for 1 min. The treated bead was left on the membrane for 2–5 min before introducing the tick to allow bead temperature to rise to that of the membrane.

A single male tick was released from a fine paintbrush onto the top of the bead. Behaviour was viewed from above and filmed at magnifications of 5× or 21× with a Canon CI-20P colour CCD video camera attached to a Zeiss operational microscope (working distance: 25 cm). Recordings were made on a JVC super VHS video recorder (HR-S5500E) and played back for analysis on a Sony Trinitron colour monitor. All males in a given experiment were tested on both the control and treated bead, half of them first on the control the other half first on the test. Behaviour was quantified using The Observer event recorder (Noldus Information Technology, The Netherlands) (Noldus, 1991). A maximum time of 180 s was allotted to each tick on the bead and/or arena. The total time spent on the bead (contact time) and on the arena around it (searching time) before the tick's first crossing of the edge of the arena to leave were taken as parameters for statistical analysis with the Wilcoxon signed ranks test on paired replicates (test vs control).

Behavioural bioassay 2: locomotion compensator and wind-borne odours

To study the walking behaviour of male *B. microplus* and its responses to wind-borne 2,6-DCP we used a servosphere apparatus which serves to keep the animal in a fixed position while permitting free displacement in the horizontal plane (Kramer, 1976). A perspex sphere (50 cm dia) with a rough painted surface is mounted between two low-inertia servo motors capable of moving it along two orthogonal axes. A tick is supplied with a *ca* 1.5 mm² piece of reflective foil (No. 7610, 3M, Switzerland) attached to its dorsum and placed on the sphere. A filtered incandescent light beam (40 mm dia, filter cut-off at 780 nm) is projected on the upper pole of the sphere. Light, reflected by the foil, hits a position sensor which continuously generates information about the displacement of the tick and this is used to drive the servo-motors that compensate for the displacement, thus holding the animal on the apex of the sphere. Two incremental pulse generators supply information about all 0.1 mm displacements of the sphere in the *X* and *Y* directions every 0.1 s and this is fed to a SAM II 68K computer (KWS Inc. Ettlingen, Germany) for track recording.

Temperature- and humidity-conditioned air was continuously blown from a water jacketed aluminium tube (35 mm i.d.) fitted with an aluminium foil tube at its mouth (70 mm long) supporting a honeycomb baffle to reduce turbulence and ending in a rectangular mouth (18 mm high, 35 mm wide) 3 cm from the sphere's apex. This air-stream (28°C, for humidity and velocity see Table 2) arrived tangentially at the top of the sphere where the tick walked. Stimuli were introduced to the air-stream from a 25 ml gas-wash bottle via a silicone tube and syringe needle inserted through a rubber septum in the wall of the aluminium tube 23 cm from its mouth. 2,6-DCP in solution was pipetted onto a 20 cm² piece of filter paper and soaked in *ca* 0.5 ml paraffin oil after evaporation of the solvent; a blank was made up in an identical way with solvent only. Voltage/pressure converters controlled the flow (240 ml/min) of the charcoal filtered air through the gas-wash bottle, and solenoid valves permitted air-stream switching from the blank to the bottle containing the stimulus.

Male ticks were placed on the sphere and allowed to adapt to the conditions for 8 min before testing. The area around the sphere was kept dark with black curtains.

TABLE 2. Treatments delivered to male *B. microplus* on the locomotion compensator and percentage upwind displacement (mean \pm SD) in control and test periods

Air conditions			Upwind displacement (%)		
Wind speed	(%) r.h.	2,6-DCP dose (ng)	Control	Test	<i>n</i>
15	70	5000	14 \pm 14	19 \pm 25	12
15	90	5000	19 \pm 19	9 \pm 12	10
30	90	5000	7 \pm 9	11 \pm 12	9
15	90	500	9 \pm 12	15 \pm 27	9
30	90	50	9 \pm 8	4 \pm 3	7

Each test consisted of a 60 s blank run followed by 60 s with 2,6-DCP. The tracks were analysed on an IBM compatible computer. Mean displacement of *B. microplus* males (2.5 mm body length) in 0.1 s was relatively low compared to the sphere's base resolution (0.1 mm), leading frequently to inaccurate description of angles associated with displacement segments. Displacement, deviation angle from wind direction and turn angle (difference between deviation angles of successive segments) were calculated instead for each 0.6 s segment (or 100 segments/min). Additionally, records of animals that walked less than 0.5 mm/s for more than 50% of either the test or control period were discarded. The following statistics were calculated for control and test walk of each animal: mean speed (displacement/time), median angular velocity (absolute turn angle/time), circular mean of the deviation angles (Batschelet, 1981), and upwind displacement (sum of all segment lengths with a deviation angle between 60° and -60° upwind, as a percentage of the total displacement). Differences between test and control responses were evaluated with a permutation test on paired replicates.

RESULTS

Electrophysiology of 2,6-dichlorophenol receptive sensilla

The spontaneous activity of cells in the d I 1 and d II 1 sensilla was highly variable and appears to be due to the absence of certain cells in some recordings. Whether this was due to cutting the tip of the sensillum is not clear. A consistent separation of action potentials into different cell classes was not possible. The overall spontaneous activity of olfactory cells in sensillum d I 1 was generally lower and a clear response to stimulation was obtained, whereas responses from the d II 1 were more difficult to analyse (Fig. 1).

