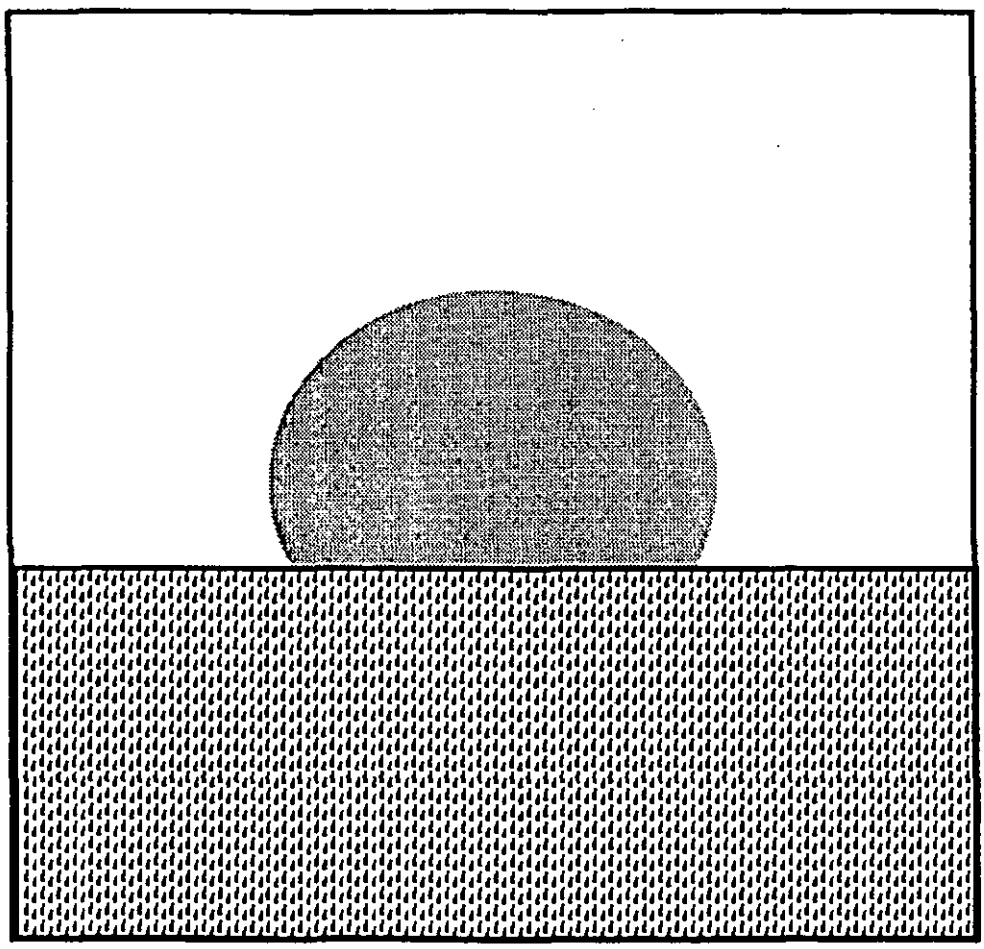
Chemical stimuli in the mating behaviour of the cattle tick *Boophilus microplus*



Marien de Bruyne

(Stimuli chimiques dans le comportement sexuel de la tique du bétail *Boophilus microplus*)

Thèse présentée à la faculté de Sciences de l'Université de Neuchâtel pour obtenir le titre de docteur ès sciences

Université de Neuchâtel 1996 Institut de Zoologie

IMPRIMATUR POUR LA THÈSE

Stimuli chimiques dans le comportement sexuel de la tique du bétail *Boophilus microplus*

de M. Marien de Bruyne

UNIVERSITÉ DE NEUCHÂTEL

FACULTÉ DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel sur le rapport des membres du jury,

Messieurs P. Guerin, P.-A. Diehl, R. Stocker (Fribourg) et J.-L. Connat (Dijon)

autorise l'impression de la présente thèse.

Neuchâtel, le 2 juillet 1996

Le doyen:

R. Dändliker

To the cover: *To most of us this looks like a rising sun over a corn field, radiating a bright day ahead of us. To a male tick this looks like a female tick, firmly attached to the skin of a vertebrate host, promising excellent chances for spreading his genes. The latter organism of course, has got it right.*

"Subjectively, first of all, we are inevitably the centre of perspective of our own observation." (... Scientists) "are now beginning to realise that even the most objective of their observations are steeped in the conventions they adopted at the outset and by forms or habit of thought developed in the course of research; so that, when they reach the end of their analysis they cannot tell with any certainty whether the structure they have reached is the essence of the matter they are studying, or the reflection of their own thought."

Pierre Teilhard de Chardin in "The phenomenon of man" 1955
In French; English translation of 1959

"We live together, we act on, and react to, one another; but always and in all circumstances we are by ourselves. The martyrs go hand in hand into the arena; they are crucified alone. Embraced, the lovers desperately try to fuse their insulated ecstasies into a single self-transcendence; in vain. By its very nature every embodied spirit is doomed to suffer and enjoy in solitude. Sensations, feelings, insights, fancies—all these are private and, except through symbols and at second hand, incommunicable. We can pool information about experiences, but never the experiences themselves. From family to nation, every human group is a society of island universes.

Aldous Huxley in "The doors of perception" 1954

The taste of tick sex

Put in plain English, the subtitle above is basically all this thesis is about,. In science we have the habit of writing in code rather than in prose and certainly not in poetry. This means that every word used should have a precisely defined meaning. So on the cover title I specify "*Boophilus microplus*" as the scientifically correct name of the particular species of tick under investigation, I use "chemical stimuli" rather than taste and I write "mating behaviour" to put my approach to sex in an ethological context rather than suggesting a genetic or a reproductive physiology approach. However, scientists live in a world of illusions just like the rest of the human species and holding to the idea that words are precise is one such illusion.

As humans we largely communicate via auditory and visual cues. We talk or write a lot and make signs and facial expressions. Auditory and visual communication exists in arthropods too but is overall less important than chemical communication. In the discipline of chemical ecology, natural compounds that carry information, used during interactions between organisms are called semiochemicals or more precisely infochemicals (Dicke and Sabelis, 1988). Much has been written about them. Substances mediating specific behaviours have been defined and redefined, ordered in classes and subclasses. Pheromones were defined as chemicals used for communication between two or more animals of a single species (Karlson and Lüscher, 1959). They can be classified in terms of the behaviours they elicit from receiving conspecifics such as aggregation, trail following, dispersal, oviposition and sexual behaviours. However, confusion occurs commonly between behaviour and the function or effect of that behaviour. For instance, the function of an alarm pheromone is clearly to transmit a state of alarm in the local population of a certain species. The actual behaviour it elicits can be different between species, ranging from dropping down from a leaf in aphids, to aggressive defense behaviour in ant soldiers, or simple dispersal. Another complication lies in the definition of behaviour. If a group of randomly placed animals aggregate over a certain amount of time in a specific spot in an arena this is often termed aggregation behaviour. However such an experiment does not say anything about the behaviours involved in each individual animal which lead to the overall effect of aggregation.

A chemical signal emitted by females or males eliciting behaviour from the opposite sex that leads directly or indirectly to mating is usually refered to as a sex pheromone (Shorey, 1973). Throughout this thesis I have avoided the use of such words where possible, precisely because they do have a more or less defined meaning. So even though the topic we are dealing with definitely suggests we are looking for pheromones, I take a careful approach and will not try to run ahead of my results. In part II of the general introduction I will therefore give a review of chemical stimuli that are tick derived or associated with ticks without consistently hanging such confusing labels like "mounting sex pheromone" on them and not rigorously ordering them into arbitrary classes.

The three plain English words used above (tick, sex and taste) do mark the biological stage that we set for ourselves. Ticks are the very peculiar group of animals that we in our department are so familiar with but that need introduction to the reader who is generally more confronted with other arthropods. Most work on pheromones has been done on insects and comparisons are often made but not always valid.

PREFACE

Sex is one of two important resources for any animal, the other being food. Most chemical signals that scientists have decoded carry information about food sources or mate quality. Arguably, sex is the most important of the two. The role of the whole biological machinery of an organism is ultimately to reproduce. Food is only the fuel for the engine. This does not mean that the chemical signals involved could not be similar or the same. Why animals have sex, *i.e.*, exchange gametes, in order to reproduce is actually still a big question to which the answer is not so well known as suggested in some first-grade text books.

With the word taste we enter into the fascinating realm of perception. Ticks live in a world of illusions just as humans do. All animals, by means of their nervous systems, respond to changes in their environment by adapting their behaviour to it. The illusion in the mind of the tick and the corresponding behavioural response can be evoked by the experimental scientist by manipulating the appropriate physical elements of the right environment. What are those elements that evoke the response that ultimately allows the individual, and with it the species, to survive in its natural environment and how does he perceive them? By writing taste I already suggest that the dominant sensory modality in tick sex is chemical. I will argue here that this is at least partly the case. Incidentally, the title might just as well have been "the smell of tick sex" were it not for the fact that I have not been able to show a behavioural function in mating of the only smelly compound I have isolated. The difference between smell and taste is anyway hard to define. Thus I have set the stage for a study on sensory input and behavioural output, involving stimuli of a chemical nature that play a role in mating of a particular species of tick.

