Archives of Insect Biochemistry and Physiology 39:65–80 (1998)

Contact Chemostimuli in the Mating Behaviour of the Cattle Tick, *Boophilus microplus*

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Mating of the cattle tick *Boophilus microplus* is mediated by chemical stimuli on the cuticle of females. Males are arrested on the dorsum of females attached to the host, frequently sample the substrate, and then tip-over to the ventrally located gonopore. These behaviours are also observed in vitro when males are placed on a small glass bead treated with a female extract. Time spent and tip-over by male ticks on dummies is used in an assay to test the behavioural significance of fractions of the extract. TLC separation yields one apolar fraction that arrests males, though much less so than the whole extract, but lost tip-over behaviour. This apolar fraction contains a series of cholesteryl esters that, when tested individually, show no arrestment activity at levels present in the extract but, when combined, are as active as the fraction. When a small silica column is used for fractionation, all biological activity is reproduced after recombining the fractions. In addition to the early eluting apolar fraction containing cholesteryl esters, a set of highly active more polar fractions is isolated. Electrophysiological recordings from gustatory sensilla on the pedipalps of male *B. microplus,* which are regularly brought into contact with the cuticle of the female during mating, provide evidence for receptors in two of them responding to the whole extract and to the behaviourally active polar fractions. Mating behaviour involving arrestment and tip-over is clearly initiated by a mixture of chemical stimuli, and tip-over behaviour is associated with the more polar material. Arch. Insect Biochem. Physiol. 39:65–80, 1998. **© 1998 Wiley-Liss, Inc.**

Key words: tick; mating behaviour; cholesteryl esters; pheromone; arrestment; palpal organ

Contract grant sponsor: Hasselblad Foundation; Contract grant sponsor: Roche Foundation; Contract grant sponsor: Sandoz Foundation; Contract grant sponsor: Swiss National Science Foundation; Contract grant numbers: 3.609-0.87, 31-28684.90, 31-37420.93; Contract grant sponsor: Ciba-Geigy-Jubilaeums-Stiftung; Contract grant sponsor: Schweizerische Mobiliar; Contract grant sponsor: Swiss Office for Education and Science.

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Received 27 March 1998; accepted 27 August 1998

INTRODUCTION

Boophilus microplus (Canestrini) is a major tick pest of cattle throughout the world's tropical and subtropical regions, causing extensive losses to the industry by weakening cattle as well as transmitting babesiosis and anaplasmosis. *B. microplus* is a one-host species, i.e., whereas most tick species drop from the host in preparation to moult, this species goes through larval, nymphal, and adult stages on the same bovine host. In spite of its obvious economic importance, very little is known about the mating behaviour and chemical ecology of *B. microplus*. On their natural host, the males moult one day earlier, are more mobile than females, and are able to recognise engorged female nymphs as potential mates and attach underneath them (Falk-Vairant et al., 1994). Females moult in situ and their fertilisation starts during the night of the third day after male moult when females are well attached to the host and have started feeding. During rapid engorgement of the female, the male stays attached to the host underneath her. Males start moving around on the host again after females have detached and dropped to the ground to start oviposition (2–4 days after copulation). For this species it is not known what stimuli guide mating behaviour.

On the host, 2,6-dichlorophenol has been described as the volatile sex pheromone in many tick species. It induces males to detach and move towards females (Sonenshine, 1985). Olfactory sensilla on the tarsi of *B. microplus* males house receptors for this compound and it was isolated from various life stages of this tick (de Bruyne and Guerin, 1994). However, no behavioural responses could be observed and the role of 2,6-dichlorophenol in the biology of *B. microplus* remains unclear. In other tick species, it has been shown that a potent non-volatile pheromone mediates mating behaviour once the male contacts the female (Hamilton and Sonenshine, 1988). Cholesteryl oleate and other cholesteryl esters have been implied as active components for *Dermacentor variabilis* and *Dermacentor andersoni* (Hamilton et al., 1989; Sonenshine et al., 1991). In these species 2,6-dichlorophenol is needed in addition to induce mating responses, whereas a third sex pheromone containing fatty acids mediates the probing of the gonopore (genital sex pheromone, Allan et al., 1988).

Very little is known about the perception of these non-volatile pheromones by sensory organs. Ticks bear gustatory terminal pore (Tp) sensilla on their legs and palps. The fourth segment of the palps in *B. microplus* adults has nine terminal pore sensilla of two structural types, three Tp A and six Tp B (see Fig. 7B; Waladde, 1978).

Here we will investigate the stimuli involved in the mating behaviour of the cattle tick *Boophilus microplus*. Are specific elements of courtship initiated by chemical stimuli? If so, what is the nature of these stimuli and where and when do they occur? Activity of components in biological extracts is shown here by quantifying behavioural elements under controlled conditions as well as demonstrating perception by sensory organs.

MATERIALS AND METHODS

Ticks

Ticks were obtained from a laboratory colony at the Ciba-Geigy Agricultural Research Station, St Aubin, Switzerland and belong to the organophosporous resistant strain Biarra from southern Queensland, Australia. They were reared on the backs of young Simmental steers for more than 30 generations in closed stables at 23°C and 60–70% r.h.. The life-cycle and occurrence of mating under these conditions are described elsewhere (Falk-Vairant et al., 1994).

Ticks were collected by carefully removing individuals from the host with forceps. In addition, unattached males were readily collected, after female drop-off had started, by brushing them off the pelage with a paintbrush. Males or engorged nymphs were transported to the laboratory in a humidified insulated container. Pharate females were kept in an incubator at 32°C and approximately 100% r.h. until they moulted, and males were put on the ears of New Zealand White rabbits, enclosed in cotton bags, to which they readily attached. Males were removed from the rabbit 20–60 min before bioassays and kept at approximately 30°C in a closed container over water to assure high relative humidity. Before electrophysiological recordings, males were kept in an incubator at 18°C on humid tissue paper for 1–4 days.

Behavioural Bioassay

A glass bead (approximately 5 mm diameter, 3 mm high, 0.1–0.2 g), roughened with a wetstone and flattened on one side to inhibit rolling, was placed in the centre of a round arena (40 mm diameter) consisting of a Baudruche® membrane (Joseph Long Inc., Belleville, NJ) stretched over

a 0.9% NaCl solution at 35 ± 1°C on a warm plate. A 40-mm-high plastic tube placed around this arena and the permeability to water of the membrane assured a constant r.h. (>80%). Two such arenas were used simultaneously on the same warm plate, one bearing a bead treated with an extract or synthetic product in solvent applied with a micro-pipette, the second treated with solvent alone as control. A single male tick (2–14 days after moult) 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 (C.E.S., Dübendorf, Switzerland) CI-20P colour CCD video camera attached to a Zeiss (Oberkochen, Germany) operational microscope (working distance: 25 cm). Recordings were made on a Panasonic super VHS video recorder (AG-7350) 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 bead, the other half first on the treated bead. Different behaviours were quantified using The Observer 2.0 event recorder (Noldus Inf. Tech., Wageningen, The Netherlands). The tick was recorded as either being on the bead, i.e., from the first moment all legs were in contact with the bead till the last leg lost contact, or on the membrane. Ticks were allowed to descend and remount the bead but a maximum of 180 s was allotted to each tick, or observation ended when the tick crossed the edge of the arena. The total time spent on the bead (contact time) was then taken as a parameter for statistical analysis with the Wilcoxon signed ranks test on paired replicates (test vs. control). Arrestment is when ticks are significantly longer in contact with the treated bead than with the control bead, i.e., when the arrestment ratio (contact time test/control) is higher than one (see Fig. 2). In addition, it was noted whether a tick showed typical "tip-over" behaviour while on the bead (see Results for definition).

Chemicals and Extracts

All cholesteryl esters, lipid standards, and MSTFA (N-Methyl-N-trimethylsilyl-trifluoroacetamide) were obtained from Sigma Chemical Co., St. Louis, MO. Fatty acid methyl esters and 2,6-dichlorophenol were obtained from Supelco, Bellefonte, PA. Palmitoleic acid was purchased from Larodan AB, Malmö, Sweden. All other fatty acids were from Fluka, Buchs, Switzerland. Cholesterol and all solvents (analytical grade) were from Merck, Dietikon, Switzerland.