Responses were obtained to a range of synthetic 2,6-DCP loads on filter paper from both the d I 1 and the d II 1 sensilla (Fig. 2). Higher doses tended to distort the signal and cause long-lasting excitation, indicating saturation. This effect occurred at lower doses in the d I 1 than in the d II 1. Though increases in global activity of cells from the d I 1 in response to increasing doses of 2,6-DCP tended to be slightly higher, the regression lines do not differ except for the fact that variation was somewhat higher in responses from the d II 1. Olfactory cells in both sensilla responded to five tick equivalents of a total extract of either females or males (Fig. 1).

Recordings from the d II 1 showed responses to 2-nitrophenol in the same dose range as 2,6-DCP (Fig. 1)

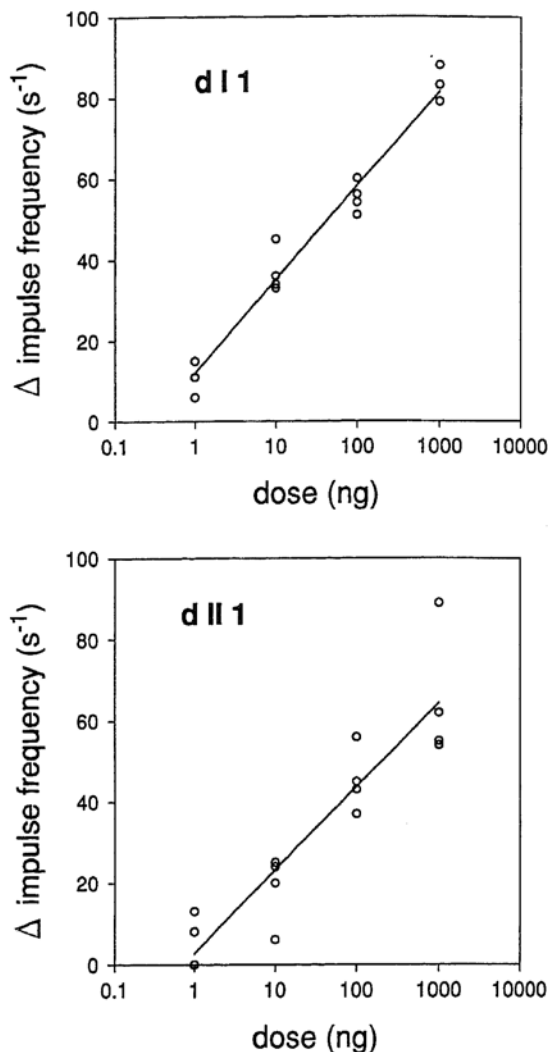


FIGURE 2. Dose-response relations for the combined activity of olfactory cells in two sensilla on the tarsus I of male *B. microplus* ticks (d I 1 and d II 1 of Fig. 1) in response to 2,6-dichlorophenol. Increase in impulse frequency was calculated by subtracting the number of impulses in the second before stimulus delivery from the number of impulses in the second after stimulus arrival. Individual data points are the means of three measurements on each of four ticks, each preparation being exposed to all the doses. Trend lines fitted by linear regression (Sigma Plot, Jandel Scientific, Germany).

and to 4-methylphenol at higher doses (> 100 ng). Both of these products only excited the d I 1 sensory cells at very high doses (> 1000 ng). Both sensilla also responded to 2,6-dibromophenol and 2,6-difluorophenol (> 100 ng).

GC, GC-electrophysiology and GC-MS analysis of extracts

The retention times and elution characteristics on the DB-wax column were determined for various compounds known from ticks such as benzaldehyde, phenol,

FIGURE 3 (Opposite.)

FIGURE 3. Capillary gas chromatography linked single sensillum tip recordings of dichloromethane/hexane extracts of females, males and larvae of *B. microplus*. Separation was done on a 30 m DB-wax fused silica column, temperature programmed from 60°C after 1 min to 200°C at $25^\circ\text{C}/\text{min}$ and to 230°C at $5^\circ\text{C}/\text{min}$ with H_2 carrier gas at 0.5 m/s, ECD detector. Recordings of d.c. and a.c. signals were made from the d I 1 wall-pore single-walled sensillum on the anterior tarsus of a male tick (cf. Fig. 1). Frequency to voltage conversion (time constant 1 s) was applied to the a.c. signal impulses while the d.c. drift was compensated with an automatic base line return (time constant 1 s). Note presence of one chromatographic peak eluting at 213°C which evokes an olfactory response from within this sensillum.