This brings us to the technical aspects of my work. Throughout, I have tried to address three differents aspects of this study. Hence a multi-disciplinary approach was inevitable, involving electrophysiological techniques for measuring sensory input, behavioural experiments for motor output and analytical chemistry for resolving complex mixtures of compounds. These techniques, each related to different domains of science have been employed here mainly to try to answer some proximate questions, asking ourselves how things work rather than why things work that way. The main body of the results arising from this approach therefore only allows me to answer such questions. In the discussion I will, however, also try to address some of the ultimate questions, *i.e.*, the why's of mating behaviour in the cattle tick and draw comparisons to what has been described for other ticks.

CONTENTS

Boophilus microplus a special kind of tick

1.1. Ticks; a group of obligate blood feeding Arthropods

Ticks (Acari: Metastigmata; see TABLE 1) are members of the Arthropod subphylum Arachnida and just like spiders, harvestmen and scorpions differ from insects and crustaceans by the lack of antenna and mandibles, but possess the characteristic scissor-like appendages called chelicera. Together with the omnipresent mites, they form one of the most species rich taxa in the Animal kingdom; the Acari. Among the Acari, that have radiated from the predatory lifestyle so common in other classes of Arachnida to almost every lifestyle and habitat imaginable, ticks stand out as a small group of specialised ectoparasites of relatively large body size that feed on blood of vertebrates. Because of their size and detrimental effect to human economy and health, they have been long since known to man and were referred to as "disgusting parasitic animals" by Aristotle in the 4th century BC. Unfortunately, man's opinion about these fascinating creatures has not changed much over the last 2300 years. The group, sometimes referred to as superfamily Ixodoidea, sometimes as suborder Ixodida or metastigmata, consists of three families. The Argasidae or soft ticks are considered most primitive. They are ectoparasites of small mammals and birds that feed intermittently, taking short (from a few minutes to several hrs) bloodmeals. The Nutalliellidae, exhibit some characters shared with either Argasidae or Ixodidae, as well as some unique features but are represented by only one species from Africa, of which only a few female specimens are described. Finally, the Ixodidae or hard ticks are characterised by the presence of a sclerotized shield (scutum), covering the dorsal side of the idiosoma either completely in males, or only partially in females (FIG 1). They are generally parasites of reptiles or mammals often developing close associations with their hosts. Each life stage takes a long (several days) bloodmeal while staying firmly attached to the host's skin.

TABLE 1: Systematic position of *Boophilus microplus*. Higher order systematics after Krantz (1970)

taxon	*name*	*characters*
Phylum	Arthropoda	Body segmented, jointed appendages
Subphylum:	Chelicerata	No antennae or mandibles
Class	Arachnida	Two pairs of mouthparts (chelicera and pedipalpi) and 8 legs in nymphs and adults, 6 legs in larvae
Subclass:	Acari	Ticks and mites, without any clear body segmentation, possessing a gnathosoma (false head)
Order	Parasitiformes	
Suborder	Metastigmata	Respiratory openings grouped behind coxa IV, Haller's organ.
Superfamily	Ixodoidea	Same as Metastigmata, *i.e.*, ticks
Family	Ixodidae	Hard ticks, posses a sclerotized dorsal shield (scutum) and mouthparts are visible from above. Palpal tarsus (segment IV) small and placed ventrally on segment III.
Subfamily	Rhipicephalinae	
Genus	Boophilus	small, inornate, no apparent festoons, palpal article I reduced, short mouthparts
Species	microplus	

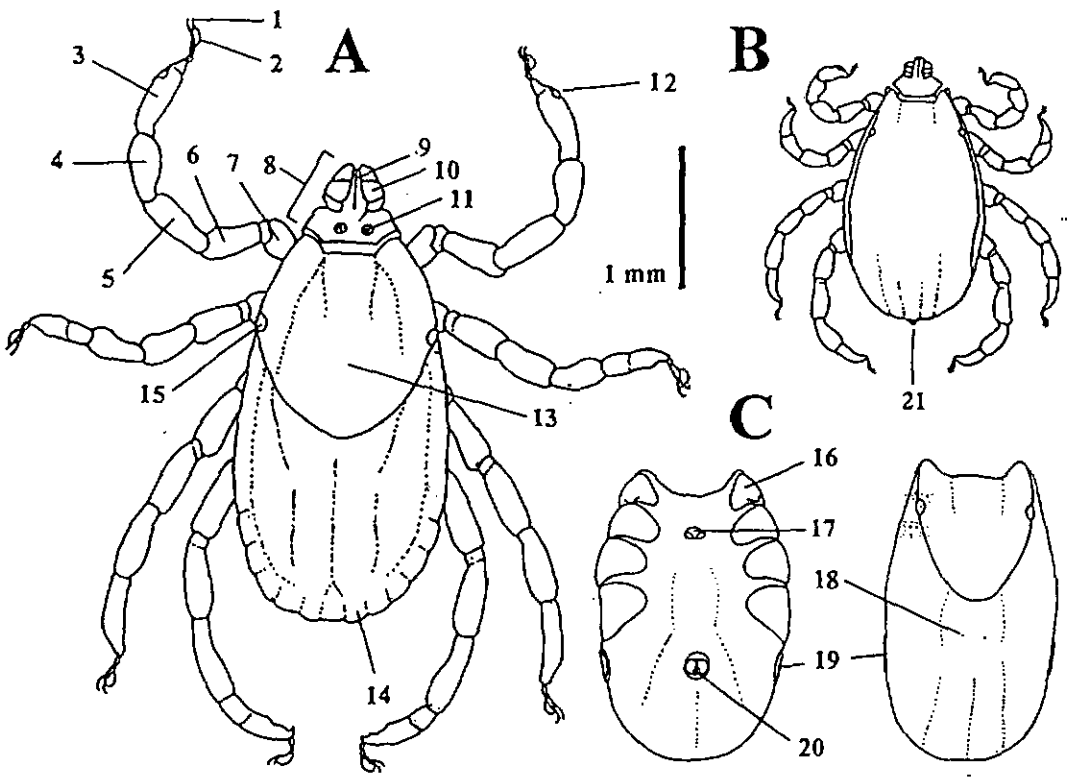


FIGURE 1. Elements of tick morphology as demonstrated on two species of the Rhipicephalinae **A:** *Rhipicephalus sanguineus* (Latreille) unfed female, dorsal view, after Pomerantzev (1959). **B:** *Boophilus microplus* (Canestrini) male, dorsal view **C:** *B. microplus* undistended female, ventral (left) and dorsal (right) views of idiosoma (body). Morphological features include: 1. claws, 2. pulvillus, 3. tarsus, 4. tibia, 5. genu, 6. femur, 7. trochanter, 8. capitulum (gnathosoma), 9. chelicera, 10. pedipalp, 11. porose areas, 12. Haller's organ, 13. scutum, 14. festoon, 15. eye, 16. coxa, 17. gonopore (genital aperture), 18. fovea dorsalis, 19. spiracular plate, 20. anus, 21. caudal process.

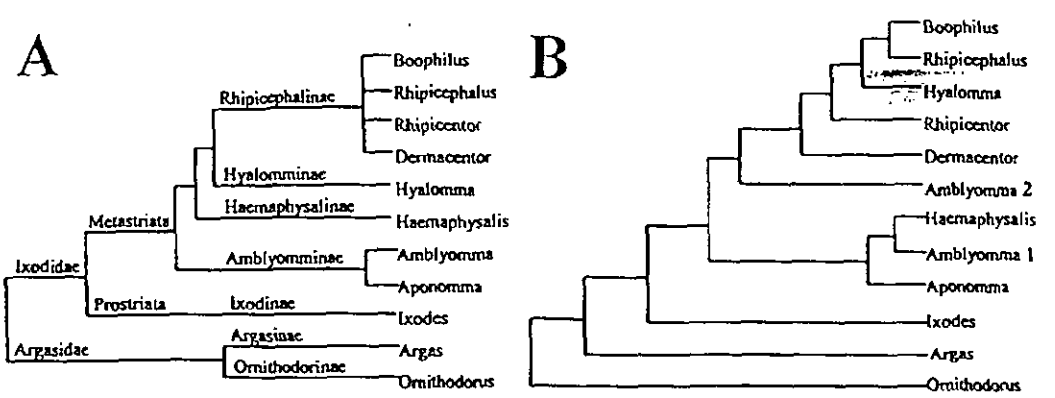


FIGURE 2. Two proposed phylogenies of important tick genera **A:** After Hoogstraal and Aeschliman (1982) based on morphological characters and host associations. **B:** After Black and Piesman (1994) based on mitochondrial DNA analysis. Species of the genus *Amblyommo* are found in two separate groups, shown by numbering them as 1 and 2.