Two methods of collection of natural products from ticks were used and termed here *tick extracts* and *tick washes.* Tick extracts were obtained by submerging freshly collected ticks (<15 min after removal from the host, 50–500 at a time) for 5–15 days at –20°C in small volumes (0.5–5 ml) of chloroform or chloroform:methanol (1:1). The extract was collected in a syringe and evaporated to dryness under a gentle stream of nitrogen, immediately redissolved in chloroform at 0.5 or 1 tick eq/µl, and stored at –20°C. Tick washes were obtained by extracting them for only 30 min at room temperature (approximately 22°C), using either hexane, chloroform:methanol, or methanol:water (1:1).

Thin Layer Chromatography and Solid Phase Extraction

For comparative chemical analysis and separation of tick extracts, chromatography was carried out on 20 × 20 cm 0.25 mm silica gel 60 thin layer chromatography (TLC) plates containing a 2.5 cm concentration zone (Merck, Darmstadt, Germany). The plates were first washed twice with chloroform:methanol (1:1) and conditioned for 60 min at 110°C. Extracts and standards were applied in <1 cm diameter spots and concentrated thrice to the bottom of the silica layer using chloroform:methanol (1:1). The plate was subsequently developed in one of the following solvent systems: (1) hexane to 17 cm, toluene to 17 cm and twice hexane:diethyl ether:acetic acid (70:30:1) to 12 cm or (2) chloroform:methanol:water (69:27:4) to 12 cm. After drying, the plates were sprayed with 50% H_2SO_4 in water and heated to 150°C in an oven. For preparative TLC, 100 female equivalents of the extract were applied in a 5 cm band and this part of the plate was not sprayed. Fractions of the resolved extract were scraped from the plate as indicated by the visualised spots on the sprayed part (see Fig. 4). The silica gel was subsequently eluted over a glass wool plug in a pasteur pipette with 2 ml chloroform for each fraction, then dried under nitrogen and redissolved in a smaller volume of chloroform.

Preparative solid phase extraction (SPE) was done on a 500 mg Silica gel in a glass Chromabond® column (Machery-Nagel, Oensingen, Switzerland) conditioned consecutively with 2 ml each of methanol:water (1:1), methanol, chloroform:methanol (1:1), chloroform, hexane:chloroform (75:25). The extract was applied as 150 female equivalents in 100 µl chloroform and subsequently eluted at approximately 1 ml/min with 4 ml hexane:

chloroform (75:25), 3 ml chloroform, 3 ml chloroform:methanol (1:1), 2 ml methanol, and 2 ml methanol:water (1:1). The ten 1 ml serial fractions, the 2 ml methanol (F11), and 2 ml methanol:water (F12) fractions were dried under nitrogen and redissolved in chloroform. These fractions were tested as sets A (F1+2+3+4), B (F5+6+7) and C (F8+9+10+11+12) in the behavioural bioassay.

Identification of Cholesteryl Esters, Cholesterol, and Fatty Acids

Cholesteryl esters were transmethylated at 85°C for 60 min in flame sealed 1 ml glass ampoules with 200 µl of 1% H_2SO_4 in methanol after adding 1 µg of tetradecane as internal standard. Fatty acid methyl esters were solvent-solvent extracted into hexane and analysed with cold on-column injection on a 30 m DB-wax capillary column (J&W Scientific, Folsom, CA; 0.25 µm film thickness, 0.25 mm ID) in a Carlo Erba HRGC 5160 gas chromatograph (GC) (Carlo Erba Instruments, Milan, Italy) with H_2 as carrier gas at 1.5 ml/min (0.5 m/s). Temperature of the column was programmed to increase from 70°C after 1 min to 90°C at 15°C/min, to 160°C at 20°C/min, to 240°C at 5°C/min, and held there fore 10 min. Identification was done by comparing retention times with standards and by matching mass spectra (see below). Quantification was made by peak area integration of the FID detector signal using a Spectra-physics SP-4270 integrator and comparison with known amounts of standards injected in the same session.

Cholesterol and free fatty acids were isolated from 70 female equivalents of extract fractionated on an SPE column in a separate procedure: After conditioning with 3 ml hexane and applying the extract, the column was extracted consecutively with 1 ml hexane, 1 ml hexane:dichloromethane (9:1), 2 ml hexane:dichloromethane (1:1), 2 ml dichloromethane, 2 ml dichloromethane:methanol (1:1), 2 ml methanol. After identifying the presence of cholesterol and free fatty acids in the dichloromethane:methanol fraction by TLC evaluation, an aliquot of this fraction was evaporated to dryness in an ampoule, and sylilated as described by Grenacher and Guerin (1994) after flame-sealing the ampoule.

Gas chromatography-mass spectrometry (GC-MS) analysis of derivatised cholesterol and fatty acids as well as underivatised cholesteryl esters was conducted with an HP-5971A mass selective detector (ionisation energy 70 eV at 180°C) linked to a Hewlett Packard (Meyrin, Switzerland) 5890 series II gas chromatograph. The sample (1 µl) was injected on-column via a 1 m precolumn into a 15 m DB-5HT nonpolar capillary column (J&W scientific, 0.1 µm film thickness, 0.25 mm ID) temperature programmed from 60°C after 5 min to 350°C at 10°C/min and held at 350°C for 15 min with helium as carrier gas at constant velocity (0.4 m/s). Mass spectra of unknowns were compared with those of standards and with spectra stored in a library using the HP CHEMSTATION program on an IBM compatible computer. Quantification of cholesterol and free fatty acids was by total ion peak integration compared with known amounts of standards injected under the same conditions.

Electrophysiology

A male tick (1–14 days after moult) was attached dorsally with double-sided adhesive tape to a small piece of perspex positioned with plasticine such as to obtain a ventrolateral view of the mouthparts under a Kombistereo microscope (1,000×, working distance 11-mm, M3Z, Wild, Heerbrugg, Switzerland). A glass reference electrode filled with 0.05% polyvinylpyyrolidone in 0.15 M NaCl was inserted caudally into the basis capitulum and pushed forward to restrict movement of the mouthparts. The palpal organ however, remained freely movable in the horizontal plane. All stimuli were dissolved in 0.1 M KCl with 1% ethanol. The recording electrode tip was broken (5–10 µm diameter), dipped in the stimulus solution, and filled from the back with electrolyte. It was then connected to a high impedance non-blocking preamplifier, mounted on a micromanipulator and brought into contact with the tip of one of the sensilla on the palpal organ. AC signals were amplified (1,000×) with a universal AC/DC amplifier (UN-03, Syntech, Hilversum, The Netherlands) and recorded via a DAS 16 analogue to digital card (Metrabite Corp., Taunton, MA) on an IBM compatible PC equipped with the spike analysis programme SAPID (Smith et al., 1990). Analysis was made on the 1 s of signal obtained 0.1 s after contact. From each preparation, three responses for each of a variable number of stimuli were recorded with at least 20 s between stimuli as well as several repeated series of controls (electrolyte only).

RESULTS

Behavioural Responses of Male Ticks to Female Cuticular Components

When male *B. microplus* ticks are placed on a small glass bead treated with an extract of female ticks (chloroform or chloroform:methanol 1:1) they stop walking and actively investigate the substrate by scraping their rostrum back and forth while the tarsi of the first legs are kept in close contact with the bead (Fig. 1). Conversely, when placed on control beads, males generally leave within 20 s and very often raise their body and wave the first pair of legs in the air just before losing contact with the bead. Arrestment can be observed with several levels of dilutions of this extract but not below 0.1 female equivalent per bead (Fig. 2A). At high doses, many males descend toward the membrane while keeping their body in close contact with the surface of the bead. Some males then try to push themselves in between the bead and the membrane. Characteristically, as viewed from above, the tick's venter becomes visible as the mouthparts and first pair of legs disappear under the bead and the bead is sometimes moved. This behaviour was observed only on treated beads and designated here as "tip-over" (Fig. 1). It was defined as a movement towards the underside of the bead while the tick slides along the surface of it, thus exposing its venter. Other males stop all locomotion and pierce the membrane with their mouthparts often attaching perpendicular to the membrane, with six legs on the bead and the first pair on the membrane. This behaviour was termed "membrane probing" (Fig. 1) and not analysed further, though it should be noted that it occasionally occurred on the control beads. Normally, on control beads, males simply continue walking, reaching away from the bead for the membrane as they descend, hence the venter is never seen (Fig. 1). The number of males showing tip-over on the test bead was dose dependent and no males tipped-over on beads treated with less than 0.3 tick equivalents.