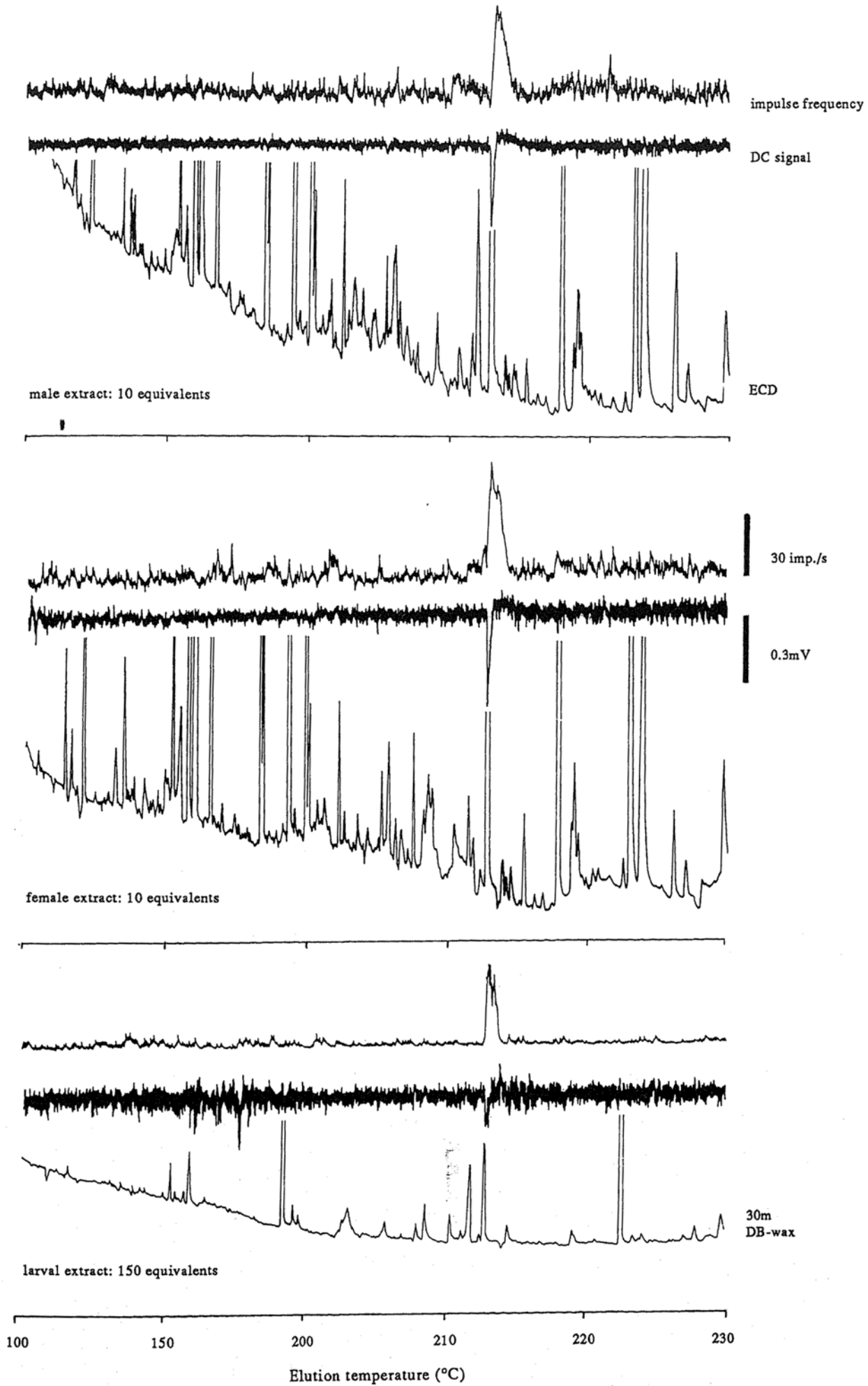


FIGURE 3. (Caption opposite.)

4-methylphenol (*p*-cresol), 2-nitrophenol (*o*-nitrophenol), methyl salicylate and 2,6-DCP, as well as for the related products 2,4-DCP, 2,5-DCP, 2,6-difluorophenol and 2,6-dibromophenol. One peak at the retention time of 2,6-DCP in extracts of *B. microplus* larvae, nymphs and adults (male and female) consistently caused a d.c. potential drop and an increase in spike frequency of receptor cells in the d I 1 or d II 1 sensillum (Fig. 3) (see also Table 1). The characteristically higher response of the ECD compared to that of the FID (not shown here) suggested a halogenated compound. The positional isomers of 2,6-DCP and 2,6-dibromophenol elute later whereas 2,6-difluorophenol elutes earlier than 2,6-DCP on this GC phase. Using either the d I 1 or d II 1 sensillum as biological detectors, no consistent responses have been observed to any other products eluting from the DB-wax column in GC-electrophysiology analysis of extracts of the different life-stages of *B. microplus*.

Identification of 2,6-DCP was based on the match between the mass spectrum of the peak at the retention time of 2,6-DCP in three different extracts and that of the synthetic product. The higher amounts of 2,6-DCP for females over males, as determined by ECD peak integration, can well be ascribed to their higher average body weight (Londt and Arthur, 1975), so that the amounts of 2,6-DCP per gram body weight are approximately the same for both sexes (Table 1). Pharate adult extracts contain this compound in quantities identical to that of engorged nymphs but freshly moulted adults already contain near adult quantities. Larval and nymphal extracts also contain 2,6-DCP but clearly less than in adults. 2,6-DCP was not present in detectable amounts in an extract of eggs (1000 equivalents injected).

Behavioural bioassay on a dummy female

Male *B. microplus* placed on top of the control dummy walked around on it for a brief period while periodically raising their front legs, but left the bead generally within 20 s. Treating the dummy with different concentrations of 2,6-DCP did not increase the total duration of male contact with the dummy, nor did it appear to influence the time spent searching in the arena after leaving the dummy (Fig. 4). Consequent visits to the bead did occur but this was evidently not related to the treatment. When the dummy was coated with a dichloromethane extract of 10 female ticks however, duration of contact was drastically increased (Fig. 4). In addition, the front legs were kept in close contact with the substrate and a behaviour typical of the first stages of mating in this species (Guerin *et al.*, 1992) could be observed with some males even crawling under the dummy. Total duration of search time on the arena was not analysed here since it was considerably reduced by the long stay of the male on the dummy.

Walking behaviour in wind carrying 2,6-dichlorophenol

After the adaptation period on the servosphere most males walked downwind in controls (Fig. 5), though occasional loops and short upwind walks were observed.

