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Behavioural and chemoreceptor cell responses of the tick, *Ixodes ricinus*, to its own faeces and faecal constituents

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Abstract. Ticks are ectoparasites of vertebrates and utilize a variety of infochemicals for host finding and acceptance as well as for intraspecific aggregation and mating responses. Individual male and female *Ixodes ricinus*, the vector of Lyme disease in Europe, readily arrest on filter paper strips contaminated with their own faeces. *I. ricinus* also responds, but to a lesser degree, to faeces-contaminated papers enclosed in metal mesh envelopes, i.e. without directly contacting the faeces, suggesting a role for volatiles in the arrestment response. The faecal constituents guanine, xanthine, uric acid and 8-azaguanine (a bacterial breakdown product of guanine) also caused arrestment of individual *I. ricinus* males and females. However, mixtures of these products induced arrestment of *I. ricinus* at doses one hundred fold lower than the lowest active dose of any of them tested singly. Saline extracts of faeces activated receptor cells in terminal pore sensilla on the first leg tarsi of *I. ricinus*. One cell in these sensilla responded in a similar dose dependent manner to guanine and 8-azaguanine, whereas a second cell was more sensitive to lower doses of 8-azaguanine. The response threshold approached 100 fM for both cells. These findings suggest that faeces and faecal breakdown products are implicated in aggregation responses of *I. ricinus*. This may account for the clumped distribution of this ectoparasite on the ground and contribute to the high proportion of mated individuals recorded prior to host colonization.

Introduction

Several tick behaviours are mediated by infochemicals. Electrophysiological and behavioural responses of ticks have been induced by volatiles from vertebrates (Steullet and Guerin 1994a; Osterkamp et al. 1999; McMahon and Guerin 2000). Products sensed on contact can mediate a range of behaviours including host acceptance (Guerin et al. 2000), aggregation between conspecifics either on or off the host (Graf 1975; Leahy et al. 1973; Leahy 1979; Gigon 1985) and mating responses (Hamilton et al. 1994; de Bruyne and Guerin 1998). These chemostimuli are perceived *via* chemoreceptor cells in specialised sensilla borne on appendages: wall-pore sensilla on the first leg tarsi carry receptor cells for the perception of exhaled

products and volatiles from vertebrate hosts (Hess and Vlimant 1986; Steullet and Guerin 1992a, 1992b, 1994a, 1994b), whereas terminal-pore sensilla on both the tarsi and palps possess receptor cells that mediate the perception of contact chemostimuli (Zolotarev and Sinitsina 1965; Waladde and Rice 1982; Guerin et al. 2000).

Ticks readily aggregate as has been documented for soft ticks (Leahy et al. 1973) as well as for both pro- and metastriate spp. (Graf 1978; Leahy et al. 1981). In metastriate ticks that mate on the host, encounters at predilection sites can be mediated by volatile aggregation attachment pheromones (Rechav et al. 1977; Schöni et al. 1984; Diehl et al. 1991; Barré et al. 1998). This facilitates mating (Rechav et al. 1977; Obenchain 1984), and co-feeding helps to overcome host defences (Wang et al. 1998). Off the host, aggregations have a survival value probably by providing a microenvironment in which the individuals forming the mass fare better (Petney and Bull 1981; Lorenzo Figueras et al. 1994; Yoder and Smith 1997). Above all, host colonisation is facilitated when such aggregations occur at sites on the vegetation regularly marked by the scent glands of vertebrate hosts (Rechav et al. 1978). Conspecific faeces has already been implicated in the aggregation responses of ticks (Leahy et al. 1973; Dusbábek et al. 1991a), as indeed in other acari (Egan 1976; Levinson et al. 1991) and insects (McFarlane et al. 1983, 1986; Lorenzo Figueras et al. 1994; Taneja and Guerin 1997). Guanine and related purines are widespread constituents of tick faeces (Hamdy 1977; Dusbábek et al. 1991b) and these products have already been implicated in the assembly responses of both soft ticks (Hassanali et al. 1989; Dusbábek et al. 1991b) and metastriate spp. (Otieno et al. 1985).

Mating in prostriates may occur off the host, so aggregation responses of adult ticks could go some way towards explaining the high proportion of mated individuals collected on vegetation (Graf 1974). Aggregation has already been documented in prostriate *Ixodes* spp. (Treverrow et al. 1977; Graf 1975; Uspensky and Emeliyanova 1980), but the products mediating the responses have not been identified. Here we account for the arrestment of individual male and female *Ixodes ricinus* on their own faeces, on the faecal constituents guanine, xanthine and uric acid, and on 8-azaguanine. In addition, we describe the electrophysiological responses of chemoreceptors in terminal-pore sensilla on the first leg tarsi of *I. ricinus* to these products.

Materials and methods

Ticks

Adult *Ixodes ricinus* (L.) reared at the Institute of Zoology, University of Neuchâtel, were stored in glass vials with polyethylene stoppers at 95% RH in constant darkness at 15 °C. It is important to keep the ticks at a high humidity to obtain good electrophysiological recordings from chemosensilla. Unmated 3–4 month-old

I. ricinus adults were held for 24 hours before behavioural tests at 20 °C, 95% RH and constant darkness in the climate chamber in which the bioassays were made.

Bioassay

A modification of the closed glass Petri dish method (Leahy 1979) was adopted using only one tick per dish (9 cm diam. and 1.5 cm high) at a time. Four filter paper strips (1.5 × 2.5 cm), one treated with the test material and three controls, were placed in each dish. Petri dishes were held in the environmental cabinet for 12 h before the test began and an activated tick was introduced into the middle of each dish. To determine the speed of a response by *I. ricinus* to faeces and faecal washes (below), records were made of arrested ticks on filter papers after 3, 5, 10, 20, 30, 60, and 120 min. In the other tests, records of tick positions were made after 360 min. Most ticks had settled down one hour after starting a bioassay. All handling of ticks and filter paper strips was done with clean forceps to avoid contamination by fingers (Grenacher and Guerin 1994). Tests were repeated between 10 and 46 times with new ticks. Petri dishes were washed between tests. The sum of ticks on the treated filter paper strips versus the sum of ticks on the three control strips was compared using the binomial test (S-Plus); ticks occurring elsewhere in the Petri dishes were ignored.