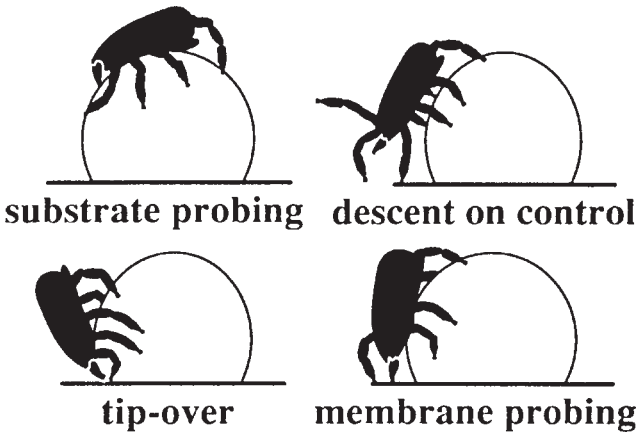

Fig. 1. Schematic drawings of typical poses characterising elements in the behaviour of male *B. microplus* ticks on a glass bead. For details see text.

Males were also significantly arrested on beads treated with 30 min washes of female ticks in chloroform:methanol (1:1) or hexane at room temperature but fewer males tipped over (Fig. 2B). The more polar methanol:water (1:1) wash was less active. Males react similarly on chloroform:methanol extracts of recently moulted female *B. microplus* (day 12) and on a similar extract of nymphal exuviae, except that they tip-over less on the exuvial extract (Fig. 2C). Arrestment is significantly less and males do not tip-over at all on a chloroform extract of *I. ricinus* exuviae. Considerable arrestment and varying levels of tip-over can be observed in male responses to extracts from female *B. microplus* of different ages: pharates (fully developed but still enclosed in the nymphal cuticle) just before moulting (12 days), before fertilisation (14 days), after most females have been fertilised and are semi-engorged (16 days), and fully engorged just before drop-off (19 days) (Fig. 3). Strongest tip-over was observed on the extract of newly moulted females. Equivalent responses can also be obtained from an extract of newly moulted males and even from 300 eq of unfed larvae. However, a bovine hair extract (in dichloromethane, 10 μg/l of low volatile mass, T. Kröber, unpublished data) at doses of 6, 20, 60, and 200 μg did not induce arrestment on the bead though some males showed tip-over on the two higher doses (results not shown).

Males Are Arrested by an Apolar Fraction But Do Not Exhibit Tip-Over Behaviour

The chloroform extract of 14-day-old female *B. microplus* separates into a number of clearly resolved spots on a TLC plate with solvent system I (Fig. 4A). The three darkest spots show up as orange under UV light (366 nm) as do cholesterol and cholesteryl oleate that co-elute with two of them. Preparative TLC of 100 tick equivalents under the same conditions was performed and ten fractions were scraped off the plate corresponding to the spots. After recombining all fractions,

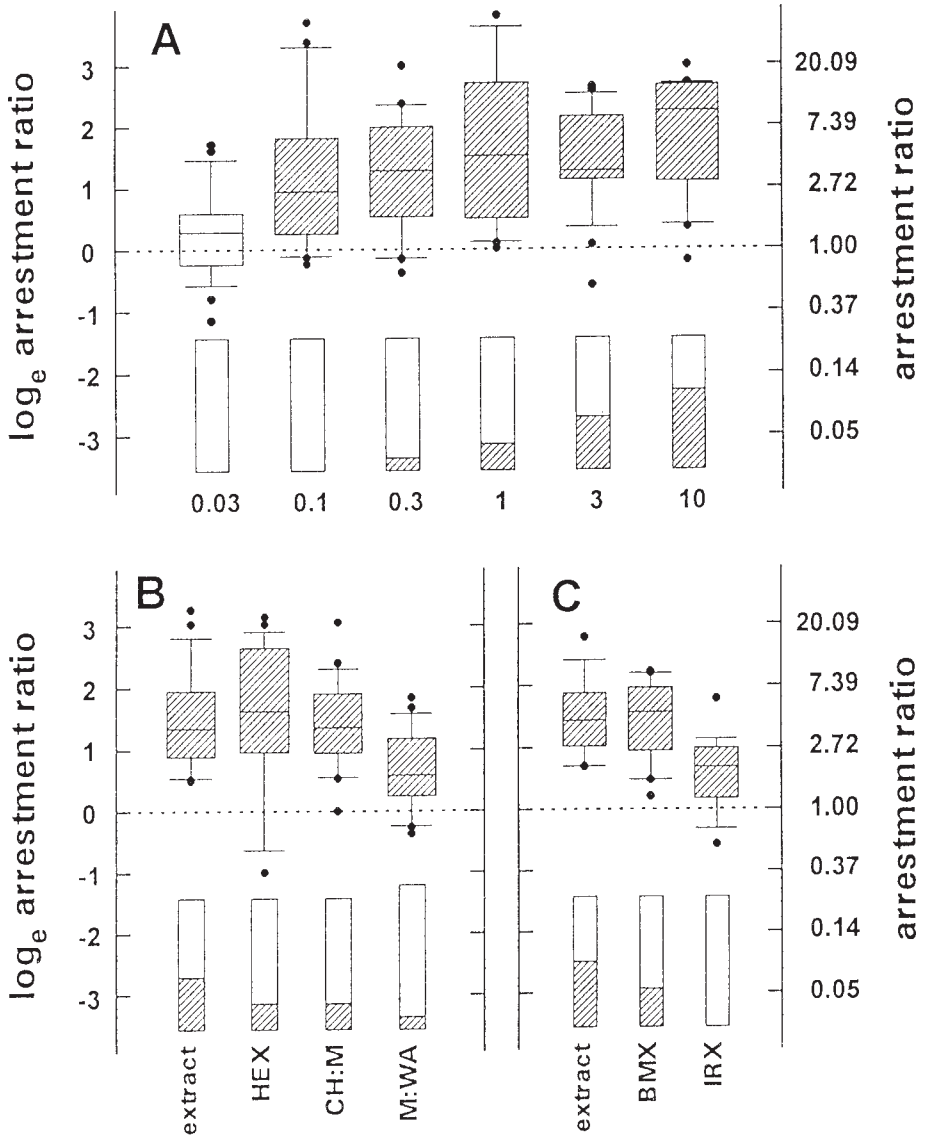

Fig. 2. Behavioural responses of individual male *B. microplus* ticks ($16 \leq n \leq 29$) on a glass bead treated with extracts of female ticks (test) or solvent only (control). Box plots show the distribution of the natural logarithm of the arrestment ratios, i.e., the ratios between the time spent on the test versus the control bead. The line within a box marks the median, the lower and upper boundaries of a box indicate 25th and 75th percentiles, error bars below and above a box are the 10th and 90th percentiles, and data-points outside the 10–90% range are shown separately. Filled boxes indicate 5% significance in Wilcoxon's paired ranks test and the dotted line marks ratio = 1, i.e., no effect. The vertical bar diagrams at the bottom of A, B, and C indicated the proportion of males exhibiting tip-over behaviour on the test bead. **A:** Different doses (in tick equivalents) of an extract of female *B. microplus* (for 7 days in chloroform:methanol 1:1 at –70°C). **B:** Three tick equivalents of the extract in A compared to 30 min washes of female ticks at room temperature in different solvents [HEX, hexane, CH:M, chloroform:methanol (1:1), M:WA, methanol:water (1:1)]. **C:** The extract in A compared to a similar extract of exuviae of *B. microplus* nymphs (BMX) and a chloroform extract of *Ixodes ricinus* nymphal exuviae (IRX), all tested at 3 tick equivalents.

the original activity at 10 tick equivalents was only partly recovered: male arrestment was considerably less and no tip-over was observed for any of the fractions (Fig. 4B). The limited arrestment was entirely associated with fraction (F) 8. TLC separation of extracts of female and male *B. microplus* resulted in a very similar pattern except that spot intensities were reduced for the male, but the pattern was different for the larval extract (results not shown).