An overview of tick systematics was given by Keirans (1992) with a complete list of valid species; there are some 850 species of ticks in the 3 families and 19 genera. Fossil evidence for the time and place of origin of ticks is virtually absent, so most theories on tick evolution are based on assumptions and extrapolations of present day distribution, host associations and morphologies. The scenario of the development of the various

tick genera that is now most commonly agreed upon is that produced by Hoogstraal and Aeschlimann (1982) (FIG 2A), although it's assumptions have recently been criticised (Klompen *et al.*, 1996). Tick taxonomy and evolutionary biology is extremely rudimentary compared to that of insects. In fact, no formal cladistic analysis had been made until Black and Piesman (1994) published a phylogeny of the important tick genera based on mitochondrial DNA. They managed to confirm largely the phylogeny proposed by Hoogstraal and Aeschlimann but with some important discrepancies (FIG 2B). Both phylogenies, however, place the genus *Boophilus* in close relation with *Margaropus* species and the genus *Rhipicephalus*. The genera *Amblyomma*, *Aponomma* and *Haemaphysalis* branch off before and the members of the genus *Ixodes* (Prostriata) are considered the most primitive Ixodidae. It is the special place of the Boophilids among the ticks that will be discussed here as it is exemplified by the experimental animal of our choice *Boophilus microplus* — a tick species that is special in more than one respect.

1.2. *Boophilus microplus:* a special case

1.2.1 Taxonomy and distribution

The members of the genus *Boophilus* Curtice 1891 (type species *B. annulatus*) are all small inornate tick species that are highly specialised parasites of Bovidae. They can be readily distinguished by the apparent absence of festoons, relatively short stout mouthparts, presence of thick ridges on the palps and reduction of article I (Arthur, 1960, FIG 1). Hoogstraal and Kim (1985) postulate that short broad mouthparts and small body size are advanced characters and *Boophilus* then represents an extreme case. This genus is probably one of the most recently evolved Ixodid genera and speciation might still not be completed (Aeschlimann, personal communication). There are now five species of *Boophilus* almost universally recognised:

TABLE 2: The five commonly recognised species of *Boophilus*.

B. microplus	(Canestrini, 1877)	Southern cattle tick (US), Asian blue tick (RSA), Common cattle tick (Arg.), Cattle tick (Aus.)
B. annulatus	(Say, 1821)	Cattle tick of the western hemisphere (US), In Russian literature also as *B. calcaratus*
B. decoloratus	(Koch, 1844)	African blue tick (RSA)
B. geigyi	Aeschlimann & Morel, 1965:	no common name known
B. kohlsi	Hoogstraal & Kaiser, 1960:	no common name known

A question has arisen as to whether *B. annulatus* and *B. microplus* are distinct species since hybridization occurs. Even though F1 females appear normal, the testes of F1 males are absent or vestigial, leading Newton *et al* (1972) to conclude they are separate biological species. *B. annulatus* and *B. microplus* can be easily separated from the members of the *B. decoloratus* group by the absence of a conspicuous seta on a protuberance of the ventromedial side of the first palpal segment. The fact that the form of the genital opening is also markedly different for the two groups (Feldman-Muhsam and Shechter, 1970) confirms their distinctness. The genital opening of *B. decoloratus, B. geigyi* and *B. kohlsi* resembles that of the *Rhipicephalus sanguineus* group.

The present day distribution of the five species is strongly influenced by man's habit of transporting cattle around the world (FIG 3). It is believed that originally *B. microplus* was indigenous to the oriental region only (Morel, 1969) whereas *B. annulatus* probably originates from the Near East. The latter species has spread

around the Mediterranean and was probably introduced in the Americas and West-Africa with Iberian cattle. *B. microplus* has been introduced into Madagascar and East-Africa on Indian cattle breeds and from Madagascar it was transported to South-Africa in 1896. It too has reached South-America from where it has spread to its present distribution. Morel (1969) also suggests that the spread of *B. decoloratus* into West-Africa's savannah was secondary. This means that originally the five species could have been distributed either parapatrically or allopatrically with *B. microplus* in tropical Asia, *B. annulatus* in the West and Central Asian steppes. and *B. decoloratus* and *B. geigyi* in East- and West-Africa respectively. *B. microplus* was probably introduced in Australia with Brahman or Balinese cattle from Indonesia in the nineteenth century (Roberts, 1965). Roberts also comments on the large variability between populations of this species in certain morphological characters such as the size and shape of the adanal plates, the caudal process in males and the scutum in females; with most characters varying considerably, even between individuals from the same host. This points to the high morphological variability generally observed in this species, complicating species determination. Incidently, Spicket and Malan (1978) noted that when Australian *B. microplus* are mated with South African ones females produce very little viable offspring suggesting the existence of sibling species.

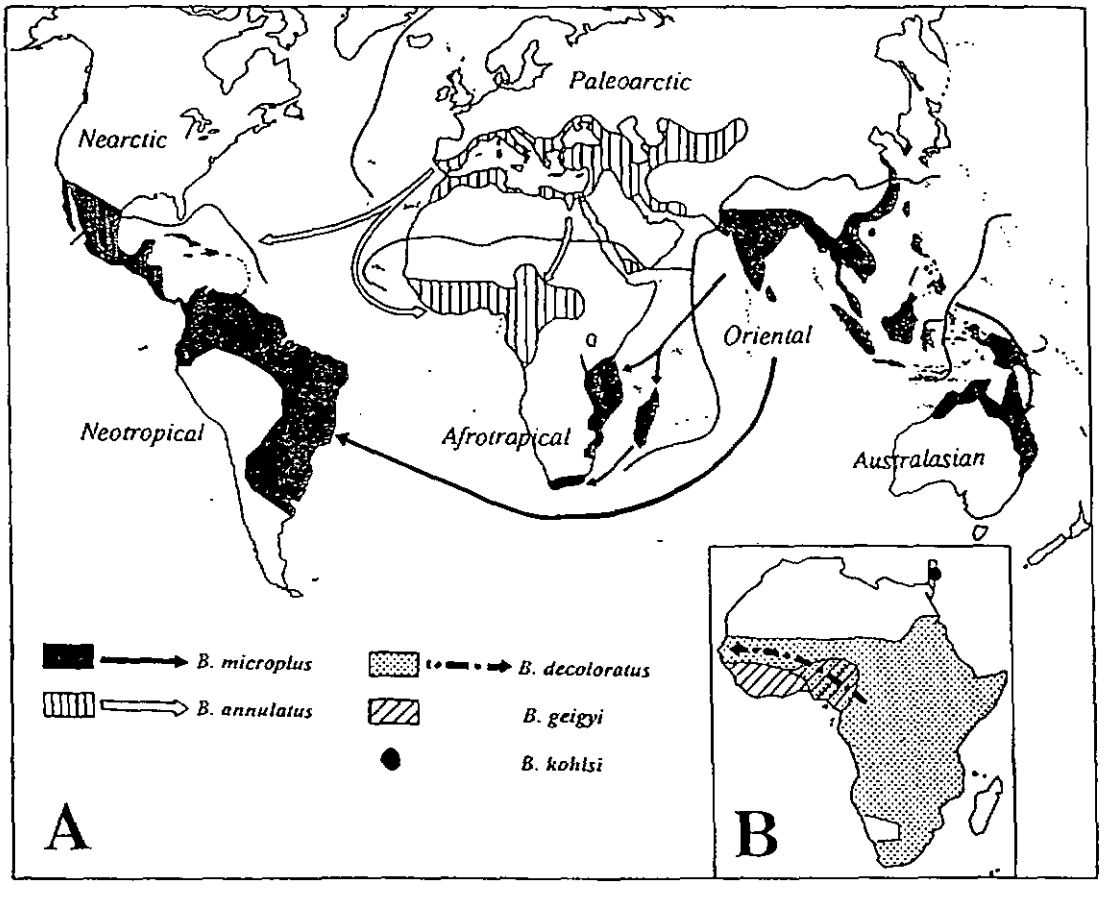


FIGURE 3. Distribution of the five recognised *Baaphilus* species in the world. A: Worldwide distribution of *B. microplus* and *B. annulatus* with arrows indicating presumed secondary spread through cattle importations. B: species of the *B. decoloratus* group in Africa and the Near East. Modified after Morel (1969), Feldman-Muhsam and Shechter (1972), Pomerantzev (1950), Roberts (1965), Wharton (1974) and Rawlings and Mansingh (1987).