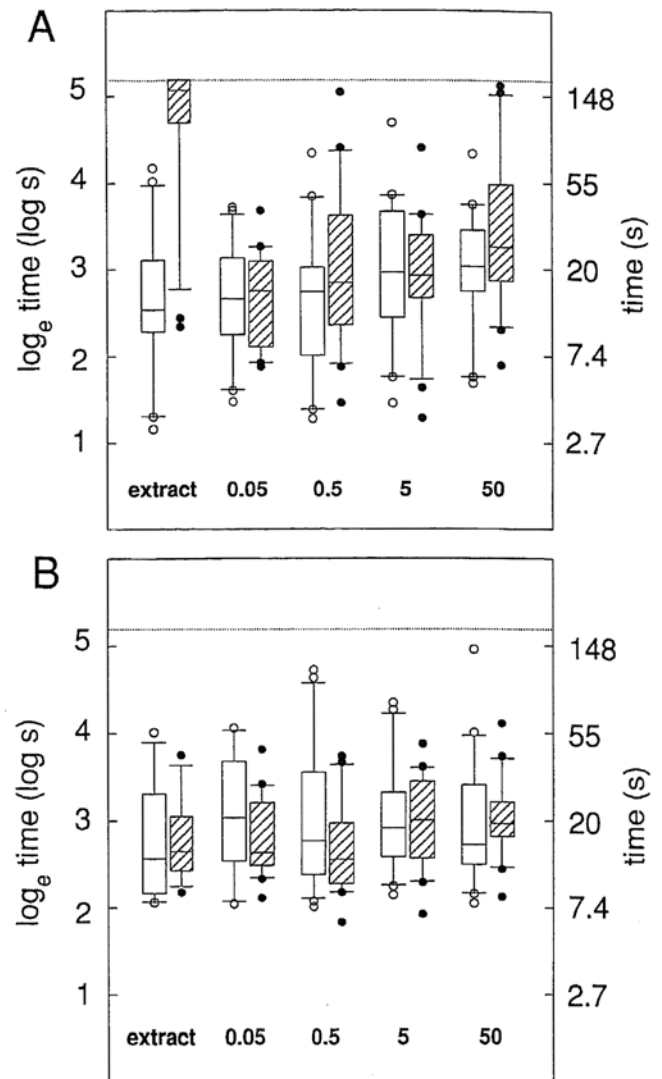


FIGURE 4. Box plots of the time spent by male *B. microplus* on a ca 0.5 mm glass bead (A) and the area around it (B) on a host-simulating arena. Open boxes are controls, hatched boxes are tests; the horizontal lines of each box represent from top to bottom the 90th, 75th, 50th, 25th and 10th percentiles of the data distribution. Other datapoints are those outside the 10th and 90th percentile range (open circles controls, solid circles tests). The horizontal dotted line indicates the maximum time allotted to each tick (i.e. 180 s). Treatments are: a CH_2Cl_2 extract of 10 two-day-old females [$n = 18$, but this is reduced for (B) to display only those ticks that left the experiment within 180 s, $n = 8$], and four doses of 2,6-dichlorophenol indicated in ng/bead ($n = 16$ for each dose).

Males were observed at different wind speeds and humidities, and at three different 2,6-DCP concentrations (Table 2). The distance walked upwind was not influenced by 2,6-DCP in any one of these treatments ($P > 0.05$). In addition, no change in overall walking direction was observed ($P > 0.05$) (Fig. 5). Walking speed was relatively low in most individuals, ranging from 0.6 to 3.4 mm/s, but usually less than one body length/s, and this was not significantly changed by switching on the stimulus ($P > 0.05$) (Fig. 6). Turn angles were normally distributed around zero, indicating no preference for a certain turning direction, and median angular velocities were also not altered by any of the 2,6-DCP concentrations offered ($P > 0.05$) (Fig. 6).

DISCUSSION

A remarkable uniformity seems to exist among metastriate ticks both in the production and perception of 2,6-DCP. In the one-host tick *B. microplus*, investigated here, this compound is extractable from all life-stages except eggs. However, we do not know whether it is also released by all life-stages. It is possible that 2,6-DCP is the phenolic product Chow *et al.*, (1972) could not identify because of the low quantities they obtained in *B. microplus* female extracts. Quantities reported here are low compared to the *ca* 60 ng found in *A. variegatum* and *A. americanum* females (Kellum and Berger, 1977), but compare better to the levels found for *D. variabilis* (Sonenshine *et al.*, 1984). Adult production in *B. microplus* is clearly higher than that of unfed larvae or engorged nymphs but differences between males and females are only marginal. *A. maculatum* Koch and *D. variabilis* males also produce 2,6-DCP in roughly the same quantities as females (Kellum and Berger, 1977; Sonenshine *et al.*, 1984). Presence of 2,6-DCP in extracts of larvae and absence from eggs is also reported for *R. appendiculatus* (McDowell and Waladde, 1986). It would seem therefore that 2,6-DCP is widely present in different life-stages of metastriate ticks.