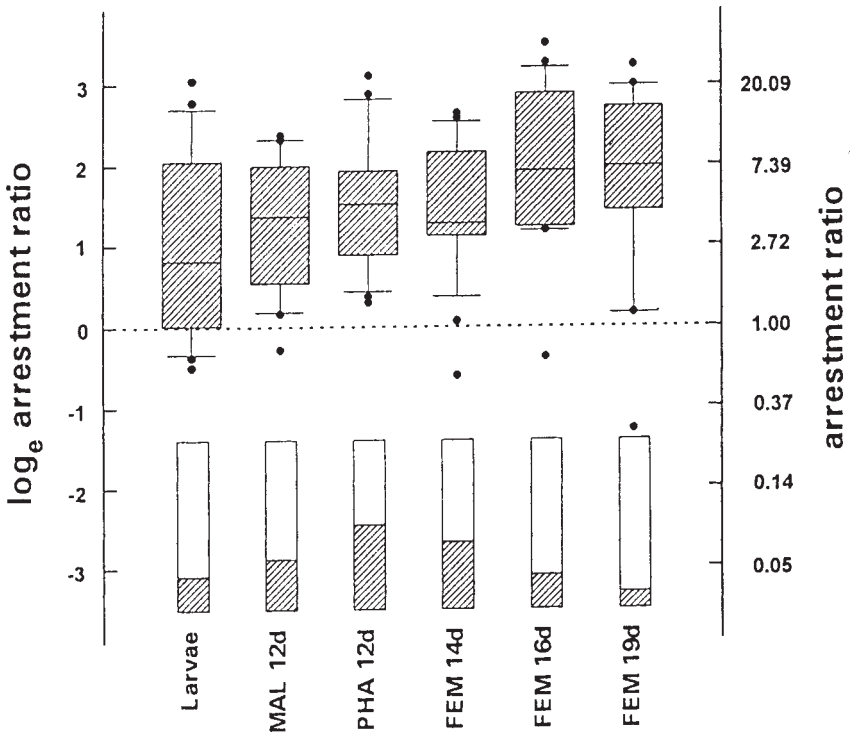

Fig. 3. Behavioural responses of individual male *B. microplus* ticks on glass beads treated with extracts (3 tick equivalents) of different life-stages and different ages (days after infestation); MAL, male, PHA, pharate female, FEM, female. Unfed larvae were tested for 300 eq. For details on presentation of data see Figure 2.

Cholesteryl Esters in the Apolar TLC Fraction Cause Male Arrestment

GC-MS analysis of the active TLC fraction F8 on a high temperature (350°C) nonpolar capillary column indicated the presence of a series of compounds with a molecular weight of 500 and higher, co-eluting with some cholesteryl ester standards. Their mass spectra contain m/z 368 and other ions characteristic of the cholesterol moiety but their molecular ions could not be determined. No other major peaks were observed under these conditions in this fraction. Transmethylation and subsequent analysis of methyl esters with an FID detector on a DB-wax capillary column indicated the presence of several fatty acid moieties (Table 1). Recovery of various synthetic cholesteryl esters from TLC plates with this method was above 80%. A comparison between extracts shows that in both the female and pharate female extracts, straight-chain saturated fatty acids predominate, notably myristic, palmitic, stearic, and arachidic and the pharate female extract is quite comparable with the female *B. microplus* extract. The identification of unknown peaks in the chromatogram was not attempted but none exceeded 1% of the most abundant peak (methyl myristate). The larval cholesteryl ester fraction shows relatively high amounts of cholesteryl oleate and an absence of the longer chain fatty acids.

The most important synthetic cholesteryl esters, present in the extract, were tested individually and as mixtures at various doses in the behavioural essay and the data are shown in Table 2. Individually, none of them arrested male ticks at doses comparable to those present in the extract. Activity was observed only at very high doses (1,000 nmol) of individual compounds. However, of the three different mixtures tested, the most complete, including both saturated and unsaturated fatty acid esters, was active at the physiologically relevant level of 10 nmol, corresponding to approximately 10 tick equivalents. This activity is comparable to that of the active apolar TLC fraction F8.

Second Group of More Polar Fractions Restores Total Activity

Because activity of the total extract after TLC separation was considerably less than before and, most notably, tip-over behaviour was

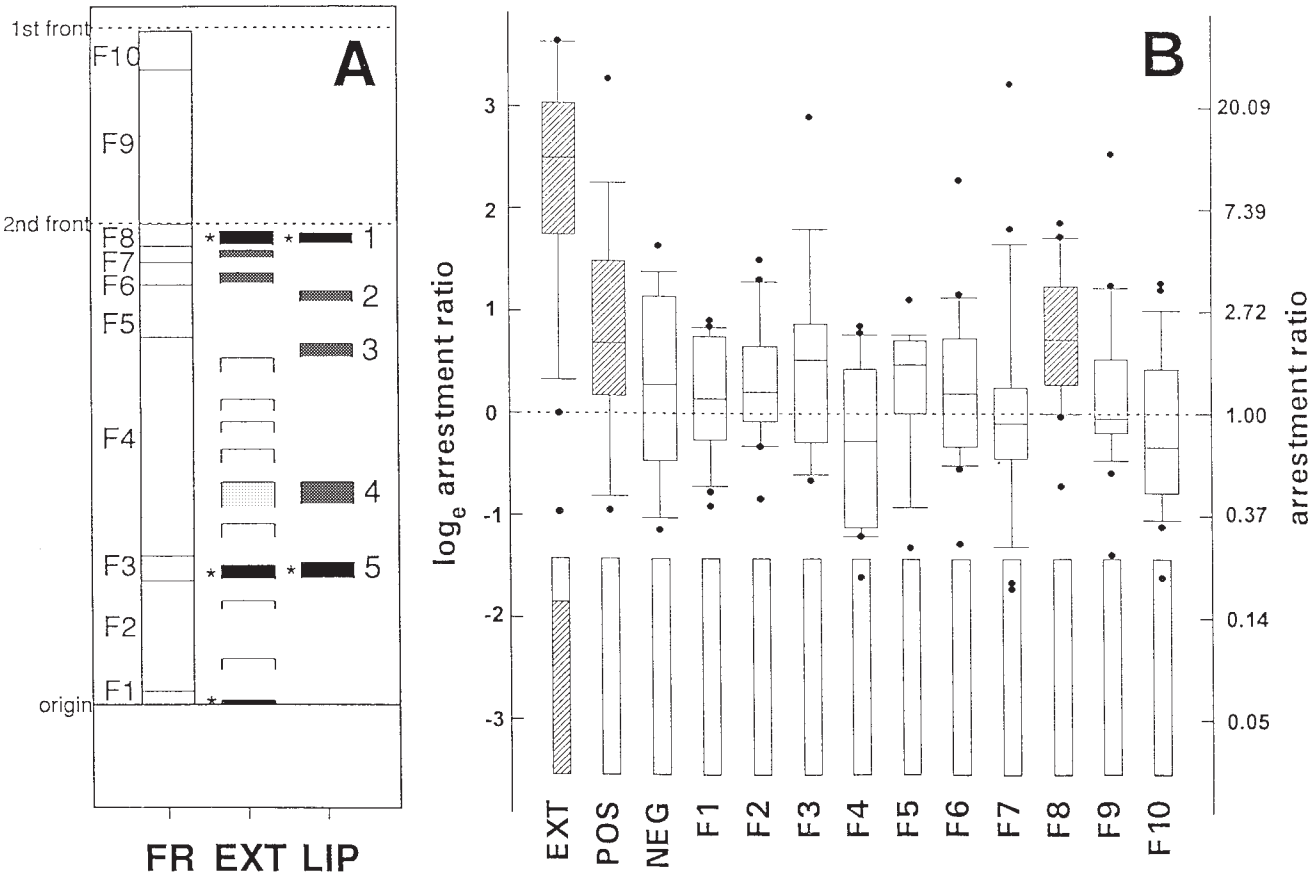

Fig. 4. Preparative thin layer chromatography (TLC) and bioassay of the fractions of a chloroform extract of female *B. microplus*. **A:** TLC separation of 0.25 mm silica gel of extract (EXT) and lipid standards (LIP). FR indicates fractions of the silica gel scraped off the plate, solvent extracted, and subsequently bioassayed (below). Solvent system I was used: hexane to 1st front, toluene to 1st front and twice hexane:diethyl ether:acetic acid (70:30:1) to 2nd front. Spots that could only be observed under UV light (366 nm) are open; the asterisk marks those spots that turn up orange in UV; the grey shading indicates intensity after charring. Standards are 1. cholesteryl oleate, 2. methyl oleate, 3. triolein, 4. oleic acid, 5. cholesterol. **B:** Behavioural responses of individual male *B. microplus* ticks on a glass bead treated with the female extract and its TLC fractions at 10 eq. EXT, extract before separation; POS, positive control, i.e., all fractions recombined; NEG, negative control, i.e., all fractions except F8. For details on presentation of data see Figure 2.

lost, we performed a column separation. After separation of 150 female equivalents of a chloroform:methanol (1:1) extract on a small SPE column, recombination of all fractions restored the full activity (Fig. 5). Some arrestment was associated with the early eluting, apolar fractions 1–4 (set A) as might be expected from the behavioural responses to the apolar TLC fraction (compare Figs. 4 and 5). However, considerable arrestment was also observed with the more polar fractions 8–12 (set C), whereas fractions 5–7 (set B) were not active. Of the different combinations, most activity was found after combining sets A with C. The tip-over on the bead appeared to be associated with C and particularly with CA. Analytical TLC using a different solvent system (II, Fig. 6) shifted and separated material, which stayed at the origin with solvent system I (Fig. 5A), whereas all spots above cholesterol elute close to the solvent front.