1.2.2 Host specificity and life cycle

Not only is the sole source of nutrients for Ixodid ticks found in vertebrate blood but their whole life history is strongly correlated with that of their host. Unlike most of the Argasidae, hard ticks attach for several days to the skin and thus pass a considerable part of their life in the particular chemical, thermal and mechanical environment of the host's skin. Most of the important processes in their life-cycle take place there, even though the time spent off the host may be much longer. Ecologically, ticks can be divided in endophilic and exophilic species (Sonenshine, 1993). The former, also called nidicolous, are closely associated with caves, nests or burrows of their host while the latter live freely in various biotopes and need to somehow locate their hosts more actively. Exophilic ticks employ one of two strategies to do this. They either actively hunt for the host or sit and wait. *B. microplus* is a typical example of the latter "ambush" type. It has considerably limited the duration of the "wait" part of the strategy by going through a non-parasitic phase only once in its lifetime, *i.e.*, as larva.

More than 90% of the known Ixodid tick species exhibit the typical three-host life-cycle (Hoogstraal and Aeschlimann, 1982) where each life-stage; larva, nymph and adult, feed on different individual host animals and drop to the ground between bloodmeals to moult. The three hosts may be from different species or from the same species, as is necessarily the case with endophilic tick species. Some species in the genera *Hyalomma* and *Rhipicephalus* have developed either an obligatory or facultative two-host life-cycle in which larvae and nymphs feed on the same individual host. The one-host life-cycle, with all stages feeding on the same host and even moulting *in situ* is almost unique to *Boophilus* and *Margaropus* species and may have evolved only once in this group. The three species of the genus *Margaropus* are closely related to *Boophilus* and are exclusively African parasites of giraffes or horses. Only a few other species show a one-host pattern, sometimes related to extreme weather conditions notably the winter or moose tick, *Dermacentor albipictus*. The larvae of *B. microplus*, being the only free living life stage, are therefore responsible for the host specificity of this species.

Most Ixodid species exhibit some form of host specificity and many species have members of the large mammalian group of Ungulates as hosts, especially in the adult stage (Hoogstraal and Aeschliman, 1982). Together with members of the genus *Aponomma*, highly specific for certain reptiles, *Boophilus* species are among the most host specific of ticks with all stages predominantly feeding on members of the Ungulate family Bovidae. *B. microplus* specialises on cattle and buffaloes, occasionally parasitising horses (Equidae). The latter phenomenon seems to be more prevalent in *B. decoloratus* which is a bit less specific, while *B. kohlsi* is almost exclusively found on goats (Feldman-Muhsam and Shechter, 1970).

The life-cycle of *B. microplus* is relatively short (Nuñez *et al.*, 1985). Under favourable conditions (warm and humid, *i.e.*, tropical) engorged females take about 14 days, after dropping from the host, to lay most of their eggs. These in turn hatch within 20 days, again depending on temperature, and larvae climb vegetation to attach to a passing host after 7 days (Davey, 1987). Development is rapid once the larvae attach to a suitable host and, most importantly, independent of climatic conditions. After 6 days on the host, nymphs appear that will moult into adults 7 days later. The first engorged females drop only 20 days after the larvae attached. By comparison, the parasitic phase of *D. albipictus* is strongly influenced by climate and takes up to 150 days in winter (Drew and Samuel, 1989). Thus, unlike most ticks which complete one life-cycle per year or less and at times include a diapause (Belozerov, 1991),

B. microplus can make it through a full cycle in just 60 days and can have up to 6 generations per year. Moreover, egg production is high, averaging 2500 per female and eclosion is usually around 80%. Even though mortality on the host can be remarkably high, this tick has a high potential for rapid population increase in pastures regularly grazed by cattle (Mount *et al.*, 1991).

1.2.3 Economic importance

Ticks are second only to mosquitoes as important vectors of disease. In fact the first demonstration of transmission of a disease by an arthropod vector was when Smith and Kilborne implicated *B. annulatus* in the causes of Texas fever in 1893 (Uilenberg, 1992). A great variety of diseases are transmitted to domestic animals. Human diseases are less prevalent, though the discovery of Lyme disease in 1977 has led to a renewed interest in tick biology. Members of the genus *Boophilus* are efficient vectors of two important cattle diseases: Babesiosis (Texas fever or red water) and Anaplasmosis (gall sickness). *B. microplus* transmits highly pathogenic agents that cause these diseases, *Babesia bigemina, B. bovis and Anaplasma marginale* world-wide. Apart from transmitting diseases, these ticks also cause anaemia, weight loss, reduced milk production, secondary infections and diminished value of hides.

The cattle tick is undoubtedly one of the most important pests of domestic animals (Wharton, 1974). It causes considerable damage to the cattle industry, particularly in areas where extensive cattle ranching is an important source of income such as the southern US, Northern Argentina, South Africa and Australia (including New Guinea and some Melanesian islands). Interestingly, there are less problems with *B. microplus* in India and other South Asian regions since Indian cattle breeds (*Bos indicus*) develop resistance to this tick and hence they occur in relatively low numbers on Zebu and Brahman cows (Wharton and Norris, 1980). The various European (*Bos taurus*) breeds used mostly in ranching are, however, highly susceptible and develop heavy tick loads. If no control measures are taken, some animals can die simply by loss of blood or secondary infection of wounds. In the US, where *B. microplus* occurs together with *B. annulatus*, heavy losses and expensive control measures led to a major campaign in the early half of this century and the two species have been eradicated from Texas and Florida. Ever since, a quarantine zone has been permanently maintained along the Mexican border to prevent reintroduction. In South Africa, *B. decoloratus* is less of a problem, but there are reports that *B. microplus* is spreading and replacing this species in certain areas (Norval and Short, 1984).

In Australia *B. microplus* is prevented from being introduced to areas outside its present range by continuous expensive control measures which are helped by climatic conditions that do not favour permanent establishment. However, since most of the methods used are chemical, the biggest problem in control of *B. microplus* is its capacity to develop resistance to the acaricides employed. This species has developed resistance against all known classes of acaricides even to the relatively recently introduced synthetic pyrethroids (Nolan, 1985). Especially in Southern Queensland, at the fringes of the tick's distribution range, numerous strains are characterised by different simple- and cross-resistance mechanisms (Wharton, 1974, Nolan, 1985). The variety of *B. microplus* that is used in our experiments is one such organophosphorous resistant strain from Biarra, Southern Queensland, discovered in 1966. Recent developments have led to the emergence of a commercial vaccine against antigens in the tick's gut cells as a new hope in the continuing struggle against *B. microplus* (Willadsen *et al.*, 1995). It is the first time that such a vaccine has been developed

against an arthropod — another illustration of the unique economic importance of this tick

1.2.4 Reproductive biology

Autogeny and repeated gonotrophic cycles occur in Argasidae but Ixodid ticks go through one single gonotrophic cycle and feeding is necessary for the females to produce a batch of eggs (Oliver, 1989). In the prostriata (genus *Ixodes*) mating can take place before or during feeding and spermatids are produced in the late nymphal and early adult stages. All metastriate ticks mate on the host during feeding and a blood-meal is necessary for males before meiosis and spermatid production can occur. Allmost all tick species are bisexual and generally produce a 1:1 sex ratio among progeny. Parthenogenesis has been reported as possible in many species of ticks including *B. microplus* (Stone, 1963, Thompson *et al.*, 1980), but is probably of little practical significance since larvae produced are barely viable. Some *Haemaphysalis* species however have parthenogenetic geographical races and it is the sole method of reproduction of at least two species of *Amblyomma* (Oliver, 1983, 1989). Copulation consists of transfering incapacitated spermatids via a spermatophore which is deposited onto the female gonopore (Feldman-Muhsam and Borut, 1983). The spermatophore is a complicated structure (Oliver, 1982) which, after being deposited, evaginates into the female tract, leaving part of it outside. It contains proteins, sugars, symbionts and a polypeptide that regulates final sperm maturation (Shepherd *et al.*, 1982) as well as a proteinaceous factor which signals the presence of sperm to the reproductive system of the female and initiates vitellogenesis (Diehl *et al.*, 1982, Sahli *et al.*, 1985). Rapid feeding and subsequent engorgement is similarly stimulated by a factor present in the spermatophore (Pappas and Oliver, 1972). In most species, males are known to be able to copulate repeatedly with the same or different females, *i.e.*, several spermatophores are regularly found in female genital tracts.

Sensory physiology of ticks

All animals need to be informed about their environment, in order to escape its dangers (desiccation, freezing, predation) and to utilise its resources (food, shelter, mates). This information is present in numerous forms (thermal, radiated, mechanical and chemical stimuli) and animals have developed various sensory systems to perceive them, concomitant with such behaviours that enable them to adapt to, or change the environment, so as to maximise their fitness. Ticks possess relatively simple sensory systems that nevertheless are sufficient for their survival. It is therefore of considerable biological interest to study their physiology and we have set about doing so in the context of the mating behaviour of *B. microplus*. At the peripheral level, arthropod sensory systems usually include a variety of small organs embedded in the cuticle called sensilla (see Zacharuk, 1985). They contain 1) sensory neurons, 2) some non-sensory associated cells and 3) a modified zone of cuticle very often in the shape of a hair, plate or pore. The typology of such structures has been described for insects (Altner and Prillinger, 1980) but analogous sensilla can be found in ticks (Hess and Vlimant, 1986). For a detailed summary of the distribution of sensilla on the legs and palps of *B. microplus* see appendix II. We can now examine the potential sensory cues that can play a role in mating by looking briefly at the different senses in ticks in general and *B. microplus* in particular.