Production of the aggregation attachment pheromone blend containing 2-nitrophenol and methyl salicylate in

A. hebraeum and *A. variegatum* only starts after feeding has taken place (Diehl *et al.*, 1991). This direct relation with feeding is not valid for the production of 2,6-DCP in *B. microplus* since unfed adults, less than 12 h after moulting, already contain nearly the same quantities of 2,6-DCP as fed adults. Its occurrence in unfed adults has also been reported in other tick species but production commences only several days after the moult (Sonenshine *et al.*, 1982, 1984). Development of *B. microplus* from larva to adult on the same host is relatively fast so production can be considered more or less continuous. An increase in synthesis associated with adults may already start in pharates. That the amount of 2,6-DCP extracted from pharates was comparable to that of engorged nymphs in this study might be due to the inability of the solvent to reach the foveal glands of the adult ticks, still enveloped in nymphal cuticle.

Our results also show that 2,6-DCP evokes responses of sensory cells in two olfactory sensilla on the tarsus of *B. microplus* males, the d I 1 and d II 1, with similar sensitivities. Responses of cells in the two homologous sensilla of *R. appendiculatus* and *A. variegatum* compare well with our results (Waladde, 1982). The increase in spike amplitude with increase in stimulus concentration reported by the latter author is also present in our recordings. Receptors for phenolic compounds seem to be widespread in ticks but olfactory receptor responses

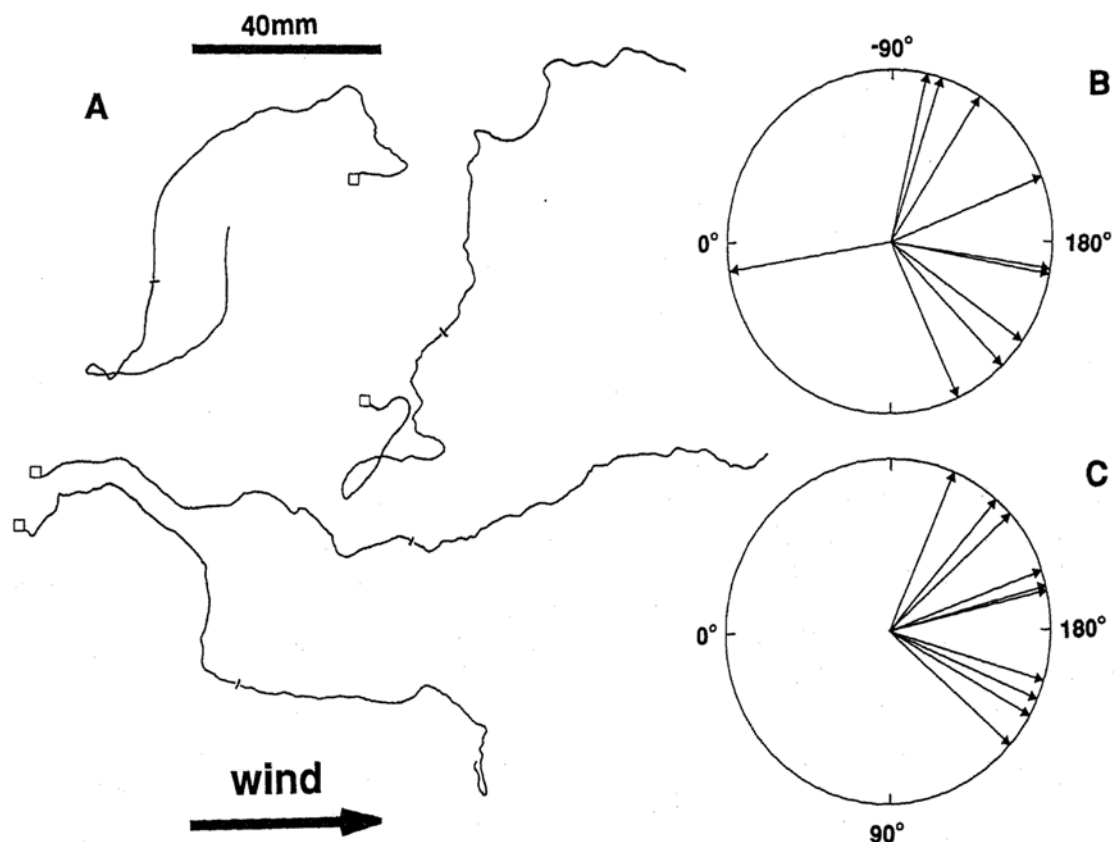


FIGURE 5. Walking behaviour of male *B. microplus* ticks on a locomotion compensator in a constant flow of air (15 cm/s, 90% r.h.). Displacement was recorded during 2 min with a resolution of 0.1 mm. The air over a 5 μ g source of 2,6-dichlorophenol on filter paper under paraffin oil was introduced into the air-stream during the second minute (test period). (A) Four examples of tracks; the starting point is indicated by an open square, the size of a male tick. Cross line indicates start of stimulus delivery. (B) and (C) Scatter diagrams of the circular means of deviation angles (0° is upwind) of 0.6 s samples of the track for control (B) and test (C) period of 10 walks.