Cholesterol, Free Fatty Acids and 2,6-Dichlorophenol Inactive at Natural Levels

GC-MS analysis of the dichloromethane:-methanol fraction from an SPE column used for purposes of identification indicated the presence of cholesterol and free fatty acids in an extract of female *B. microplus* at approximately 3 nmol cholesterol and approximately 0.5 nmol of total free fatty acids per female. A small peak at the retention time of cholesterol was also observed in the polar SPE fraction F8 (part of set C) used in the behavioural bioassays, as were free fatty acids.

TABLE 1. Fatty Acid Moieties of Cholesteryl Esters Identified by Gas Chromatographic Analysis After Transmethylation of an Apolar Fraction From a TLC Separation of Chloroform Extracts*

Cholesteryl esters (trivial name)	Code	Fatty acid methyl ester (FID peak area) (pmol/tick)						Synthetic mixtures (nmol)		
		Female	%	Pharate	%	Larva	%	I	II	III
Saturated fatty acid moieties										
Caprate	10:0	29	2	107	20	1	6	—	—	—
Laurate	12:0	28	2	24	4	1	5	—	—	3
Myristate	14:0	325	27	134	25	5	26	40	—	30
Palmitate	16:0	166	14	76	14	7	35	20	—	15
Stearate	18:0	127	11	49	9	4	19	20	—	15
Arachidate	20:0	205	17	81	15	—	—	20	—	15
Behenate	22:0	25	2	5	1	—	—	—	—	5
Lignocerate	24:0	54	5	16	3	—	—	—	—	5
Cerotate[a]	26:0	137	12	28	5	—	—	—	—	—
Unsaturated fatty acid moieties										
Palmitoleate	16:1	12	1	—	—	—	—	—	33	3
Oleate	18:1	49	4	17	3	2	9	—	33	3
Linoleate	18:2	29	2	—	—	—	—	—	33	3
Total		1,182	99	536	99	19	100	100	99	97

*Synthetic mixtures of these products tested in behavioural bioassays with male ticks are indicated on the right. —, not present or not detected.
[a]Tentative identification of this fatty acid.

The presence of cholesterol and fatty acids in F8 could be expected from the TLC analysis (Fig. 5A, compare with standards in Fig. 4A).

Since cholesterol and free fatty acids were present in the active SPE fractions in set C and are chemically related to cholesteryl esters, we tested them in the behavioural assay. Cholesterol induces arrestment in male *B. microplus* at doses just above the level present in 10 female equivalents (Table 2). A synthetic mixture

TABLE 2. Arrestment of Male *B. microplus* on a Glass Bead Treated With Synthetics and Their Mixtures[†]

Synthetic product		Median arrestment ratio dose (nmol/bead)*			
Trivial name	Code	1	10	100	1,000
Cholesteryl esters: saturated fatty acid moieties					
Laurate	12:0	—	—	n.s.	—
Myristate	14:0	n.s.	n.s.	n.s.	2.02
Palmitate	16:0	n.s.	n.s.	n.s.	2.06
Stearate	18:0	n.s.	n.s.	n.s.	1.72
Arachidate	20:0	n.s.	n.s.	n.s.	n.s.
Behenate	22:0	n.s.	n.s.	n.s.	—
Lignocerate	24:0	—	—	n.s.	—
Cholesteryl esters: unsaturated fatty acid moieties					
Palmitoleate	16:1	n.s.	n.s.	1.37	1.37
Oleate	18:1	n.s.	n.s.	n.s.	2.92
Linoleate	18:2	n.s.	n.s.	n.s.	1.36
Cholesteryl esters: mixtures					
Saturated moieties[a]	mix I	n.s.	n.s.	1.75	—
Unsaturated moieties[a]	mix II	n.s.	n.s.	n.s.	—
Combination[a]	mix III	n.s.	1.73	1.28	—
Other products					
Cholesterol	CHol	n.s.	n.s.	1.72	2.19
Fatty acid mix[b]	FA	n.s.	n.s.	n.s.	—
Complete mix + 2,6-dichlorophenol[c]	mix III+Chol+FA+26DCP	—	1.54	—	—

[†]Data are medians of the arrestment ratios, i.e., the ratio between time spent on the test vs. control bead for individual males ($13 \leq n \leq 20$). —, not tested.
[a]Mixtures as described in Table 1.
[b]See text for composition.
[c]Dosed as present in 3 tick eq.
*n.s., not significant (Wilcoxon's paired ranks test, $P > 0.05$).