Heat

A source of warmth is generally accepted to be the major cue in inducing feeding for different species of ticks. It also plays an important role in host location. Ticks readily orient to warm objects (Totze, 1933, Krijgsman, 1937, Lees, 1948) but these reactions have been observed over relatively short distances. This, and some experiments with low emissive material, led Lees (1948) to believe that only air temperature was involved (convected or conducted heat) while radiated heat was not perceived. Experiments with *A. variegatum* however, indicate heat perception over much larger distances where infrared radiation might play a role (Robert, 1985, Poffet, 1988). A cold receptor was first described in a terminal pore (tp) sensillum on the anterior tarsi of *R. appendiculatus* (Waladde *et al.*, 1981) and later warm and cold receptors were characterised in two no-pore (np) sensilla more distally on the anterior tarsi of *A. variegatum* (Hess and Loftus, 1984). The resolving power of these units for abrupt temperature changes was below 1°C. Although the first pair of legs was known to play a prominent role in orientation to heat sources (Lees, 1948), it has also been observed that masking or ablation of the anterior tarsi does not lead to complete insensitivity to heat (Totze, 1933). As mating of *B. microplus* takes place on the host, a relatively high temperature and possibly a steep gradient are part of the natural environment for this tick.

Humidity

The importance of humidity for the survival of ticks is well documented. As small animals, exposed for long periods to differing climatic conditions, ticks need to deal with the problem of desiccation. Oriented responses to humidity have been shown to be greatly influenced by physiological state. Lees (1948) recorded a marked avoidance (negative taxis) of sudden increases in humidity. Longer term reactions include kinetic

effects, which, depending on the hydration state, resulted in arrestment of ticks in areas of higher humidity. Hygroreceptors have so far not been demonstrated in ticks but may be present in the anterior pit of Haller's organ. Attached ticks and those moving through the pelage of their bovine hosts are permanently exposed to high humidities (Kuhnert, 1995).

Light

Ticks do not possess compound eyes like insects, nor do they have big simple eyes with well developed lenses as spiders do. Some ticks are reported as eyeless because no apparent eye-like structures are present on the cuticle (*e.g. Ixodes* and *Haemaphysalis* species). However, Binnington (1972) has shown that even such eyeless species have photoreceptors at several points under the cuticle and optical nerves with corresponding centres in the synganglion. Clear responses to direct light have been shown in eyeless ticks such as *Ixodes ricinus* (Lees, 1948). Light perception is probably monochromatic, with sensitivity peaking around 500 nm (blue/green) and a marked low sensitivity beyond 600 nm (red), as has been demonstrated by electroretinogram (ERG) recordings in eyes of *D. variabilis, A. variegatum* and *H. dromedarii* (Carrroll and Pickens, 1987, Kaltenrieder *et al.*, 1989). Many ticks, including *B. microplus*, have been shown to orient away from light sources and respond to changes in overall light intensity (Totze, 1933). Lees (1948) demonstrated that this negative phototaxis disappears with ageing in unfed *I. ricimus* but is restored in engorged ticks. A sudden decrease in light intensity (shading) leads to questing activity in host seeking ticks in repose position. In addition, a marked orientation toward areas of lower intensity of reflected light (*i.e.* darkness, skototaxis) was present in this species. Some hunting ticks such as *Hyalomma asiaticum* and *H. dromedarii* apparently use this to focus on a silhouette during the approach to a host (Leonovich, 1986, Kaltenrieder *et al.*, 1990). The eyes of *B. microplus* are not well developed but are visible under the microscope as a slightly elevated and modified zone of cuticle at the edge of the scutum. There is no apparent order in the arrangement of photoreceptive cells. A certain sensitivity in *B. microplus* larvae to shading has been demonstrated (Waladde and Rice, 1982) and can therefore be expected in adults too. However, it is unlikely that visual perception of mates plays a major role in mating behaviour.

Mechanical stimuli

Waladde and Rice (1982) have observed that sound (*i.e.*, air borne oscillations) in the 80-800 Hz range activates *B. microplus* larvae. However, chordotonal organs have never been found in ticks and the only convincing report for orientation to sound is from an argasid tick species, *Ornithodorus concanensis* that is attracted to the chirps (3-8 kHz) produced by its bird host (Webb *et al.*, 1977). These authors imply that the first pair of legs is involved in the perception. Vibrations (*i.e.*, substrate borne oscillations) are an important means of communication and prey location in spiders and scorpions. These stimuli are perceived by so-called slit sensilla, small intracuticular mechanoreceptive sensilla (Brownell and Farley, 1979). A number of different but similar structures has been found on the legs of ticks (Hess and Vlimant, 1984 & 1986). These structures may be involved in the perception of vibrations and/or sounds, as was previously suggested by Schulze and Schröder (1949). Unfortunately, very little research has been done on the perception of vibrations by ticks. *A. variegatum* can perceive strong vibrations in much the same range as described by Waladde and Rice

(30-300 Hz; Stämpfli-Pauchard, 1989) but reports that *Hyalomma* species respond to vibrations in the sand produced by the tread of camels and other large mammals have so far not been confirmed (Leonovich, personal communication). Another possible role of these mechanoreceptor organs is in the perception of gravitational pull (Robert, 1985). Interesting responses have been described by Lees (1948) in *I. ricinus* when climbing up and down vertical structures. These ticks are capable of evaluating the difference between upward and downward direction and get arrested just below the tip of vertical rods after several up and down walks. However they show no preference for top or bottom end of a tube with closed ends, confirming results obtained by Krijgsman (1937) with *B. annulatus* larvae.

Other tactile stimuli such as physical disturbance, substrate contact or texture probably play a major role in tick sensory ecology. Ticks questing for hosts on vegetation respond to mechanical stimuli and attach to passing adequate substrates (Lees, 1948). This is the principle behind the flagging method so often used to collect free-living stages of ticks. Ticks show distinct preferences when offered substrates with different textures (Totze, 1933). Another commonly observed phenomenon is the tendency to push into crevices and assemble at edges and obstacles (thigmotaxis) noted by many authors but never properly quantified. Male *B. microplus*, when removed from their host, are arrested by and attach to any rough surface or in corners and edges (personal observations and Kuhnert, 1995), whereas larvae form clumps by holding on to one-another. However, some of these effects can be explained without the explicit need for sensory perception. Ticks do bear many mechanoreceptive hairs with uninnervated shafts (np sensilla) on legs and other body parts (Hess and Vlimant, 1986, Vlimant unpublished). Like those described in insects, they posses the enlarged dendritic structures called tubular bodies, associated with the flexible membrane of the sensillar socket at the base (Altner, 1977). They differ from insect mechanoreceptors by having two tubular bodies instead of one which is characteristic for Arachnida (Foelix, 1985). These bodies are placed on opposite sides of the sensillum and might thus be more sensitive to the direction of deflection of the hair. Ticks generally show negative anemotaxis (Lees, 1948). Although the receptors involved in this orientation to wind are not known they probably are mechanoreceptive seta.

Chemical stimuli

The discrimination between olfaction and taste (gustation) is based on definitions that differ widely between various fields of interest (Chapman, 1995). Henceforth we will define olfaction as the sense carried by wall-pored sensilla (wp) with shafts innervated by sensory cells that probably project directly to a defined group of nerve cells in the glomeruli of the central nervous system. In contrast taste sensilla are typically terminal pored (tp) and the first synapses of their receptor cells are in the region of the brain directly linked with the body region on which they lie (palpal lobe, pedal lobes etc.). No assumptions are made about criteria used in definitions elsewhere such as the medium of transport of chemical cues, the nature of the chemicals perceived, the transduction mechanisms involved or the specificity/sensitivity spectrum of receptors.