Biological substrates for behavioural assays with ticks

Filter papers strips were contaminated with *I. ricinus* faeces by placing them in polyethylene capped glass vials (4 cm h., 2 cm o.d.) containing ten male and ten female *I. ricinus* of both sexes for between 1 and 6 months after moulting (termed culture-vial filter papers below). To transfer faeces to test filter paper strips, faeces from 13 contaminated filter papers held for 1 month in the company of ten male and female *I. ricinus* was removed by mixing the contaminated strips in a 0,9% NaCl solution (nanopure water, 1 strip/ml) for 5 min. Test filter paper strips were dipped in this solution for a few seconds and left to dry at room temperature. Controls were dipped in a 0,9% NaCl solution.

To test synthetic chemicals (all $\geqslant$ 98% pure), guanine and NH_4Cl (Merck), 8-azaguanine (2-amino-6-hydroxy-8-azapurine; Aldrich), xanthine and uric acid (Fluka), and urea (Sigma) were emulsified by sonication in 0,9% aqueous NaCl or dissolved in nanopure water and 20–40 μl of solutions of different doses were applied with a disposable micropipette to the test filter paper strips. Controls were treated correspondingly with 0.9% aqueous NaCl or nanopure water.

To test for responses to possible vapours from faeces, faeces-contaminated filter paper strips were wetted with 3 drops of nanopure water. (Wetting may allow ammonium salts present on faeces-contaminated filter papers to form gaseous ammonia or facilitate the release of NH_3 through microbial action.) These strips were enclosed in a bronze envelope (2.5 × 1.5 cm, 0.5 mm thick bronze, 0.4 mm mesh) to prohibit contact by ticks with the test substrate. The enclosed faeces-contami-

nated filter paper strips were tested in the Petri dish assay as above against controls in similar bronze envelopes to which water only had been applied.

Analysis of tick faecal constituents

To analyse for guanine in samples of *I. ricinus* faeces by gas chromatography linked mass spectrometry (GC-MS, below), faecal samples and guanine were derivatized. Faeces samples were obtained by removing the white deposits visible on 3 faeces-contaminated filter paper strips that had been enclosed with 10 engorged nymphs moulting to adulthood for 1 month. The filter papers were dipped for 1 min in 3 ml 1N HCl in a 10 ml conical flask. This solution was concentrated under a stream of N_2 to 100 μl and transferred to a 1 ml tapered vial (Chromacol, U.K.). It was brought to dryness under N_2 and 10 μl N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, puriss Fluka) were added to the white faecal deposit; 10 μl MSTFA were added to 1 μg synthetic guanine in a separate vial. The mixtures were sealed for 1h sealed under N_2 at room temperature. [In this manner, all products with hydrogen on a polar atom such as alcohols, acids and amines are derivatized as trimethylsilyl esters, and these less polar products are more efficiently resolved by capillary gas chromatography (below)]. The samples were again brought to dryness under N_2 and collected from the tapered 1 ml vials in 2 μl $CHCl_3$ using a 10 μl syringe for GC-MS analysis. This was done in a Hewlett Packard 5890 series chromatograph linked to a HP 5971A mass selective detector. The derivatized sample was immediately injected on-column to a DB-5HT non-polar high resolution fused-silica capillary column (15 m, 0.25 mm i.d., 0.1 μm film thickness; J. & W. Scientific, Ca., U.S.A.) equipped with 1 m deactivated fused-silica as pre-column and connected via 1 m of the same deactivated capillary to the MS (ionisation chamber temperature 180 °C; ionisation energy 70 eV). The detector, operating in the EI mode, scanned *m/z* 30 to 650. Helium was used as carrier gas under constant flow (velocity 40 cm/s at 60 °C). Identification was by comparing the retention times of the analytes with standards, and by comparing the mass spectra of unknowns with those of known synthetics in the computer-based library of the GC-MS associated HP Chemstation (Wiley version C.01.05).

Faeces recovered as above from filter paper strips contaminated by adult male or female *I. ricinus* and 100 ng guanine as reference were dissolved separately in 1N HCl and analysed with a UV/VIS spectrophotometer (UVIKON 810) from 200 to 320 nm.

A potassium tetraiodomercurate (II) based colorimetric test (Merck) with a detection limit of 0.05 ppm/filter paper strip was used to assay for ammonia in *I. ricinus* faecal deposits. For this, filter paper strips were held for six months in 20 culture vials (above) with 25 newly moulted adult females per vial. The electrophysiological response of olfactory receptors on antennae of *Triatoma infestans* nymphs (Taneja and Guerin 1997) was also used to assay for ammonia in *I. ricinus* faeces deposits. Ten wetted faeces-contaminated filter paper strips from the culture vials (above) were tested for presence of ammonia by passing charcoal-filtered air over

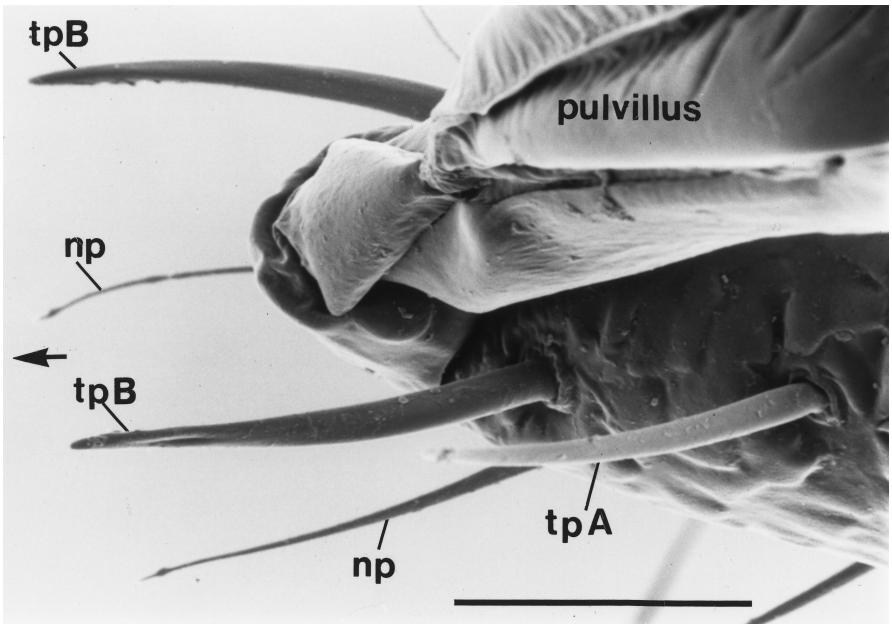

Figure 1. External dorsolateral view by SEM of the left first-leg tarsus tip of *Ixodes ricinus* with the pulvillus folded dorsally (arrow points distally). The micrograph shows paired tpA (second tpA obscured by the tarsus), tpB, and a no pore (np) sensilla. Responses to purines and 8-azaguanine were recorded from the tpB sensilla, scale bar 50 μm.

them in a 5-ml polypropylene syringe that served as stimulus cartridges for the electrophysiological assay on *T. infestans* (Taneja and Guerin 1997).