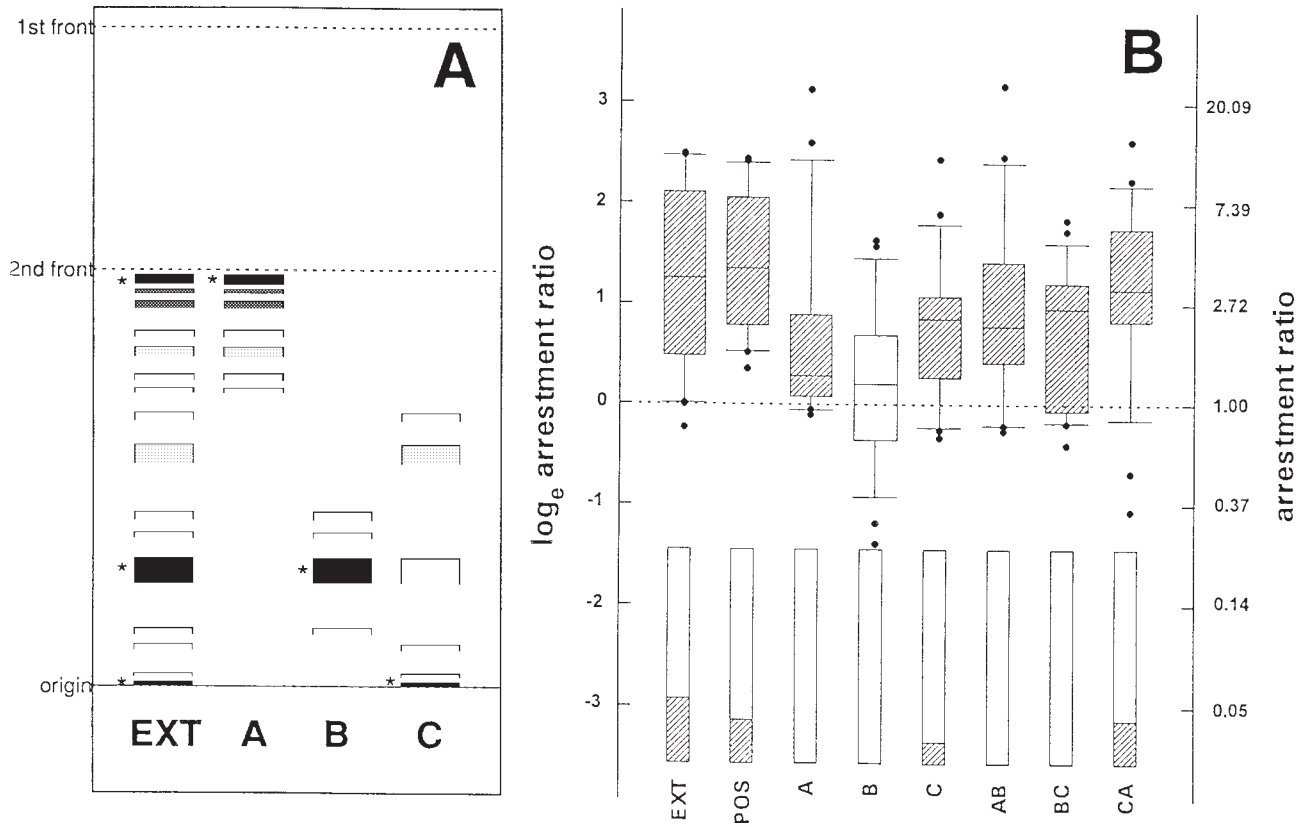

Fig. 5. Preparative solid phase extraction (SPE) on a small silica gel column of a chloroform:methanol (1:1) extract of female *B. microplus* and bioassay of fractions. SPE elution was with 4 ml hexane:chloroform (75:25), 3 ml chloroform:methanol (1:1), 2 ml methanol, and 2 ml methanol:water (1:1). The ten 1 ml serial fractions and methanol (F11) and methanol:water (F12) fractions were recombined in sets A (F1+2+3+4), B (F5+6+7), and C (F8+9+10+11+12). **A:** Thin layer chromatography (TLC) of the whole extract (EXT) and SPE fraction sets A, B, and C. For details on solvent system I employed and presentation of data see Figure 4A. **B:** Behavioural responses of individual male *B. microplus* ticks to a glass bead treated with the female extract and its SPE fraction sets at 3 eq tested both alone and combined. EXT, extract before separation; POS, positive control, i.e., all fractions A, B, and C combined. For detailed on presentation of data see Figure 2.

of fatty acids corresponding to that found in the extract (myristic:palmitic:palmitoleic:stearic:oleic:linoleic acids at 6:10:2:6:50:30) was not active at doses near natural levels. No tip-over behaviour was observed in any of these tests. Adding fatty acids and cholesterol to the cholesteryl esters at doses comparable to 3 tick equivalents did not significantly increase arrestment (results not shown), nor did it influence tip-over. In addition, no effect was observed when 2,6-dichlorophenol was added to this complex mixture at naturally occurring amounts (i.e., 1.5 ng for 3 eq, see de Bruyne and Guerin, 1994) (Table 2). None of these products are, therefore, likely to be the active components of the more polar SPE fraction.

Gustatory Sensilla on Male Palps Respond to the More Polar SPE Fractions

In order to further characterise male responses, we have recorded electrophysiologically from gustatory sensilla of *B. microplus*. It was very difficult to record reliably from the two Tp B sensilla on the distal first tarsi while it was impossible to obtain electrical contact with the two Tp A sensilla nearby. Since males scrape their mouthparts over the bead during prolonged stays on the bead, we decided to study the electrophysiology of sensilla on the palpal organ.

With the tip recording method used here we could not obtain noticeable electrical contact with Tp A sensilla 3, 4, and 9 on the palps (Fig. 7A,B). Sensillum 6 was difficult to reach and, therefore,

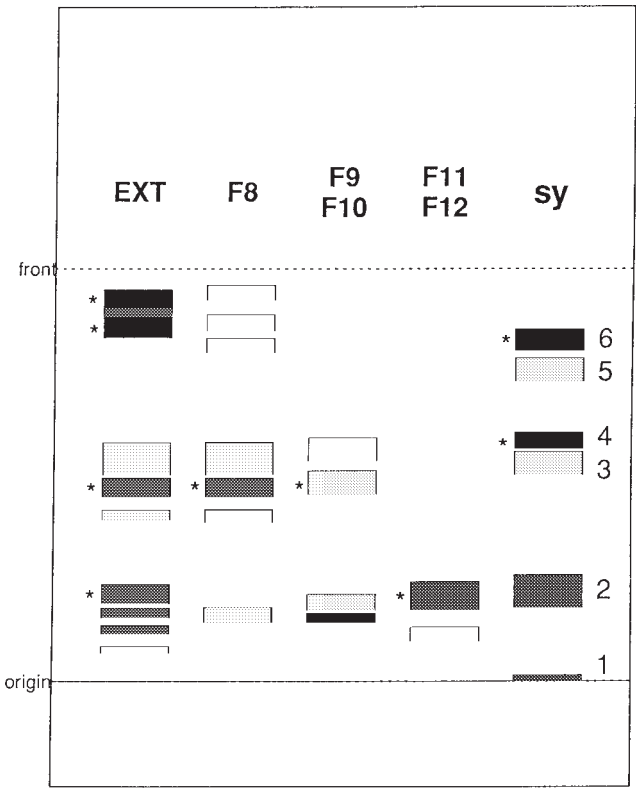

Fig. 6. TLC separation on 0.25 mm silica gel of a chloroform:methanol (1:1) extract (EXT), the polar SPE fractions (that make up set C in Fig. 5A) and some synthetics (sy) with solvent system II (chloroform:methanol:water 69:27:4 to 12 cm). Spots that could only be observed under UV light (366 nm) are open; the asterisk marks those spots that turn up orange in UV; the grey shading indicates intensity after charring. Standards are (1) caffeine, (2) lecithine, (3) 9,10,16-trihydroxypalmitic acid, (4) ecdysone, (5) monoolein, and (6) cholesterol.

not studied. A few recordings were made from sensilla 1, 5, and 8 but no consistent responses were obtained to the control (1% ethanol in 0.1 M KCl), and since this could, therefore, not be used as a test for the condition of the preparation, further analysis of these sensilla was abandoned. However, sensilla 2 and 7 house a receptor that consistently responded with a homogenous tonic spike train of 30–60 spikes/s to the control (Fig. 7C). This response is similar in both sensilla and dose dependent for KCl (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1 M tested). In both sensilla, there was a clear change in this pattern, with an increase in the total number of action potentials recorded when stimulated with 0.1 eq/μl of SPE fractions F8, F9, and F11 (Fig. 8). Fraction F10 was active but to a lesser extent, whereas some inhibition occurred with fraction F5. Responses to different doses of fraction F8 were obtained (0.001, 0.003, 0.01, 0.03, and 0.1 eq/μl) and the response to F9 resembles that to 0.01 eq of F8 (Fig. 7). Clearly a receptor or receptors other than the one responding to KCl is/are involved in the perception of these fractions which are part of the active set C. No such change was observed when testing other fractions. A response was also obtained from 0.01 and 0.1 eq/μl of the unseparated extract but not from 0.1 μg/μl of bovine hair extract. The cholesteryl ester mix III (see Table 1) when tested at a level comparable to that present in 0.1 tick eq/μl did also not evoke responses distinctly different from the control.

DISCUSSION

Male Behavioural Responses to Chemical Stimuli on the Cuticle of Females

The mating of *Boophilus microplus* is clearly mediated by chemical stimuli associated with the cuticle of female ticks. The behaviour of males in vitro on a glass bead treated with extracts of females in various solvents is similar to that observed on females in vivo (Falk-Vairant et al., 1994) but distinctly different from that on an untreated bead. Responses are obtained from extracts of whole ticks and exuviae at naturally occurring levels, so important chemical cues are associated with the cuticle. Several elements are present in the behaviour observed on the glass beads, which also occurs during mating on the host, such as the strong drive to tip-over and crawl under the bead, which normally leads to the male to the ventrally located gonopore of the attached female (Falk-Vairant et al., 1994). The absence of this behaviour when bovine hair or *Ixodes* extracts are employed demonstrates the specific nature of the stimuli involved.