Ticks are characterised, among other things, by Haller's organ, a compound sensory organ consisting of a capsule and an anterior pit on the dorsal aspect of the tarsus of leg I. Originally described by Haller in 1881 as an auditory organ, its structure has since been accurately investigated and found to comprise various sensilla with a relatively stable number of sensory cells and a gland associated with the capsule (Foelix and Axtell, 1972, Balashov and Leonovich, 1976, Hess and Vlimant, 1986). An

olfactory function for this organ was first suggested by Lahille (1905) based on observations on *B. annulatus*. Hindle and Merriman (1912) then unequivocally established the location of olfaction on the tarsus of the fowl tick *Argas persicus* (Argasidae). It has since been established that more olfactory sensilla are present on the surface of the anterior tarsus outside Haller's organ but none have been found elsewhere. These olfactory wall-pored (wp) sensilla are of two types: single-walled (wp-sw) and double-walled (wp-dw). The former have branched dendrites innervating the shaft whereas the latter have unbranched dendrites and cuticular canals running between the two walls (spoke wheel structure, Foelix and Axtell, 1971). Binnington (1987) showed that sensory nerves from the anterior legs of *B. microplus* pass the pedal ganglion and project onto olfactory glomeruli. Thus the olfactory system seems analogous to that of insects with leg pair I functioning like antennae. This is also clear from behavioural observations (Lees, 1948) where leg waving suggests a function of air-sampling similar to that shown by insect antennae. Lees (1948) also demonstrated the important role of olfactory stimuli in host seeking by *I. ricinus*.

In spite of its considerable importance, the entire olfactory system in ticks comprises only *ca* 90 peripheral nerve cells (Hess and Vlimant, 1986) as compared to *ca* 40.000 sex pheromone receptors alone in male moths (Boeckh *et al.*, 1965). Roughly half of these are located in Haller's organ. The physiological responses of some of these cells have been characterised (Sinitsina, 1974, Haggart and Davis, 1979, 1980, 1981, Waladde, 1982, Steullet,1993) and in *A. variegatum* responses were found to CO_2, H_2S, NH_3, short fatty acids, ketones, lactones, and aldehydes which have all been isolated from host odours (Steullet,1993). In addition, various phenolic compounds as well as benzaldehyde- and salicylaldehyde-based compounds which can be both host- and/or tick-produced are perceived by olfactory hairs on the tarsus. It seems that the tick olfactory system uses a minimum amount of cells in an optimal way with very little replication of receptor types.

Responses to gustatory stimuli have been poorly investigated. In fact, early authors suggested that a sense of taste was absent in ticks (Totze, 1933). However, several authors have since shown that terminal pore (tp) sensilla are present on the tarsi, especially of leg I, and on the pedipalps. In addition, such sensilla are located on the idiosoma as well (Vlimant, personal communication). A role in the mating behaviour of three ixodid ticks has been suggested for the so-called claw sensilla at the tip of the tarsus 1 (Phillips and Sonenshine, 1993) but physiological data for these and other tarsal contact chemoreceptors are lacking except for reports on responses to salts of one of the paired claw sensilla in two *Ixodes* and one *Hyalomma* species (Elizarov, 1963, Guerin *et al.*, 1992). The compact group of tp sensilla on the apical surface of the palpal segment IV has been suggested to play a role in perception of an off-the-host contact pheromone in some Argasid ticks and *A. hebraeum* (Leahy *et al.*, 1975a, Rechav *et al.*, 1977). This "palpal organ" is probably a complex mechano-gustatory organ capable of perceiving many different contact stimuli (Grenacher, personal communication). Additional gustatory receptors are described in pores in the hard cuticle of the cheliceral digits of *B. microplus* (Walladde and Rice, 1977) and electrophysiological recordings from these sensilla in *Dermacentor* species revealed responses to 20 hydroxy-ecdysone (Taylor *et al.*, 1991).

Summarising we can state that the signals most likely involved in the mating behaviour in *B. microplus* are of chemical and/or mechanical nature perhaps modified by thermal and humidity cues.

Tick associated chemical stimuli in Tick biology*

Many tick species have been investigated for the role of chemical signals in their behaviour but research has mostly concentrated on economically important species most notably *Dermacentor variabilis* and *D. andersoni*, two American ticks that infest dogs, various cattle pests such as *Amblyomma* species and *Rhipicephalus appendiculatus*, the camel tick *Hyalomma dromedarii* and the fowl tick *Argas persicus*. With regard to the use of infochemicals in intraspecific communication no clear picture has emerged of the distribution, variety and common principles within the Ixodidae. There is practically no knowledge available for *Boophilus microplus*, excepting an early report by Chow et al. (1972). In view of its economical importance, but maybe more because of its special position in tick biology, an investigation into chemically mediated behaviour of this species is clearly needed. Before proceeding with such a study it is appropriate to give a brief introduction to chemical signals mediating behaviour in other species of ticks, restricted to those stimuli associated with ticks rather than their hosts or their environment.

3.1. Volatile signals

For most Ixodid tick species the first and major task to accomplish in life is to locate a host. It is on the host that they will find both food and mates. Volatile signals from the host play a crucial role in helping ticks to reach this goal and various host odours have been shown to excite olfactory receptors in the tick *Amblyomma variegatum* (Steullet, 1993). It is in this same genus of ticks that volatile signals, produced by ticks already feeding on the host, were found to play a role in host seeking when combined with CO_2 (Norval *et al.*, 1989). This signal, produced by male ticks, is now commonly referred to as "aggregation attachment pheromone" or also "attraction-aggregation-attachment pheromone" and was first described as attracting female and male *A. maculatum* on the host to feeding males. Its presence was necessary for females to attach to a host (Gladney 1971, Gladney *at al.*, 1974). Such a signal is also present in *A. hebraeum* and attracts nymphs as well (Rechav et al., 1976, 1977). Schöni *et al.* (1984) isolated 2-nitrophenol, methyl salicylate and nonanoic acid from extracts of fed male *A. variegatum* and showed that this mixture of compounds induced aggregation as well as pairing of males and females in a bioassay off the host. It is probably produced in dermal glands on the ventro-lateral cuticle of male ticks and only after a few days of feeding (Diehl *et al.*, 1991). Apps et al. (1988) isolated benzaldehyde instead of methyl salicylate and 2-methyl propanoic instead of nonanoic acid in the headspace of *A. hebraeum* which might explain the species specific behavioural responses to this pheromone (Rechav, 1978). Olfactory receptors for components of the pheromone of both species are found in Haller's organ of *A. variegatum* (Hess and Vlimant, 1986, Diehl *et al.*, 1991, Steullet and Guerin, 1994a&b) and field experiments confirm earlier findings that 2-nitrophenol attracts females of this species over a considerable distance whereas methyl salicylate and nonanoic acid play a role in pairing and attachment (Hess and de Castro, 1986, Schöni *et al.*, 1984). Dongus and Gothe (1995) demonstrated the existence of a volatile signal from fed male *Hyalomma truncatum* that attracts conspecific ticks. Consequently, a signal, combining attraction

* for information on chemical compounds in this and following chapters refer to appendix I

to the host with aggregation and sexual pairing on the host, may not be exclusive for *Amblyomma* species.

In the 1970's Berger identified a compound which apparently induced male ticks on the host to detach and move towards females (Berger *et al.*, 1971, Berger, 1972). It was promptly declared a female produced sex attractant in agreement with ideas that were then developing in research on moth pheromones. However the compound, 2,6-dichlorophenol, as well as the behaviour it causes were controversial (Oliver, 1974). The rather unusual phenomenon of the biosynthesis of a chlorinated organic compound by an arthropod was confirmed when radiolabelled Cl injected into nymphs of *A. americanum* was found incorporated in 2,6-dichlorophenol produced by the adults (Berger, 1974) and the compound has since been found in 14 species in the genera *Amblyomma, Haemaphysalis, Hyalomma, Dermacentor* and *Rhipicephalus* (Sonenshine, 1985). However, it was consistently found in males as well as in females whenever it was properly investigated (Kellum & Berger, 1971, McDowell & Waladde, 1986, Sonenshine *et al.*, 1984, Price *et al.*, 1994). Only in *A. americanum* and *H. dromedari* is there evidence that males produce considerably less of this compound (Kellum & Berger, 1971, Silverstein *et al.*, 1983). A complex of dermal glands, associated with two small pored plates on the alloscutum of metastriate ticks, the foveae dorsales, was subsequently linked with biosynthesis and emission of this compound (Sonenshine *et al.*, 1981). These foveal glands are present in all life-stages of metastriate ticks but are not present in prostriata (Schulze, 1942, Dinnik and Zumpt, 1949).

Behavioural responses evoked by 2,6-dichlorophenol include leg movements of attached males and subsequent detachment (*e.g.* Berger, 1972, Chow *et al.*, 1975), effects which it has been shown can be evoked by many other non chemical stimuli such as an airstream, heat or physical disturbance. In *H. dromedarii* it attracts males on the host over a distance of 1 cm but appears not to be attractive when offered in an airstream off the host (Khalil *et al.*, 1981) whereas *A. variegatum* and *A. hebraeum* males in the field are attracted over considerable distance to sources of 2,6-dichlorophenol when combined with CO_2 (Norval *et al.*, 1991). Orientation to a wide range of concentrations of 2,6-dichlorophenol applied to the skin of a rabbit was observed in fed males of *D. variabilis* and *D. andersoni* (Sonenshine *et al.*, 1976). Unfed males and unfed or fed females did not show such responses. However some of these tests were done in the presence of "preserved" female ticks and the statement that 2,6-dichlorophenol is sufficient to elicit orientation and copulation is therefore not fully convincing. The role of this compound in mating behaviour of tick species is still not fully resolved.