Electron microscopy

I. ricinus adults were fixed in 70% EtOH, dehydrated with 70% to 100% acetone, critical-point-dried in CO_2, whole mounted on a stub, coated with gold in a sputtering apparatus and observed under a Philips XL 20 scanning electron microscope (SEM).

Electrophysiology

The responses of chemoreceptors in terminal pore (tp) sensilla on first leg tarsi of *I. ricinus* (Figure 1) to test substances were made using the method first used by Hodgson et al. (1955) to record from contact chemosensilla of flies. This consists of placing a glass electrode (1.5 mm o.d.), the drawn-out tip of which contains a solution of the test substance, over the extremity of the contact chemosensillum. For this, male *I. ricinus* lay attached by the venter on double-sided adhesive tape on a Plexiglas® stub with the first pair of legs outstretched. In this manner the tips of the tarsi were clear of any obstacle to facilitate contact with the recording elec-

trode. The stub was mounted on plasticine to facilitate orientation of the preparation under the microscope for recordings. Preparations were mounted under a combistereo microscope (M3Z, Wild, Switzerland) with a working distance of 10 mm at 800-fold magnification. The preparation was maintained at 20 °C and 90% RH in a conditioned air stream (Steullet and Guerin 1992b).

A glass reference electrode (2 mm o.d., 10 μm tip diam.) filled with 10 mM KCl and connected *via* a chloridized silver wire to ground was placed with a Leitz micromanipulator on the coxa-trochanter joint of the first leg from which recordings were made. All organic stimuli were dissolved in either 100 mM KCl or 100 mM NaCl with 1% ethanol, except for the inorganic salts (only 1% ethanol added). The salts were all $\geqslant$ 99% pure: NaCl and LiCl (Fluka, p.a.), RbCl (Sigma, pure) and KCl (Merck, extra pure). Faecal extracts and dilutions thereof were made as above in 0,9% NaCl. The recording electrode with a 10 μm tip diam. was dipped in the stimulus solution, back-filled with electrolyte, and immediately brought into contact with the contact chemosensillum under test. This electrode was connected via a chlorinated silver wire to a high impedance non-blocking preamplifier (Syntech, The Netherlands) mounted on a second Leitz micromanipulator. AC signals were amplified (500x) with a universal AC/DC amplifier (UN-03, Syntech) and recorded via a DAS 16 analogue to digital card (12 bit, 10 KHz, Metrabyte Corp., USA) to a PC equipped with the spike analysis software SAPID (version 16.0; Smith et al. 1990). Spikes were analysed from the first 500 ms of signal obtained 50 ms after contact. From each preparation, two to three responses for each of a variable number of stimuli were recorded with at least 20 s between stimulations. Repeated stimulations with the same stimulus were made in succession to control for reproducibility (van Loon 1990). Electrolyte alone was applied to the sensillum tip between treatments to check for any residual test material on the sensillum. When this was observed, the sensillum tip was bathed in a succession of electrodes containing electrolyte only. Contact time between the test solution and the sensillum was kept as short as possible (ca. 1 s). Individual dose effects on spike frequency were compared with their respective controls using the Mann-Whitney U test. Responses as a function of dose were subjected to the nonlinear least squares regression (see Figure 5).

Results

Identification of I. ricinus *faecal constituents*

Guanine was identified as a constituent of *I. ricinus* faeces by GC-MS and spectrophotometry. The most abundant product identified in the extract of the *I. ricinus* faecal extract analysed by GC-MS was urea followed by guanine, 12-hydroxyoctadecanoic acid and cholesterol (Figure 2). The retention time of derivatized synthetic guanine matched that of the product with the same mass spectrum in the faecal extract. The adsorption spectrum of the faecal deposits recovered from male

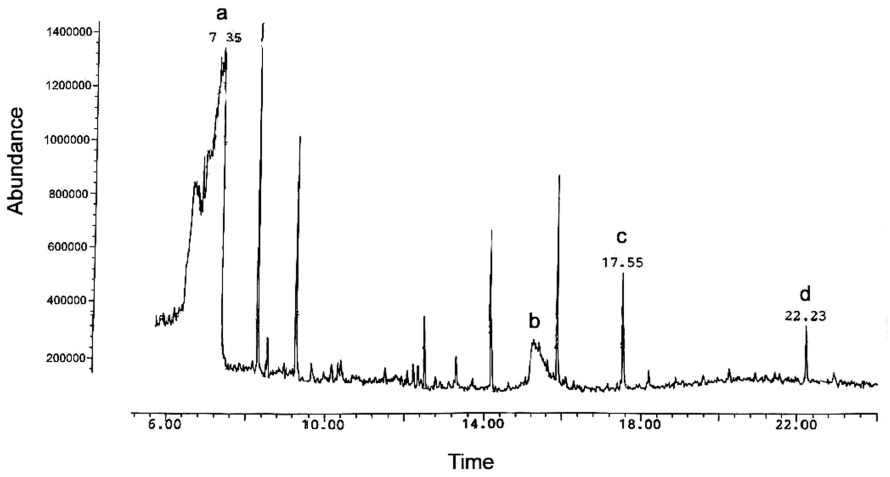

Figure 2. Gas chromatography linked mass spectrometric analysis of an N-methyl-N-trimethylsilyltrifluoroacetamide derivatized extract of *Ixodes ricinus* faeces. Trimethylsilyl (TMS) ester peaks identified were urea-bis-TMS (a), guanine-tri-TMS (b), 12-hydroxyoctadecanoic acid-bis-TMS (c) and cholesterol-TMS (d).

and female *I. ricinus* corresponded to that of guanine (Figure 3). Ammonia was identified from faeces-contaminated filter paper strips by colorimetry, indicating a mean amount of 0.03 ppm/filter paper strip ($n = 20$). This was confirmed when vapours from wet faeces-contaminated filter papers evoked a response from the NH_3 receptors on the antenna of fifth instar *T. infestans* nymphs.