The first effect of the extract is an arrestment. Males spend more time investigating the treated bead. We have defined the arrestment ratio in relation to location of an individual male tick, i.e., on the bead or on the surrounding membrane-arena and it was measured in time (duration of contact). The second effect, namely tip-over, was defined in behavioural terms and only its incidence was scored for each male, i.e., he either engages in it or not. Whereas the arrestment is the end result of a number of behavioural elements, tip-over is one such element. The two parameters are thus related because males that tip-over will spend more time on the bead while

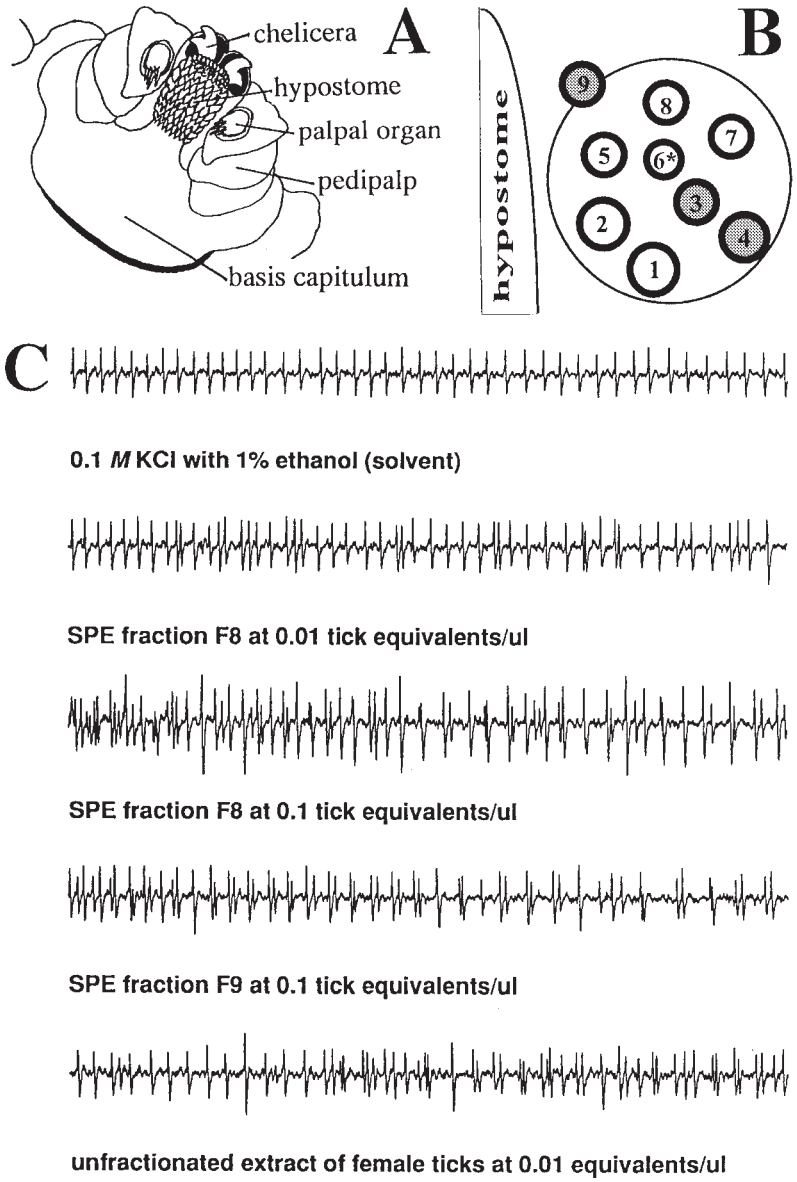

Fig. 7. Electrophysiological responses from contact chemoreceptors in terminal pore sensilla on the palps of male *B. microplus* in response to stimulation with an extract of female ticks and fractions thereof (SPE, solid phase extraction). **A:** Ventral view of the mouthparts showing the palps and the modified fourth segment (palpal organ) in the folded position. **B:** Schematic drawing of the position (relative to the hypostome) and numbering of the sensory hairs on the apical surface of the palpal organ when in the extended position. Filled circles are Tp A type sensilla, open circles are Tp B type sensilla. **C:** Recordings of action potentials (0.5 s) obtained 0.1 s after bringing a glass capillary containing the stimulus dissolved in 0.1M KCl plus 1% ethanol into contact with the tip of sensillum 7. Spike counts are 43, 54, 75, 61, and 57 from top to bottom.

doing so. This could explain the slight increase in arrestment with extract dose. However, other behaviours caused arrestment without, or before tip-over.

Since arrestment and tip-over involve distinct behaviours, these can then also be mediated by different cues. This view is supported by the fact that the tip-over response disappeared when TLC fractions of the female extract were tested, whereas arrestment was still significant. The primary effect of cholesteryl esters, isolated and identified by TLC from the apolar part of the extract, was therefore on other motor patterns which cause arrestment. A second chemical cue is necessary to induce a complete response, i.e., increased arrestment and tip-over behaviour.

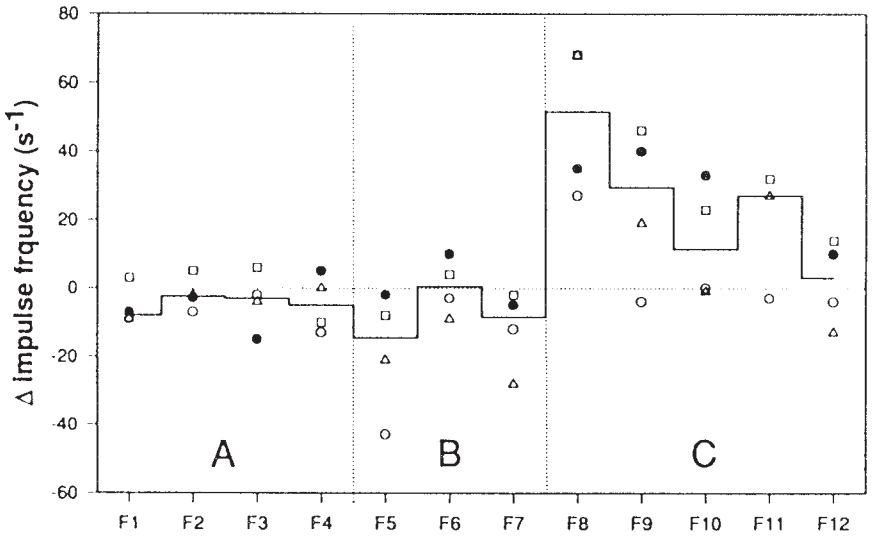

Fig. 8. Electrophysiological responses of sensillum 2 (black dots) and 7 (open symbols) on the palpal organ of five male *B. microplus* to twelve fractions obtained after separation of a female extract on a small silica column (SPE, solid phase extraction). The letters A, B, and C indicate the combined fraction sets tested in the behavioural assay (see Fig. 5). Responses are calculated from 1 s recordings by subtracting the total number of impulses in controls (0.1M KCl + 1% ethanol) from the total number of impulses recorded in response to stimulation with the individual fractions (0.1 female eq/μl in 0.1M KCl + 1% ethanol). Individual data points are the means of three measurements on a single sensillum and the solid line connects the medians of these points. In one of the data sets F11 was not tested.

The tipping over is a more mating-specific behaviour. With increasing dose of the female extract, more males show this response. Since tip-over behaviour was never observed in controls, we conclude that it is triggered by a chemical cue even though mechanical stimuli like the shape of the bead probably play an important role in guiding it (see Hamilton and Sonenshine, 1995). It can, and probably will be, only performed on an appropriately shaped object. The key stimulus inducing this behaviour is present in the polar part of the extract.

Role of Cholesteryl Esters and Cholesterol in Arrestment

We isolated and identified several cholesteryl esters from tick extracts that mediate arrestment of male *B. microplus* on glass beads. In addition, substantial amounts of cholesterol were found in extracts of female *B. microplus*. Cholesterol and cholesteryl esters have been shown to be very abundant components of cuticle lipids of female *B. microplus* (Cherry, 1969). The relatively high proportion of saturated straight chain fatty acids found here on the female cuticle is also typical for bovine skin lipids (Lindholm et al., 1980; Downing and Lindholm, 1982) and differs from the free fatty acid profile of the same extract. Cholesterol and cholesteryl esters might be, at least partly, contaminants from bovine skin secretions. The different fatty acid composition of cholesteryl esters in the *B. microplus* larval extract could stem from the fact that they have not been in contact with a bovine host and that their cuticle is synthesised from material present in the egg.

Our results also show that a behavioural response comparable to that of the active TLC fraction containing the cholesterol esters at "physiologically relevant" levels could not be obtained from any of the synthetic analogues of the major components of this fraction on their own, nor from the mixtures of the major unsaturated or saturated synthetic esters. Only when saturated and unsaturated esters were mixed was a response obtained at natural levels. We do not know, however, to what extent the exact ratios between individual cholesteryl esters are relevant. One of them, cholesteryl oleate, has been described as the "mounting" sex pheromone of *D. variabilis* and is a major component in extracts from several tick species (Sonenshine et al., 1991), but it is not a major component of the cuticle extract of *B. microplus*. Even though arrestment response of males was highest to synthetic cholesteryl oleate, it was not different from the other cholesteryl esters in requiring a very high dose. Most importantly, the activity of the apolar part of extracts of tick cuticle in this species is

due to a mixture of cholesteryl esters, not a single compound.