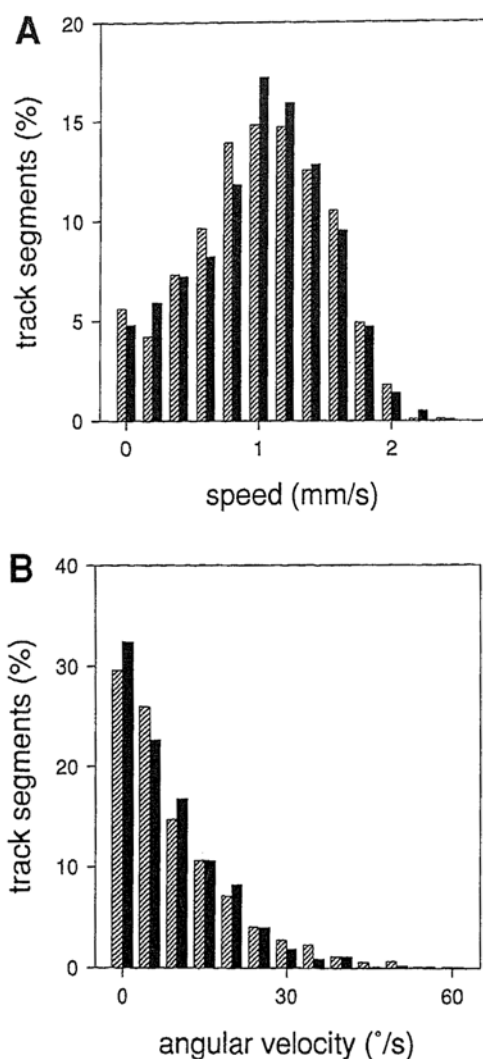


FIGURE 6. Frequency distributions of speed (A) and angular velocity (B) of 0.6 s segments of male *B. microplus* walking tracks on a locomotion compensator. Displacement of ticks was first recorded for 1 min to the conditioned air-stream (15 cm/s, 90% r.h.) with air from a blank stimulus bottle added (controls with hatched bars) followed for a second minute with air over 5 μ g 2,6-dichlorophenol in a stimulus bottle added to the conditioned air-stream (tests with dark bars). Pooled data of walks by 10 males.

do not necessarily imply behavioural activity. Haggart and Davis (1981) for example observed no difference in responses from receptors of male and female *A. americanum* to 2,6-DCP, yet only males show behavioural responses to this product (Kellum and Berger, 1977). Even *Ixodes ricinus* L., a prostrate tick species which does not possess foveal glands and is known to show no oriented responses to 2,6-DCP (Graf, 1975), bears a receptor for this product in the homologous d I 1 sensillum (cf. Guerin *et al.*, 1992). Similarly, the d II 1 sensillum in *B. microplus* showed responses to 2-nitrophenol, a component of the aggregation attachment pheromone of *Amblyomma* spp., but neither this nor indeed any product other than 2,6-DCP was detected by GC coupled electrophysiology in any of the extracts investigated here.

Since 2,6-DCP is both produced and perceived by *B. microplus* a role in the behaviour of this species would seem plausible. However we could not find any evidence

to support this hypothesis in the responses of adult males. Although the total female extract containing 2,6-DCP caused male arrestment on a glass dummy, 2,6-DCP alone on the bead failed to induce this behaviour. It could be argued that 2,6-DCP may function as an attractant rather than an arrestant. As a concentration gradient can be assumed to exist immediately around the treated bead, males leaving the bead would be expected to turn back to higher concentrations of an attractant but this was not observed. It cannot be excluded that 2,6-DCP may play a role in courtship in combination with other tick related compounds but on its own it clearly does not contribute to arrestment of males near or on potential mates.

We also could not demonstrate an oriented response on the part of walking male *B. microplus* to 2,6-DCP in an air-stream on the servosphere. Other tick species, Bruchid beetles and Triatomine bugs do show oriented responses to semiochemicals in the same experimental set-up (Guerin unpublished; Taneja unpublished). The air-stream can apparently be perceived by the ticks since they show an overall downwind walking behaviour, a phenomenon also noted for some of the other arthropods cited above. We therefore conclude that 2,6-DCP does not evoke anemotactic responses in male *B. microplus* ticks guiding them to the stimulus source. Some kind of anemotactic response however, is likely to be involved in the orientation of *A. hebraeum* and *A. variegatum* males to a combined source of CO₂ and 2,6-DCP in the field (Norval *et al.*, 1991) though the role of CO₂ in the blend could be decisive. The doses tested on the locomotion compensator were equivalent to those evoking strong responses in electrophysiology. Unoriented responses such as a change in walking speed or angular velocity (rate of turning) indicating kinetic orientation mechanisms (ortho- and klinokinesis, respectively) were also excluded by our experiments.

Orientation and/or arrestment of other tick species to 2,6-DCP have been demonstrated in off-host experiments with a Petri-dish bioassay (Leahy and Booth, 1983) and a four choice olfactometer (Yunker *et al.*, 1992). The behavioural mechanisms underlying the orientation in these non-discriminating experiments should have been detected in our experiments had they been part of a response to 2,6-DCP by *B. microplus* males. Experiments demonstrating attraction to 2,6-DCP for a number of other tick species with doses of 2,6-DCP applied on hosts (Berger, 1972; Kellum and Berger, 1977; Khalil *et al.*, 1981) include factors such as host odour and certain mechanical stimuli not included in our laboratory experiments. In a single experiment we did aim to register any major influence of these factors on the responses by *B. microplus* to 2,6-DCP. Two rubber septa treated with 1 mg 2,6-DCP and another two with dichloromethane alone (solvent) were stapled on the hips of a young steer. The animal was heavily infested with *B. microplus* males and females, and the dispensers were placed on the 14th day of the infestation—just prior to when fertilization of females begins

(Falk-Vairant *et al.*, 1993). The area around the dispensers was investigated after 24, 48 and 96 h but no newly attached or moving males were observed in the vicinity of the dispensers.