I. ricinus *arrestment on conspecific faeces, purines and 8-azaguanine*

Individual male and female *I. ricinus* showed significant arrestment on conspecific faeces in the Petri dish assay after 20 min, a response that increased with time (Table 1). Arrestment on older (10 months) faeces-contaminated filter paper strips was not so fast (9 on test papers, 11 on control papers, after 60 min; $P < 0.01$). In the assay where *I. ricinus* was deprived of contact with the faeces-contaminated filter paper strips by enclosing them in bronze envelopes, there was no significant response by *I. ricinus* males ($n = 15$) in the first hours of the assay, although arrestment was noted after 24 hours (7 on test papers, 8 on control papers, $P < 0.05$). The arrestment stimulus associated with faeces-contaminated filter papers could be transferred to new filter paper strips by dipping them in the 0.9% NaCl solution in which the tick-contaminated strips had been steeped (Table 1).

Guanine caused arrestment of both male and female ticks on treated filter papers at a range of doses at and above 10 μg, but lost activity at 1 μg (Table 2). Doses higher than 1 mg of guanine were not tested due to the difficulty of properly dissolving such amounts of the product in water. 8-Azaguanine caused arrestment of male and female *I. ricinus* at 1 μg on the filter paper strips, but lost activity at a dose ten times lower. Xanthine was active only at 10 μg (Table 2), but biological

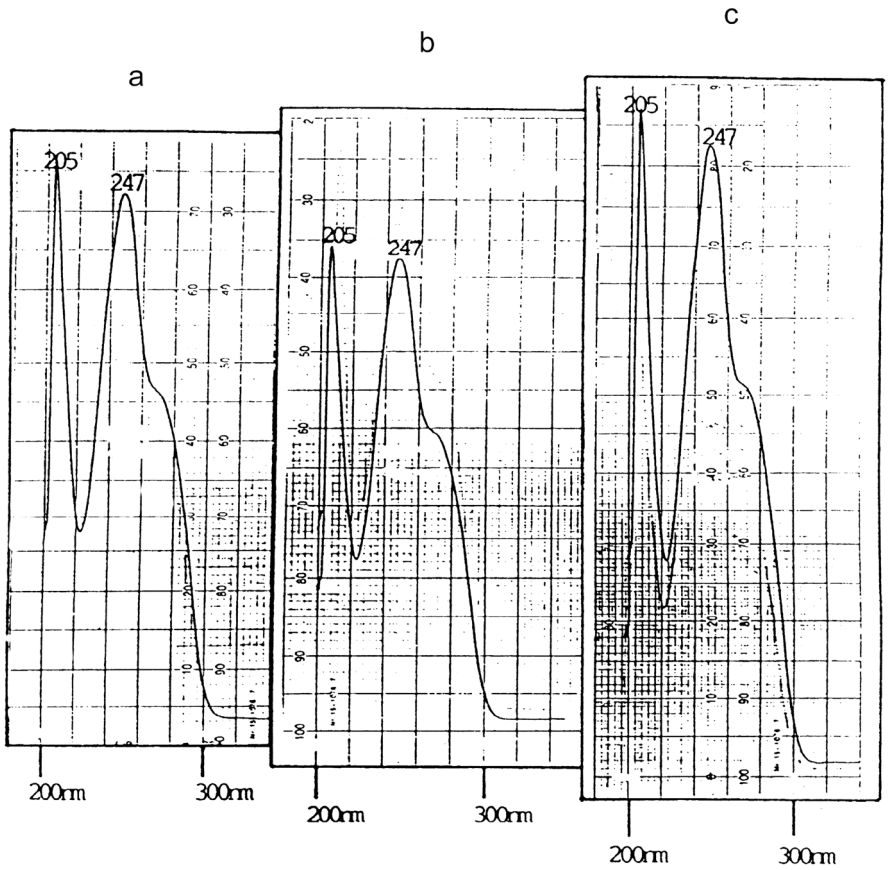

Figure 3. Absorption spectra of faecal deposits of female (a) and male (b) *Ixodes ricinus*, compared to that of guanine (c). All three solutions (see text) showed an absorption maximum at 247 nm, typical of guanine.

activity of guanine was restored when combined with xanthine at a dose where neither compound was any longer active on its own (1 μg and 100 ng, respectively). Uric acid acted as an arrestment stimulus on its own for both sexes of *I. ricinus* at 1 μg, but did not increase activity of the guanine:xanthine mixture of 1 μg:100 ng at this dose (Table 2). A mixture of the three most active compounds, i.e. 8-azaguanine, xanthine and uric acid induced arrestment by males at 10^{-5}:10^{-4}:10^{-5} mg, respectively. Females reacted only at a ten times higher dose of this mixture. Urea evoked no behavioural response at a range of doses.

I. ricinus *contact chemosensilla on first leg tarsi*

Ten terminal-pore contact chemosensilla occur on the first-leg tarsi of *I. ricinus* (Figure 1). According to their ultrastructure, these sensilla are classified as type A

Table 1. Arrestment of male and female *Ixodes ricinus* on filter paper strips treated with faeces of conspecifics as a function of time. Test filter paper strips were contaminated with faeces by either enclosing them in glass vials with freshly moulted adult *I. ricinus* for one month or by application of a faecal wash. Arrestment on a test strip was compared, using the responses of individual ticks, to three control strips in a Petri dish assay (see Materials and methods).

Sex tested	Test material	Time (min)							No. of ticks on test and controls at 120min
		3	5	10	20	30	60	120	
Male	Faeces	4*	4*	6**	11***	15***	16***	18***	22
Female	Faeces	2	3*	3	7**	11***	17***	16***	22
Male	Faecal wash	1	1	3	3	7	13**	14**	32

* = P < 0.05, ** = P < 0.01, *** = P < 0.001

or B (Hess and Vlimant 1986; Thonney 1987). The 8 tpA contact chemosensilla have an external and internal lymph cavity, the latter enclosing the unbranched dendrites of the sensory cells that extend to the tp. The 2 tpB contact chemosensilla at the tip of the tarsus possess a single lymph cavity that is contiguous with the terminal pore and encloses the dendrites of the sensory cells (Guerin et al. 2000). In addition, both types of tp sensilla may possess mechanosensory units at the base of the shaft. Pairs of tpA and tpB sensilla point ventrally at the tips of the first leg tarsi and come into contact with the substrate as *I. ricinus* walks (Figure 1; Kröber and Guerin 1999). Sensory cells in tpA sensilla on the tarsus of *I. ricinus* number between 3–4, whereas the tpB sensilla always have 4 units (Thonney 1987). In this study electrophysiological recordings could only be made from tpB sensilla.