Olfactory receptors for 2,6-dichlorophenol have been found in five species of Ixodid ticks in two sensilla: one identical to the one containing 2-nitrophenol receptors and the second more distal from Haller's organ on the tarsus of leg I (Chow,1979, Haggart and Davis, 1981, Waladde, 1982, Thonney, 1987). This product has remained the only identified volatile implicated in sexual behaviour in ticks. However, Wood et al. (1975) report the presence of two other phenolic products, 4-methyl phenol and phenol in some *Rhipicephalus* species and their potential to cause detachment and attraction of male ticks. In other tick species, various distinct chemical signals have been described to influence behaviour of male ticks from a distance but have so far not been identified (Gothe 1987, Leonovich, 1981, Petney and Bull, 1981, Andrews and Bull, 1982a, Rechav, 1983. Andrews *et al.*, 1986)

3.2. Low-volatile, cuticle associated signals

Lipophilic chemical signals of low volatility, present on the cuticle are known to play various roles in arthropod behaviour. Cuticle hydrocarbons have especially been implied in the mating behaviour of members of a wide variety of insect orders. They also mediate host recognition in parasitoids and play a major role in the social organisation of bees (Howard and Blomquist, 1982) as well as in the biology of its acarine parasite *Varroa jacobsoni* (Rickli, 1994). A variety of long chain hydrocarbons have been reported to be present on the cuticle of ticks (*e.g.* Estrada-Peña *et al.*, 1992) including *B. microplus* (McCamish and Cannell, 1980) but have so far not been implicated in tick behaviour. In *D. variabilis* and *D. andersoni*, mating behaviour is mediated by chemicals on the cuticle since hexane extracts applied to previously washed and biologically inactive females induce typical courtship behavioural responses after the male contacts the female (Hamilton and Sonenshine, 1988). Cholesteryl oleate and other cholesteryl esters have been isolated from these extracts and their activity in combination with 2,6-dichlorophenol has been demonstrated in this bioassay (Hamilton *et al.*, 1989, Sonenshine *et al.*, 1991). Cholesteryl esters have also been reported to induce mating responses in *R. appendiculatus* (Hamilton *et al.*, 1994). However, this signal is not considered to be very specific.

In the two *Dermacentor* species another chemical signal inside the female gonopore induces male ticks to copulate, *i.e.*, produce a spermatophore and insert it (Sonenshine *et al.*, 1982) and prevents interspecific copulations. Since mating behaviour is aborted when the anterior reproductive tract is surgically removed, but restored when the gonopore is treated with extracts of this tissue, this signal must not normally be present on the exterior parts of the cuticle. Allan *et al.* (1988) suggest that fatty acids, predominantly myristic (14:0), palmitic (16:0) and stearic (18:0) acid are responsible since they are present in an extract of the anterior reproductive tract and, at least in *D. variabilis*, are as active as the extract. However these compounds are present on other parts of the cuticle as well, and the methyl esters of these free fatty acids, though not present in the extract, can induce the same activity. Taylor *et al.* (1991) describe male copulatory responses to 20-hydroxy-ecdysone and other steroids deposited on the gonopore and account also for electrophysiological responses from gustatory receptors in the chelicera to this hormone. The exact composition of this chemical signal as well as the relevance of its components for species specific behavioural responses remain to be established. In two African camel ticks *H. dromedarii* and *H. anatolicum excavatum* such a genital signal is present but apparently not species specific (Khalil *et al.*, 1983). There is some evidence for a similar signal in *A. americanum* but the regulation of genital probing behaviour appears to be different for *A. maculatum* (Allan *et al.*, 1991).

In Argasidae the coxal organ produces a secretion that induces male mating responses when smeared over the cuticle of nymphs (Schlein and Gunders, 1981). The coxal organ has an osmoregulatory function in argasid ticks and is absent in Ixodidae. However an exocrine gland associated with it is present and is thought to play a role during moulting in argasid as well as ixodid ticks (Binnington, 1975).

3.3. Substrate deposited signals

Many arthropod species are known to aggregate and several cases are known in which a chemical signal deposited on the substrate is involved (Ishii and Kuwahara, 1968, Schofield and Patterson, 1977, Mumcuogli *et al.*, 1986), often in combination with other stimuli such as mechanical contact with objects or conspecifics. An association

with faeces and/or other excretory products is commonly demonstrated *e.g.* in flour mites (Levinson *et al.*,1991) and the acarine bee parasite *V. jacobsoni* (Donzé and Guerin, 1994). In ticks, aggregation of free living life stages on filter papers impregnated with tick derived material was first demonstrated in Argasidae (Leahy *et al.*, 1973, 1975b, Leahy, 1979). This water or saline (0.9% NaCl) soluble non-volatile interspecific signal is commonly, though not consistently, referred to as "assembly pheromone". The arrestment of groups of ticks after 1 hr on such contaminated filter paper discs has been observed in petri-dish bioassays in numerous species of Argasid ticks. Off-host aggregation is induced by products from all life-stages and all stages appear to respond, though strongest responses are often recorded from males responding to products of females. Fed ticks appear to produce more of this material whereas unfed ticks respond better. Similar effects were observed in four *Ixodes* species (Graf, 1975, Treverrow *et al.*, 1977, Uspensky and Emelyanova, 1980, Hájkova and Leahy, 1982), two *Dermacentor* species, *A. americanum* and *Heamaphysalis leporispalustris* (Leahy *et al.*, 1983) as well as in *H. dromedarii* (Hájkova *et al.*, 1980). In the latter species, significant responses were only obtained from males responding to female contaminated papers. It would seem therefore that arrestment in response to substrate deposited material from ticks is a widespread phenomenon in argasid and ixodid ticks though the existence of such a pheromone in the *Dermacentor* species was later denied (Taylor *et al.*, 1987). The immediate locomotory and other behavioural responses have never been recorded, so knowledge on the mechanisms involved is lacking and end results are apparent only after 30 min. In addition, the role of this behaviour in tick ecology is unclear. It has been suggested that ticks aggregate to prevent loss of water and increase the chances of locating hosts or mates. George (1981) observed that even though responses in bioassays of two argasid species were interspecific their clusters in nature were usually separated.

In *Argas walkerae* the active material is associated with tick excreta (Gothe *et al.*, 1984). However, in *Ixodes* and *Aponomma* species, papers brought in contact with nymphal exuviae produced similar effects (Treverrov *et al.*, 1977, Uspensky and Emelyanova, 1980) which might indicate a secretory instead of excretory origin of some chemicals involved. Otieno *et al.* (1985) demonstrated the presence of guanine in active fractions and its role in aggregation of two argasid species and also showed responses from *R. appendiculatus* to this compound. In addition many other purines as well as ammonium salts induce arrestment. Xanthine and hypoxanthine were shown to be present in pellets of five species of Argasidae and xanthine increases arrestment to guanine (Dusbábek *et al.*, 1991a & b). Guanine has long since been known as the primary end product of the nitrogen excretion cycle of many arachnids including mites and ticks (Kitaoka, 1961, Hamdy, 1972, Hamdy and Sidrak, 1982). It is excreted in the form of white pellets from the anus and distinct from the dark faeces which mainly contain haematin. Perception of this chemical signal is eliminated after ablation or masking of the palps (Leahy *et al.*, 1975a, Gothe *et al.*, 1984) so contact chemoreceptors on the palpal organ are likely to be involved but mechanical factors (Dusbábek *et al.*, 1991a) and humidity (Hassanali *et al.*, 1989) are reported to modify behavioural responses.