The apparent absence of a behavioural response to 2,6-DCP in males of *B. microplus* is contradictory to conventional knowledge about the role of this product as a pheromone in ticks. Though behavioural responses have been reported in a number of species of metastriate ticks from various genera, no convincing evidence has been presented for any one-host species. The detachment response of male *B. microplus* and *Rhipicephalus sanguineus* reported by Chow *et al.* (1972) to a "phenolic" compound eluting from the GC is not fully convincing since the conditions of the air-stream in which the compound was delivered from the chromatogram to the attached ticks were not described and data on adequate controls was not presented. Moreover, when 2,6-DCP was subsequently identified by Chow *et al.* (1975) in extracts of *R. sanguineus* no further reference was made to *B. microplus*. It could be that males of this species show behavioural responses to 2,6-DCP only during a specific physiological state not present in any of our test males. It can also not be excluded that 2,6-DCP in combination with other volatiles perceived by receptors other than those tested here may play a role in male courtship behaviour. Finally, since all life stages of *B. microplus* succeed each other on the same bovine host, aggregation of larvae and nymphs could account for females normally being in the immediate vicinity when males moult, thus reducing the need for a long range attractant in this species. 2,6-DCP may have an additional function in ticks other than that of sex pheromone.

In conclusion, although 2,6-DCP is produced by *B. microplus* and can be perceived by olfactory receptors in males of this species, we have no evidence that it plays a role in their behaviour. The total extract of females on a glass dummy did evoke a strong arrestment response from males. It is likely therefore that other chemical constituents of this extract play a crucial role in male behaviour.

REFERENCES

- Apps P. J., Viljoen H. W. and Pretorius V. (1988) Aggregation pheromones of the bont tick *Amblyomma hebraeum*: identification of candidates for bioassay. *Onderstepoort J. Vet. Res.* **55**, 135-137.
- Batschelet E. (1981) *Circular Statistics in Biology*. Academic Press, London.
- Berger R. S. (1972) 2,6-Dichlorophenol, sex pheromone of the lone star tick. *Science* **177**, 704-705.
- Berger R. S., Dukes J. C. and Chow Y. S. (1971) Demonstration of a sex pheromone in three species of hard ticks. *J. med. Ent.* **8**, 84-86.
- Bull C. M. and Andrews R. H. (1984) Two different mating signals used by female reptile ticks. In *Acarology VI* (Eds Griffiths D. A. and Bowman C. E.), pp. 427-429. Ellis Horwood, Chichester.
- Byrne K., Gore W. E., Pearce G. T. and Silverstein R. M. (1975) Porapak-Q collection of airborne organic compounds serving as models for insect pheromones. *J. chem. Ecol.* **1**, 1-7.
- Chow Y. S., Lu F. M., Peng C. T. and Cheng P. C. (1972) Isolation of lipids and sex pheromone from hard ticks. *Bull. Inst. Zool. Acad. Sin. (Taipei)* **11**, 1-8.
- Chow Y. S., Wang C. B. and Lin L. C. (1975) Identification of a sex-pheromone of the brown dog tick *Rhipicephalus sanguineus*. *Ann. ent. Soc. Am.* **68**, 485-488.
- Diehl P. A., Guerin P. M., Vlimant M. and Steullet P. (1991) Biosynthesis, production site, and emission rates of aggregation-attachment pheromone in males of two *Amblyomma* ticks. *J. chem. Ecol.* **17**, 833-848.
- Dinnik J. and Zumpt F. (1949) The integumentary sense organs of the larvae of Rhipicephalinae (Acarina). *Psyche (Camb.)* **56**, 1-17.
- Falk-Vairant J., Guerin P. M., de Bruyne M. and Rohrer M. (1994) Some observations on mating and fertilisation in the cattle tick *Boophilus microplus*. *Med. Vet. Ent.* **8**. In press.
- Gladney W. J., Grabbe R. R., Ernst S. E. and Oehler D. D. (1974) The gulf coast tick: evidence of a pheromone produced by males. *J. med. Ent.* **11**, 303-306.
- Gödde J. (1989) Vibrating glass stylets: tools for precise microsurgery on cuticular structures. *J. Neurosci. Meth.* **29**, 77-83.
- Graf J.-F. (1975) Ecologie et éthologie d'*Ixodes ricinus* L. en Suisse (Ixodoidea: Ixodidae), cinquième note: mise en évidence d'une phéromone sexuelle chez *Ixodes ricinus*. *Acarologia* **17**, 436-441.
- Guerin P. M., Steullet P., Kröber T., Diehl P. A., Vlimant M., de Bruyne M., Cordas T., Falk-Vairant J., Kuhert F. and Lösel P. M. (1992) The chemical ecology of ticks at the host vector interface. In *Proc. 1st Int. Conf. Tick-borne Pathogens at the Host-vector Interface: an Agenda for Research* (Eds Munderloh U. G. and Kurtii T. J.), pp. 314-323. Minnesota University, Saint Paul, MN.
- Haggart D. A. and Davis E. E. (1981) Neurons sensitive to 2,6-dichlorophenol on the tarsi of the tick *Amblyomma americanum* (Acari, Ixodidae). *J. Med. Ent.* **18**, 187-193.
- Hess E. and Vlimant M. (1986) Leg sense organs of ticks. In *Morphology, Physiology, and Behavioural Biology of Ticks* (Eds Sauer J. R. and Hair J. A.), pp. 361-390. Ellis Horwood, Chichester.
- Kellum D. and Berger R. S. (1977) Relationship of the occurrence and function of 2,6-dichlorophenol in two species of *Amblyomma*. *J. med. Ent.* **13**, 701-705.
- Khalil G. M., Nada S. A. and Sonenshine D. E. (1981) Sex pheromone regulation of the mating behaviour in the camel tick *Hyalomma dromedarii* (Ixodoidea: Ixodidae). *J. Parasit.* **67**, 70-76.
- Kramer E. (1976) The orientation of walking honeybees in odour fields with small concentration gradients. *Physiol. Ent.* **1**, 27-37.
- Leahy M. G. and Booth K. S. (1983) Attraction of metastriate ticks (Acari: Ixodidae) to sex pheromone 2,6-dichlorophenol and to substituted phenols. *J. med. Ent.* **1**, 104-105.
- Leonovich S. A. (1981) Occurrence of a sex pheromone in the Ixodid tick *Hyalomma asiaticum* (Ixodidae). *Parazitologiya (Leningr.)* **15**, 159-156.
- Londt J. G. H. and Arthur D. R. (1975) The structure and parasitic life cycle of *Boophilus microplus* (Canestrini, 1888) in South Africa (Acarina: Ixodidae). *J. Ent. Soc. S. Afr.* **38**, 321-340.
- McDowell P. G. and Waladde S. M. (1986) 2,6-Dichlorophenol in the tick *Rhipicephalus appendiculatus* Neumann: a reappraisal. *J. chem. Ecol.* **12**, 69-82.
- Noldus L. P. J. J. (1991) The observer: a software system for collection and analysis of observational data. *Behav. Res. Meth. Instrum. Comput.* **23**, 415-429.
- Norval R. A. I., Peter T., Yunker C. E., Sonenshine D. E. and Burridge M. J. (1991) Responses of the ticks *Amblyomma hebraeum* and *A. variegatum* to known or potential components of the aggregation-attachment pheromone. I. Long-range attraction. *Exp. Appl. Acarol.* **13**, 11-18.
- Schöni R., Hess E., Blum W. and Ramstein K. (1984) The aggregation-attachment pheromone of the tropical bont tick *Amblyomma variegatum* Fabricius (Acari: Ixodidae): isolation, identification and action of its components. *J. Insect Physiol.* **30**, 613-618.
- Schulze P. (1942) Die Rückensinnesfelder (Foveae dorsales) der Zecken. *Z. Morph. Oekol. Tiere* **39**, 1-20.
- Smith J. J. B., Mitchell B. K., Rolseth B. M., Whitehead A. T. and Albert P. J. (1990) SAPID tools: microcomputer programs