Electrophysiological responses of contact chemosensilla

An *I. ricinus* faecal wash in 0,9% NaCl evoked a response from a receptor cell (no. 1) characterised by high amplitude action potentials in the tpB sensilla of the tarsi (Figure 4). Whereas the major part of the electrophysiological recordings were made with males, responses of females were similar (n = between 4 and 15 for the different treatments tested on females). Guanine also evoked a response from this receptor when presented in the same electrolyte (Figure 4). The guanine-sensitive cell fired at a frequency that was dose dependent with a maximum firing rate at 10 pM (Figure 5). The threshold for the response approached 100 fM, and responses to doses higher than 1 mM were not different from the control. Although cell 1 still fired when guanine was presented in KCl as electrolyte (Figure 6), presence of KCl did modify the response of this receptor to guanine. No response was observed below to 1 mM guanine in 100 mM KCl. Furthermore, the response frequencies recorded at 10 mM guanine in KCl were significantly higher (median frequency of 11 spikes/500 ms, n = 51 sensilla) than those recorded with similar concentrations of guanine in NaCl (median frequency of 0.5 spikes/500 ms, n = 18; P < 0.001). 8-Azaguanine also evoked a response from receptor cell 1 at concentrations be-

Table 2. Arrestment effect of purines from tick faeces and the purine analogue 8-azaguanine on *Ixodes ricinus*. A test filter paper strip was treated with either a product or a mixture. Arrestment on a test strip was compared, using the responses of individual ticks, to three control strips in a Petri dish assay (see Materials and methods).

Sex	Substance(s)	Amount(s) mg	Ticks on treated filter paper	Total No. of ticks on test and controls after 360 min
Female	Guanine°	1	6*	12
Male	Guanine°	1	10**	18
Female	Guanine°	10^{-1}	14*	34
Male	Guanine°	10^{-1}	13***	19
Female	Guanine°	10^{-2}	10*	22
Female	Guanine	10^{-2}	19*	46
Male	Guanine	10^{-2}	14*	33
Female	8-Azaguanine	10^{-3}	11**	18
Male	8-Azaguanine	10^{-3}	10*	15
Female	Xanthine	10^{-2}	15**	32
Male	Xanthine	10^{-2}	15***	28
Female	Uric acid	10^{-3}	19***	58
Male	Uric acid	10^{-3}	14*	34
Female	Guanine: xanthine	10^{-3}:10^{-4}	21***	42
Male	Guanine: xanthine	10^{-3}:10^{-4}	13	39
Female	Guanine: xanthine: uric acid	10^{-3}:10^{-4}:10^{-3}	15**	29
Male	Guanine: xanthine: uric acid	10^{-3}:10^{-4}:10^{-3}	14*	34
Female	8-Azaguanine: xanthine: uric acid	10^{-4}:10^{-3}:10^{-4}	16***	19
Male	8-Azaguanine: xanthine: uric acid	10^{-4}:10^{-3}:10^{-4}	16***	20
Male	8-Azaguanine: xanthine: uric acid	10^{-5}:10^{-4}:10^{-5}	10**	17
Female	8-Azaguanine: xanthine: uric acid	10^{-5}:10^{-4}:10^{-5}	4	17

Guanine inactive at 10^{-3} mg; 8-azaguanine inactive at 10^{-4} mg; xanthine inactive at 10^{-1}, 10^{-3} and 10^{-4} mg; uric acid inactive at 10^{-4} mg; guanine: xanthine inactive at 1: 10^{-1} mg and 10^{-1}: 10^{-2} mg; 8-azaguanine: xanthine: uric acid inactive for males at 10^{-6}: 10^{-5}: 10^{-6} mg. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, unilateral binomial test; ° in 0.9% NaCl, otherwise in nanopure water.

tween 100 fM and 100 nM (Figure 5). The dose response pattern indicated an effective concentration range similar to that of guanine. Receptor cell 1 also responded to uric acid in 100 mM NaCl at concentrations between 10 nM to 10 mM, with a maximum at 1 mM (Figure 5). Uric acid did not significantly affect the activity of cell 2. Evidence was obtained to suggest that both guanine and uric acid affected receptor cell 1 in the tpB sensilla since a combination of 100 nM guanine and 100 (μM uric acid did not activate cells with other spike amplitudes ($n = 20$). Xanthine only evoked a response from receptor cell 1 at the higher dose of 1 mM ($P \leqslant 0.05$).

A second receptor cell (no. 2) in the tpB sensilla characterised by small amplitude action potentials was activated by purines, 8-azaguanine and KCl, and to a

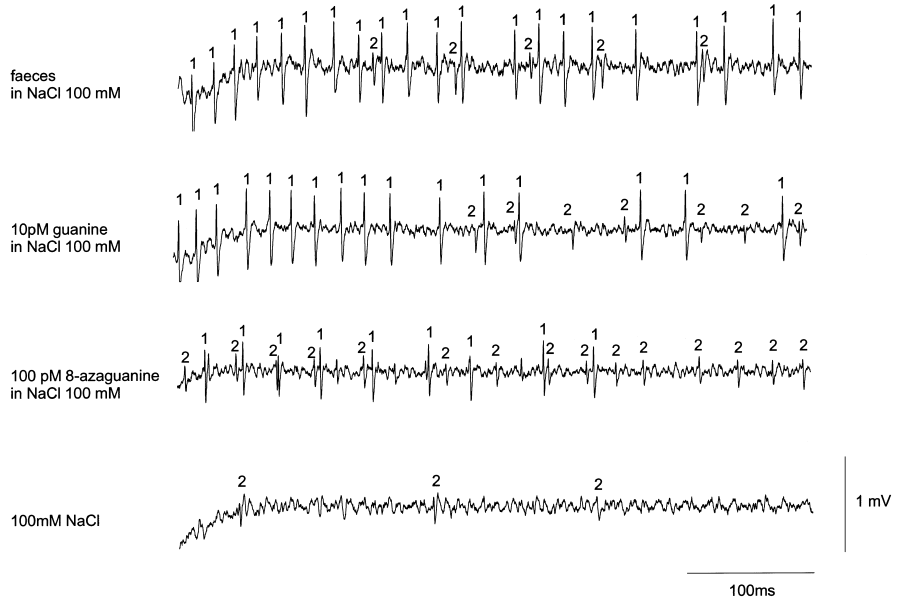

Figure 4. Electrophysiological responses of contact chemoreceptors in a terminal-pore B contact chemosensillum (see Figure 1 and text) on a male first leg tarsus of *Ixodes ricinus* to a saline wash of conspecific faeces diluted ten times (see text), to guanine and to 8-azaguanine all in 100 mM NaCl as electrolyte. Cell 1 is characterized by a high amplitude and cell 2 by a small amplitude spike. Cell 1 responds strongly to the faecal wash, but cell 2 only weakly. Guanine and 8-azaguanine evoke responses from both cells. Cell 2 also responds to KCl and RbCl in a dose dependent manner (see Figure 6 and Figure 7). Neither cell responds to NaCl.