Multicomponent Mating Signal: A Second Chemical Signal Mediating Tip-Over

The cholesteryl esters in the apolar TLC fraction are only responsible for initial arrestment and do not initiate the tip-over behaviour. After recombining the fractions eluting from the solid phase extraction (SPE) column, the second fractionation method used here, complete activity of the extract was recovered including tip-over behaviour on the bead. This suggests that a second chemical cue present in the cuticle extract must have been lost in the TLC procedure, reducing the activity of the extract to merely the arrestment effect of the cholesteryl esters. Both methods use solid-liquid chromatography on silica gel, but detrimental effects could have arisen in the procedure from (1) the concentration zone, (2) drying in between successive elutions, (3) the use of toluene, diethyl ether, acetic acid, or any impurities in these solvents, or (4) the UV fluorescence indicator. The result of the bioassays after SPE fractionation confirms the presence of two distinct chemical signals, one in the apolar fractions, containing cholesteryl esters, and a second in the more polar fractions, the two separated by non-active neutral fractions. Moreover, this second fraction causes arrestment as well as tip-over on its own, but its activity is above all evident when admixed to the apolar fractions. Hence, the complete chemical message in mating is a multicomponent one.

It has not been possible to identify the active constituent(s) of these polar fractions of the female extract. We can only state that they must be more polar than cholesteryl esters and wax esters and probably less easily extracted from the cuticle since the 30 min washes of females in the same solvent as used for extracts were slightly less effective in inducing tip-over behaviour. Nevertheless, the low activity of the methanol:water wash points to the essential contribution of the hydrophobic components of the extract. The polar SPE fractions as recombined in C still contain many different compounds or classes of compounds as shown by TLC. The electrophysiological responses demonstrate activity that reduces from fractions F8 to F9 to F10 and then increases slightly for F11 again. This could point to a more complex composition of the adequate stimulus for these receptors than just a single compound or class of compounds.

Role of the Palpal Organ in Mating Behaviour

The palpal organ is known to regularly come into contact with the substrate during tick locomotion as the body goes up and down. This is referred to as "bobbing" (Jorgensen, 1984) and could be observed on control beads as well as on the membrane after leaving the bead in this study. On test beads, males cling to the substrate with the legs, bringing the palps into close contact with it, and make vigorous probing movements with the mouthparts. Responses to the more polar fractions of the female extract eluting from the silica column in two of the nine gustatory sensilla on the palpal organ provides further evidence for a role of components of these fractions in the biology of male *B. microplus*. Correspondence between the activity of these polar fractions on palpal sensilla and their activity in the bioassay strongly suggests that palpal receptors perceive chemical compounds mediating male mating responses. The rostrum, bearing the palps, is brought into more intensive contact with the substrate during mating and is also often flexed at 90° on extract-treated beads. Evidence for the involvement of mouthparts in mating responses of *B. microplus* has been provided by Falk-Vairant (cited in Guerin et al., 1992) who showed that masking the palps with wax increased male probing of the female dorsum with the mouthparts in an effort to compensate for loss of stimulation. Electrophysiological investigations of the palpal organ's gustatory sensilla have been rare, so little information is available on the relevant chemical stimuli ticks are able to perceive via these sensilla. Behavioural studies indicate they perceive the assembly pheromones (Leahy et al., 1975), which were identified to be excreted purines such as guanine (Otieno et al., 1985; Dusbábek et al., 1991). The role of palpal sensilla in a crucial element of mating behaviour, the tip-over, begs for a more elaborate description of their sensitivity and specificity to various chemicals.

We should mention here that various authors have pointed to the role of tarsal chemoreceptors in mating behaviour in this and other species (Guerin et al., 1992; Phillips and Sonenshine, 1993). In addition to the palpal organ sensilla, *B. microplus* also carries twelve gustatory sensilla on the tarsus of the first pair of legs (Hess and Vlimant, 1986). Two Tp B and two Tp A are located distally just behind the claws, and ten other Tp A sensilla are located around Haller's organ. Little is known about the physiology of these sen-

silla. Masking experiments suggest that terminal pore sensilla on the tarsi perceive the contact sex pheromone of *Dermacentor* (Phillips and Sonenshine, 1993). However, we have not been able to make satisfactory recordings from these "claw sensilla" in *Boophilus*. It is likely that these sensilla play an essential role in mating and probably other behaviours as well. They are essential for inducing the initial arrestment response (Guerin et al., 1992) and could, therefore, perceive cholesterol esters but they may also be sensitive to the polar compounds in fraction C. Our recordings clearly demonstrate perception of these compounds via sensilla on the palps but it would be wrong to conclude that these hairs house the only receptors for them.

Specificity of Chemical Stimuli in Tick Mating

Mating responses of male ticks to tick cuticular extracts show differing degrees of specificity. Generally the mounting sex pheromone is judged not to be highly specific although differences in cholesteryl esters do occur between species (Sonenshine et al., 1991). The lack of tip-over and reduced arrestment to the cuticle extract of *I. ricinus* suggests that at least in this species the signal relevant to *B. microplus* is either absent or different. However, it is known that interspecific mating occurs between *B. microplus* and *B. annulatus* (Graham et al., 1972) and *B. decoloratus* (Spickett and Malan, 1978). Furthermore, there is no indication that the second signal is specific to any one life stage of *B. microplus*, since tip-over was observed in response to larval and male extracts as well. It may simply be that it is particularly prominent in females shortly after moulting since extracts of the latter proved most active.

In tick species previously investigated, a role for a second more polar signal in mating behaviour in addition to the isolated cholesteryl esters cannot be excluded. The bioassay, employing "delipidised" female ticks, as used by Hamilton and associates (1988, 1989) to isolate and describe cholesteryl oleate as a mounting sex pheromone, has the advantage that the full mating behaviour can be observed, whereas in our bioassay the males cannot locate a gonopore after tipping-over and, therefore, the mating sequence cannot be completed. However, use of delipidised females carries the inherent danger that, while the solvent rinse they employed was effective in removing the cholesteryl ester fraction responsible for arrestment, it may not have succeeded in removing additional products acting synergistically. The mating behaviour was then only restored when cholesteryl esters were added to them. Alternatively, it could be that mating behaviour of *B. microplus* is guided differently from that of *D. variabilis* for which cholesteryl oleate alone in concentrations near those present on the female apparently accounts for all observed behaviour (Hamilton et al., 1989). It seems that in *B. microplus*, 2,6-dichlorophenol is not essential for the arrestment response to cholesteryl esters as is the case for *Dermacentor* species. We have shown here that this product need not be present for the arrestment response and when present does not modify the behaviour. This confirms earlier findings of absence of any evidence of a behavioural response by males to 2,6-dichlorophenol in this species, despite its presence in all life-stages (de Bruyne and Guerin, 1994).

In conclusion, we can postulate that mating in this one-host tick species is guided by a multicomponent chemical stimulus. It involves cholesteryl esters but other more polar chemical signals present on the cuticle of females are more important in inducing the complete repertoire of mating behaviours. Gustatory sensilla on the palps of male ticks probably play a role mediating these responses.

ACKNOWLEDGMENTS

We are indebted to the Hasselblad, Roche, and Sandoz Foundations as well as to the Swiss National Science Foundation (grants 3.609-0.87, 31-28684.90, and 31-37420.93), 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 Misters Rohrer, Jonczi, and Cesari of the Ciba-Geigy Agricultural Research Station, St. Aubin, Switzerland, for supplying us with ticks. We are grateful to Mrs. Knutti for taking care of the rabbits, to S. Grenacher for the *I. ricinus* exuvial extract, and to T. Kröber for the bovine hair extract and for his help with the development of the bioassay. We are also grateful to Miss M. Vlimant for data from microscopic studies on the morphology of tick palpal sensilla and Dr. A. Rawyler for an introduction to the transmethylation method. This paper is part of the Ph.D. thesis of Marien de Bruyne at the University of Neuchâtel.

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