- for analysis of multi-unit nerve recordings. *Chem. Senses* **15**, 253-270.
- Sonenshine D. E. (1985) Pheromones and other semiochemicals of the Acari. *A. Rev. Ent.* **30**, 1-28.
- Sonenshine D. E., Silverstein R. M., Plummer E. C., West J. R. and McCullough T. (1976) 2,6-Dichlorophenol, the sex pheromone of the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles and the American dog tick, *Dermacentor variabilis* (Say). *J. chem. Ecol.* **2**, 201-209.
- Sonenshine D. E., Gainsburg D. M., Rosenthal M. D. and Silverstein R. M. (1981) The sex pheromone glands of *Dermacentor variabilis* (Say) and *Dermacentor andersoni* Stiles, sex pheromone stored in neutral lipid. *J. chem. Ecol.* **7**, 345-357.
- Sonenshine D. E., Silverstein R. M. and Rechav Y. H. (1982) Tick pheromone mechanisms. In *Physiology of Ticks* (Eds Obenchain F. D. and Galun R. L.), pp. 439-468. Pergamon, Oxford.
- Sonenshine D. E., Silverstein R. M. and West J. R. (1984) Occurrence of the sex attractant pheromone, 2,6-dichlorophenol, in relation to age and feeding in the American dog tick, *Dermacentor variabilis* (Say) (Acari, Ixodidae). *J. chem. Ecol.* **10**, 95-100.
- Waladde S. M. (1982) Tip recording from ixodid tick olfactory sensilla: responses to tick related odours. *J. comp. Physiol.* **148**, 411-418.
- Wood W. F., Leahy M. G., Galun R., Prestwich G. D., Meinwald J., Purnell R. E. and Payne R. C. (1975) Phenols as pheromones of ixodid ticks: a general phenomenon? *J. chem. Ecol.* **1**, 501-509.
- Yunker C. E., Peter T., Norval R. A. I., Sonenshine D. E., Burridge M. J. and Butler J. F. (1992) Olfactory responses of adult *Amblyomma hebraeum* and *A. variegatum* (Acari: Ixodidae) to attractant chemicals in laboratory tests. *Exp. Appl. Acarol.* **13**, 295-301.

Acknowledgements—We are indebted to the Hasselblad, Roche and Sandoz Foundations as well as to the Swiss National Science Foundation (Grant Nos 3.609-0.87 and 31-28684.90), the Ciba-Geigy-Jubilaeums-Stiftung, Schweizerische Mobiliar and the Swiss Office for Education and Science for funding studies on tick sensory physiology at Neuchâtel. We thank Messrs Bouvard, Rohrer, Jonczi and Cesari of the Ciba-Geigy Agricultural Research Station, St. Aubin, Switzerland for supplying us with ticks. We are grateful for the programming expertise of Mr T. Beyens, University of St Etienne, France and of Dr E. Kramer, Max-Planck-Institute, Seewiesen, Germany. We are thankful to Mrs Knutti for taking care of the rabbits and we acknowledge the input from Mr Falk-Vairant in some initial work on this project. This paper is part of the Ph.D. thesis of Marien de Bruyne at the University of Neuchâtel.