lesser extent by the *I. ricinus* faeces wash (Figures 4 and 6). Guanine activated this cell over the concentration range 10 pM to 1 mM (Figure 5) but not at concentrations higher than 1 mM. The highest increase in frequency was recorded to 8-azaguanine at a dose of 1 nM, with a threshold at 100fM (Figure 5). Xanthine and uric acid only significantly affected spike frequency of cell 2 at the higher doses of 1 mM and 10 mM, respectively. A 10^{-1} and 10^{-2} times diluted *I. ricinus* faecal wash in saline served to consistently increase spike frequency of receptor cell 2 (increase of 4 and 2 spikes over the control values, $n = 26$ and 20 sensilla, $P < 0.001$ and $P < 0.05$ respectively; Figure 4).

Receptor cell 1 in the tpB sensilla was not activated by NaCl, nor by any other salt over a range of doses (Figure 4). Receptor cell 2 responded in a dose dependent manner to KCl (slope $a = 6{,}69$, $r = 0{,}69$, $P < 0.001$; Figure 7) and to RbCl ($a = 7$, $r = 0{,}79$, $P < 0{,}001$; Figure 7) but with an overall lower response to the latter salt. Neither NaCl nor LiCl significantly affected spike frequency of this cell (Figure 7), hence the choice of NaCl as electrolyte for treatments (above). The response of cell 2 to KCl was inhibited by the presence of guanine (median frequency of 16 spikes/500 ms to 100 mM KCl, $n = 16$, compared to a median frequency of 11spikes/500

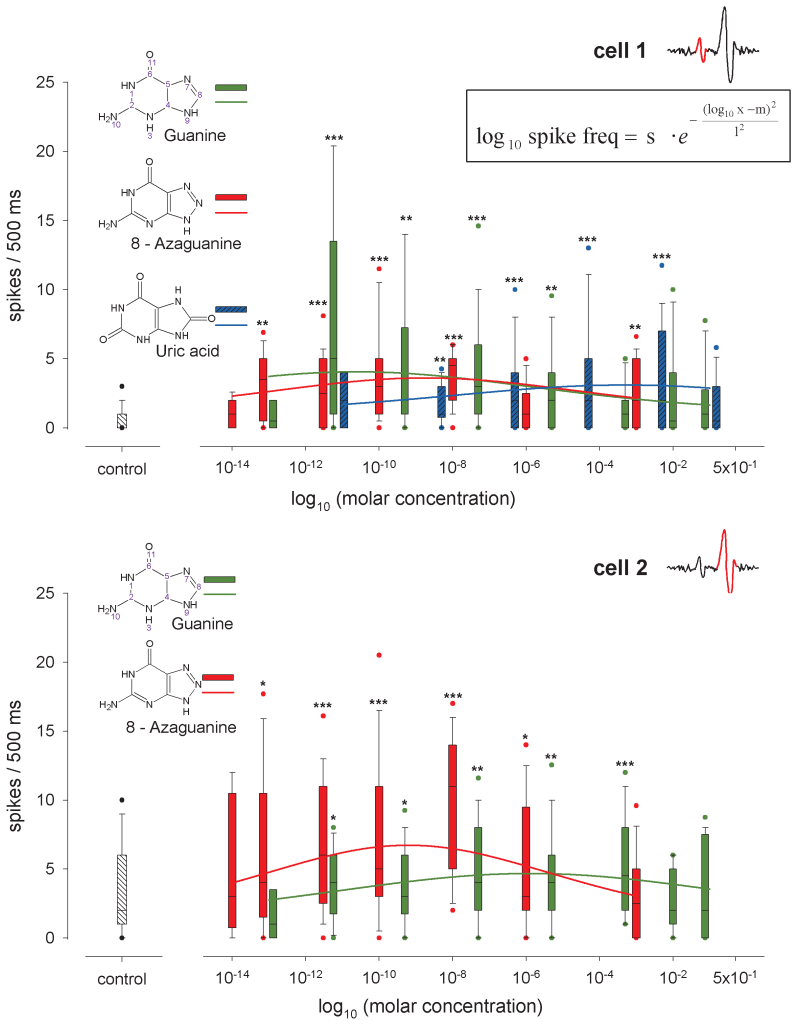

Figure 5. Electrophysiological responses of contact chemoreceptors in a tpB sensilla on the first leg tarsus tip of *Ixodes ricinus* males to purines and to a purine analogue 8-azaguanine. (A) Guanine (green), 8-azaguanine (red) and uric acid (blue) modify the spike frequency of cell 1 characterised by a high amplitude spike (see also Figure 4). No significant difference between the response pattern of this cell to guanine and azaguanine was detected under three parameters, i.e. maximal effective concentration m, maximal response evoked s, and spread l (see equation in box) of the fitted nonlinear least squares regression. The concentration causing maximal response for uric acid is 10^6 times higher than for guanine and 8-azagunanine ($P \leqslant 0.05$, method of double S.D.). (B) 8-Azaguanine (red) and guanine (green) stimulate cell 2 characterised by a low amplitude spike (see also Figure 4), but this cell did not respond to uric acid. Sensitivity for 8-azaguanine was clearly higher: the most effective concentration for 8-azaguanine was 10^{-4} times lower than for guanine. The limits of the boxes indicate the twenty-fifth and seventy-fifth percentiles, the solid line in the box is the median, the capped bars indicate the tenth and the ninetieth percentiles and the fifth and ninety-fifth percentile are plotted as circles. A significant difference in spike frequencies between test and control at a given concentration is marked above the box: U-test, *$P<0.05$, **$P<0.01$, ***$P<0.001$; $n=4$ to 118 sensilla per treatment. Data at 10^{-13} M, $5{\cdot}10^{-12}$ M and 0.1 M are displaced slightly for clarity.

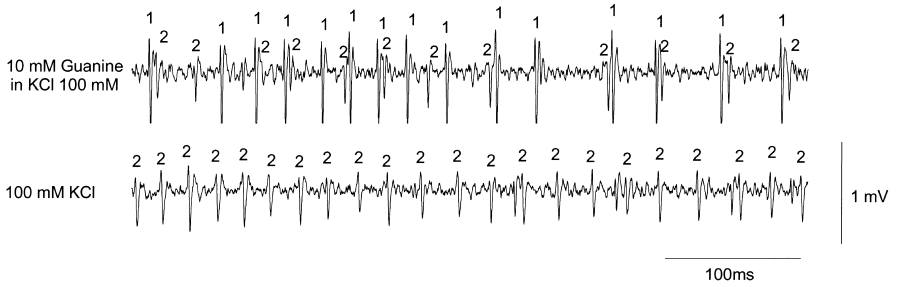

Figure 6. Electrophysiological response of receptors in a tpB contact chemosensillum on the first leg tarsus of *Ixodes ricinus* to 10 mM guanine in 100 mM KCl. Cell 1 responds in a dose dependent manner to guanine, whereas cell 2 responds in a dose dependent manner to both guanine (see also Figure 5) and KCl (see also Figure 7).

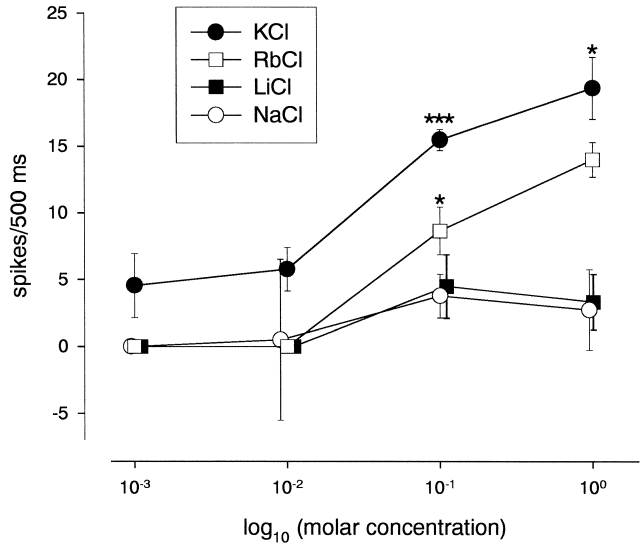

Figure 7. Electrophysiological responses of receptor cell 2 in terminal pore B contact chemosensilla on first leg tarsi of *Ixodes ricinus* to increasing doses of four monovalents cations of chloride (n = 4 to 116 sensilla; * $P < 0.05$, *** $P < 0.001$).

ms to 10 mM guanine in 100 mM KCl, n = 51, P = 0; Figure 6). Adequate stimuli for the other two receptor cells in the tpB sensilla were not identified in this study.

Discussion

This report provides evidence for a role of conspecific faeces as an arrestment stimulus for *I. ricinus* and characterises contact chemoreceptors in terminal pore sensilla on the tarsi of this species for the perception of faecal constituents and a related product, 8-azaguanine. Male and female *I. ricinus* respond individually to

faeces from freshly moulted adult ticks of both sexes in behavioural assays, and faeces-contaminated filter papers still caused arrestment after 10 months. The polar nature of the active faecal constituent(s) is indicated by the fact that the biological activity for behaviour and electrophysiological assays could be transferred in ionised water.

The polar product guanine was identified here as a constituent of *I. ricinus* faeces as collected from engorged nymphs moulting to adulthood that had passed all stages of digestion (Tatchell 1964) in contact with the filter papers. Guanine caused arrestment of adults of both sexes on guanine-treated filter paper strips. This complements the established role of guanine in the assembly responses of a range of other soft and hard tick spp. (Leahy et al. 1973; Leahy 1979; Dusbábek et al. 1991b). The threshold for the arresting effect of guanine on *I. ricinus* was found here to be 2.7×10^{-3} mg per cm^2, compared to 1.21×10^{-6} mg/cm^2(8×10^{-12} M/cm^2) for *Argas persicus* (Otieno et al. 1985) and 0.28 mg/cm^2 in five argasid species (Dusbábek et al. 1991b). Guanine was presented on filter paper in all these cases. The behavioural threshold for guanine of *I. ricinus* established here is below the amount of about 1 mg found by GC-MS on individual faeces-contaminated filter paper strips. The electrophysiology data indicate responses at levels below 10 pM (1.5 pg/ml). However, there was quite some variation in the response at the concentrations evoking maximal electrophysiological activity for guanine and 8-azaguanine. This may be explained by the fact that guanine, like other purines with a secondary amine on a carbon adjacent a carbon with a ketone group, can be present in two forms in aqueous solution, *i.e*. the enol and keto forms. Nevertheless, the low threshold indicated for guanine is not surprising since it is only one of a series of constituents contributing to the arrestment effect of faeces.

I. ricinus showed a lower behavioural and electrophysiological threshold for 8-azaguanine than for any other of the products tested in electrophysiological and behavioural assays. This product also contributed to the arrestment effect of purine mixtures tested. 8-Azaguanine is a bacterial breakdown product of guanine with an extra nitrogen in position 8 (Hirasawa and Isono 1978). Dusbábek et al. (1998) already accounted for microbial conversion of purines into uric acid in faecal deposits of *Argas walkerae* and in a mixture of the associated products guanine, xanthine and hypoxanthine in saline.

Other purines contributing to arrestment of *I*. ricinus in our behaviour assays, i.e. xanthine and uric acid, have already been identified as faecal constituents of argasid and metastriate ticks (Otieno et al. 1985; Dusbábek et al. 1991b) and cause aggregation in argasid and ixodid spp. (Otieno et al. 1985; Dusbábek et al. 1991b). In this regard, xanthine and uric acid may well have been present in our faecal extracts as analysed by GC-MS but were present below the detection limit of the detector. Dusbábek et al. (1991b) reported uric acid and xanthine from trace levels to 0.1–5% and 8.9%, respectively, in the excretion of *A. persicus*. Uric acid is shown here to activate receptor cell 1 in *I. ricinus* tpB tarsal sensilla only at concentrations 10^6 times higher than for guanine and 8-azaguanine. However, uric acid caused arrestment of *I. ricinus* at doses below that effective for guanine. It is possible that a receptor sensitive to such doses of uric acid reside elsewhere, such as in

palp sensilla. Likewise, xanthine only activated receptor cell 2 at the higher dose of 1 mM ($p < 0.001$, $n = 15$), but xanthine restored biological activity in a mixture when guanine was presented at too low a dose. Mixtures of these products caused arrestment of *I. ricinus* at doses one hundred times lower than the lowest active dose of any of them tested singly.

I. ricinus responded faster to faeces-contaminated papers (20 min) and to faecal washes applied to filter paper (60 min) than to guanine-treated ones (60–120 min), suggesting a role for other products in faeces in the induction of arrestment. A similar scenario has been described for *A. walkerae* (Gothe et al. 1984; Gothe 1987) where guanine was suggested to be just one of the constituents of the aggregation pheromone, as the response to this chemical was lower than that to the naturally occurring aggregation pheromone. Urea was identified as a major constituent of *I. ricinus* faeces in this study. The product is also present in human sweat (Geigy Scientific Tables 1981) and is an excretory product in some insects (Cochran 1985). However, when urea was applied to filter paper strips in our bioassays it did not evoke a response at 0.1 mg / filter paper strip ($n = 13$). Other than guanine and urea, the behavioural or electrophysiological roles of the additional products indicated by GC-MS in *I. ricinus* faeces were not investigated in this study.

Ammonia was detected in filter papers with *I. ricinus* faecal deposits in this study using colorimetry, and its presence was confirmed in the vapours over such papers using the antennal NH_3 receptors of *Triatoma infestans*. As ammonia receptors have been described for ticks (Haggart and Davis 1979, 1980; Steullet and Guerin 1994b), we endeavoured to establish if faeces could still arrest *I. ricinus* when the faeces-contaminated filter papers were enclosed in a bronze mesh. It did occur, but only after a long delay compared to faeces-contaminated filter papers that the ticks could contact, showing the weak but significant role of volatiles from faeces in inducing arrestment in *I. ricinus*. C. McMahon has shown with the use of solid-phase microfibers that filter paper strips contaminated with *I. ricinus* faeces release a range of volatiles (unpublished data, this laboratory). Graf (1975) already showed that biological activity of female *I. ricinus* for males could be transferred with a humidified air stream to filter paper strips. NH_4Cl treated filter papers had no effect on *I. ricinus* at the dose tested in the present study (0,05 mg per filter paper), but a saturated solution of this product on filter paper did cause assembly of *Ornithodorus porcinus porcinus* (Otieno et al. 1985). Neitz and Gothe (1984) reported the importance of faecal volatiles for *A. walkerae* where vapours alone were shown to induce aggregation.

Our behaviour tests with different doses and ratios of faecal products in mixtures were not exhaustive, and further tests are required to refine the arrestment stimulus for *I. ricinus*. This will most probably include other as yet unidentified and identified faecal components. However, this study suggests that the arrestment responses of the prostriate *I. ricinus* follow the pattern already established for a range of other metastriate and argasid tick spp. (Leahy 1979; Leahy et al. 1973, 1975, 1981; Steullet and Guerin 1992a). Ticks, like other acari, excrete excess nitrogen in the form of purine pellets that are only poorly soluble in water. This has a double advantage for ticks such as *I. ricinus* as this mode of excretion reduces

water loss to a minimum, and serves to distribute markings associated with self in the humid environment provided by the litter zone where *I. ricinus* survives. Certainly the purines will be subjected to breakdown by bacteria in the litter zone, and we have shown here that receptor cell 2 of the tpB sensilla is even more sensitive to 8-azaguanine than to the parent product guanine. The patchy or clumped distribution recorded for *I. ricinus* on the ground (Gigon 1985) may well be mediated by responses to conspecific faeces at such loci, thus facilitating the off-host matings recorded for this prostriate species (Graf 1975).

In addition to the sensitivity of cell 2 in the tpB tarsal sensilla to purines and related products, this receptor also responded to KCl and RbCl in a dose dependent manner from 10 mM to 1 M concentrations, with KCl serving as the best stimulus. Cell 1 did not respond to NaCl or KCl. Acarine faeces may also contain salts with Na^+ or K^+ cations (Kaufman and Sauer 1982). Presence of NaCl was not required to induce the arrestment effect of guanine on *I. ricinus* in our behavioural assay. KCl did influence the manner in which guanine activated cell 2 electrophysiologically. In the presence of KCl, receptor cell 1 responded poorly to lower doses (< 1 mM) of guanine but stronger to a 10 mM concentration of guanine than when presented in NaCl. Sensitivity of receptor cell 2 to K^+ seems to be a general phenomenon in ticks as it was recorded by electrophysiology during the course of this study in tarsal sensilla of *I. scapularis, I. hexagonus* and *Amblyomma variegatum* (n between 2 and 20). Furthermore, potassium predominates in the sweat of herbivorous mammals, hosts common to these tick species (Guerin et al. 2000).

While previous studies on the assembly responses of ticks to their own faeces and faecal products have concentrated on behaviour, this report takes the story a step further by identifying the receptors involved in faecal recognition on the first leg tarsi. Few studies have been made on the role of these sensilla in the chemical ecology of ticks, although it has been suggested many times (Lees 1948; Balashov 1983). Previous researchers have found that contact chemosensilla on both the tarsi and palps are necessary for a range of behaviours such as host acceptance (Foelix and Chu Wang 1972; Guerin et al. 2000), mating (Feldman-Muhsam and Borut 1971; Oliver 1974; Phillips and Sonenshine 1993; Falk-Vairant et al. 1994; de Bruyne and Guerin 1998) and aggregation stimulus recognition (Leahy et al. 1975; Graf 1975). Aggregation responses of *Ornithodorus moubata* on filter papers in Petri dishes could be reduced by masking or removing the sensilla on tarsus 1 (Leahy et al. 1975). Tp_B sensilla have four sensory cells in *I. ricinus* (Thonney 1987) as in *Amblyomma americanum* (Chu Wang and Axtell 1973), *Boophilus microplus* (Waladde and Rice 1982; Jorgensen 1984), and argasid spp. have five (Chu Wang and Axtell 1973). This study indicates a high degree of conservatism between the prostriate *I. ricinus* and the other tick species cited in terms of both the number of receptors in tarsal tp sensilla and the physiological and behavioural responses that these contact chemoreceptors mediate.

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