The on-host sexual behaviour of *Boophilus microplus* males and their *in vitro* responses to females and dummies in a host-simulating arena

MARIEN DE BRUYNE *unpublished results*

INTRODUCTION

Mating behaviour in *Boophilus microplus* follows the general pattern described for other Ixodid tick species. To permit transfer of a spermatophore to the genital aperture of a female the male needs to accomplish four things: 1) he has to detach himself from the host's skin, 2) locate the female, 3) pass to her ventral side and 4) locate the gonopore. How these goals are achieved, and particularly the types of signals which play a role in inducing and guiding the male's behaviour, is still a matter of dispute. Sonenshine (1985) describes the mating behaviour in *Dermacentor variabilis* as follows: 1) attached, feeding males detach under the influence of the sex pheromone 2,6-dichlorophenol. 2) initial random searching behaviour leads to detection of pheromone which is followed by short-range orientation to the female, 3) after making contact, males mount and palpate the female dorsum then turn to crawl under the female via the posterior end, and 4) ventral positioning then leads to location of the gonopore, insertion of the chelicera and deposition of the spermatophore after some 10 to 20 min. Females are described as fairly passive in this process. Though they often raise their bodies to facilitate male passage, they are apparently not capable of hindering males by refusing this move. It is not entirely clear in what way the first two phases are regulated by the pheromone, especially the distinctness of the *de novo* detection of 2,6-dichlorophenol in phase 2, after mediating detachment in phase 1. Hamilton and Sonenshine (1988) score 6 phases in their behavioural bioassay with *D. variabilis* and *D. andersoni*: orientation, contact, mounting, turning, move to female's venter and location of gonopore. They conclude that 2,6-dichlorophenol regulates the first two phases and a contact sex pheromone the remaining four. In both these descriptions, however, it is not clear which events mark the transition between the different phases or how they were quantified. A third chemical signal associated with the gonopore had already been described for these same two species (Sonenshine *et al.*, 1982)

The mating behaviour of *Aponomma hydrosauri* and other Australian reptile ticks follows a similar pattern (Andrews and Bull, 1980, 1982a&b). However, these authors discriminate between two volatile signals, one responsible for detachment of fed males inducing unoriented movements on the host and another affecting fed and unfed males for attraction at short range. In addition, males of different species show courtship behaviour after contact with females of all three species, presumably mediated by a third signal, but mating is successful only with conspecific females. They argued that this is because the female body lift occurs only in response to conspecific males

(Andrews, 1982). They also observed that differences in body size and leg orientation during the ventral positioning make it impossible for males of one species to insert their mouthparts into the gonopore of females of other species and suggest that such mechanical factors play an important role in mating.

B. microplus is different from other tick species previously investigated for mating behaviour, by being a one-host tick, *i.e.*, larvae locate and colonise the host and nymphs moult into adults on the host so that in the latter life-stages there is no host seeking and host colonising phase prior to the meeting of the sexes. It has also been shown that males are capable of recognising potential mates while they are still in the nymphal stage (Falk-Vairant *et al.*, 1994), and form pairs of venter to venter feeding ticks. Under the conditions described by these authors, when a steer is infested once with a large quantity of larvae, the males emerge 12 days later whereas females appear on day 13. By day 14 most ticks are in pairs but fertilisation starts only on day 15. As in other tick species, fertilisation is followed by rapid feeding and weight increase of the females, resulting in the first engorged ticks dropping off the host on day 18. These results are briefly summarised in Fig. 1.

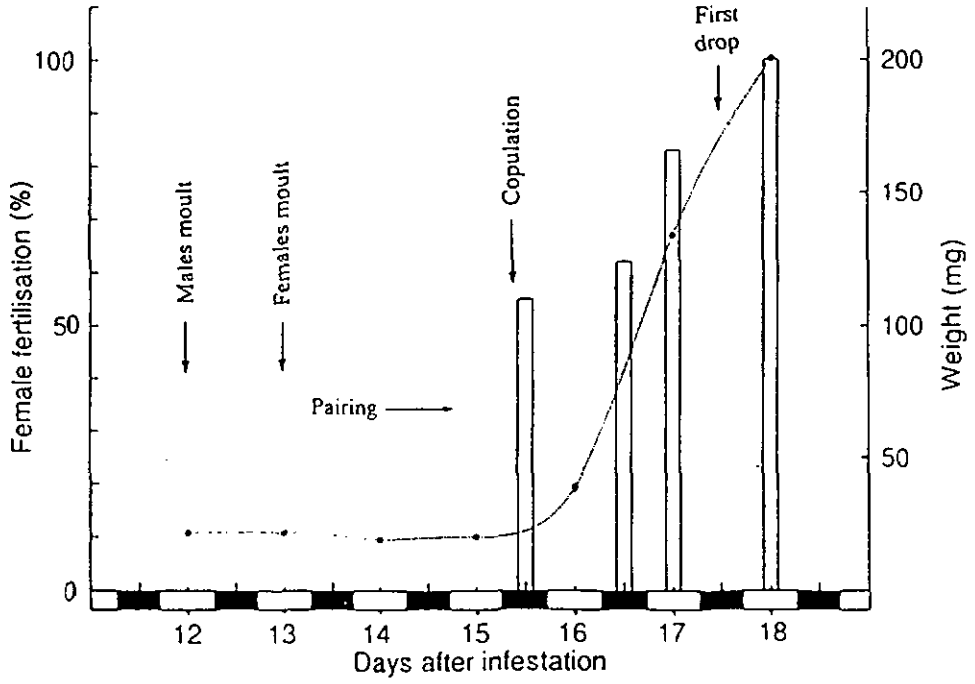


FIGURE 1. Development of *B. microplus* females on a young steer and the incidence of important events in their life history (modified after Falk-Vairant *et al.*). Female engorgement is indicated by weight (•——•) and bars represent the percentage of sampled females found to contain a spermatophore. Black marks on the x-axis indicate scotophase.

It has been shown in choice experiments on the host that *B. microplus* males mate indiscriminately with *B. microplus* and *B. decoloratus* females even though the two species do not produce offspring after interspecific mating (Spicket and Malan, 1978). Likewise, under on-host experimental conditions, *B. microplus* males copulate with *B. annulatus* females (Graham *et al.*, 1972). This lack of specificity in mating preference has also been observed in the two *Dermacentor* species and even between *D. variabilis* and *Rhipicephalus sanguineus* (Sonenshine *et al.*, 1974). It is questionable whether behavioural and/or pheromonal mechanisms of reproductive isolation are present in ticks. Allan *et al.* (1989) argue that in *D. variabilis* and *D. andersoni* a

chemical signal at the level of the gonopore can provide species recognition. In the three Australian reptile ticks the chemical signal causing males to detach in the presence of females is apparently species specific (Bull and Andrews, 1984). According to Bull (1986) ecological mechanisms can also explain the maintenance of parapatric boundaries that exist between species without wasteful interspecific mating.

I have observed mating behaviour on rabbit ears to determine the ability of male ticks to locate females and to characterise the different behaviours involved in mating. I have also studied mating under various *in vitro* conditions and developed a bioassay for isolating chemical cues mediating this behaviour. In order to understand the development of sex-ratio and tick density on the bovine host I also report on some population sampling during the days before copulation occurs.

MATERIALS AND METHODS

Ticks and tick sampling from a steer

Boophilus microplus (Canestrini) of the Biarra strain was reared on young Simmental steers at the Ciba-Geigy Agricultural Research Centre, St Aubin, Switzerland, under conditions corresponding to those described by Falk-Vairant *et al.* (1994). For studying the development of the tick population on the bovine host, all attached ticks in square areas (55 cm^2) located on the shoulder of a steer roughly 20 cm down from the centre or on the back were sampled with forceps. Male mobility was estimated by brushing the back, flank and neck area on one side of a steer with a paintbrush and collecting ticks in a plastic tray working on alternate sides of the steer between days. It was assumed that in this way only unattached ticks were collected: Some fully engorged females (<20) were also collected with this method on day 19 but not counted. In order to obtain newly moulted unfed ticks, engorged nymphs were collected by carefully removing them from the host with forceps and transported to the laboratory in a humidified container. They were kept in glass vials in an incubator at 28°C and 90% r.h. and, after moulting, adult males and females were put separately on the ears of New Zealand White rabbits enclosed in cotton bags where they readily attached. In addition, males were collected on different days from the bovine host and transferred to rabbit ears in the laboratory.

Behaviaural observations on rabbit ears and in an artificial feeding system

Observations of male and female behaviour was filmed at magnifications of 5x or 21x with a Canon C1-20P colour CCD video camera attached to a Zeiss operational microscope (working distance: 25 cm) equipped with a coaxial cold-light source. Light intensity in these and other observations was between 2000 and 7000 lux. Recordings were made on a Panasonic super VHS video recorder (AG-7350) and played back for analysis on a Sony Trinitron colour monitor. A rabbit was held (90 min max.) in a closed wooden restrainer leaving the head and ears exposed. The ears were flattened out and lightly restrained. A male *B. microplus* was released on the ear between 3 and 12 mm away from an attached male or female tick and allowed 3 min to commence walking (*i.e.*, displace itself >2 mm). Attached female ticks were classified according to size: 1) undistended (3 mm), 2) semi-engorged (5 mm) and 3) gorging (8 mm) The following behaviours were scored: male contact with attached tick, first tip-over of male, duration of dorsal exploration until first tip-over (latency), location of first tip-over, and incidence of female body lift (see results for definitions)

Using the same observation method, male behaviour was observed *vis-a-vis* females feeding in an artificial feeding chamber. Female *B. microplus* had been allowed to moult in the laboratory and were placed on a silicon membrane treated with an extract of steer pelage according to the method described by Kuhnert *et al.* (1995). The ticks were allowed to attach and fed for 2 days on defibrillated bovine blood enriched with ATP and glutathione, while non attached females were removed. Individual males were released and their behaviour was scored as above.

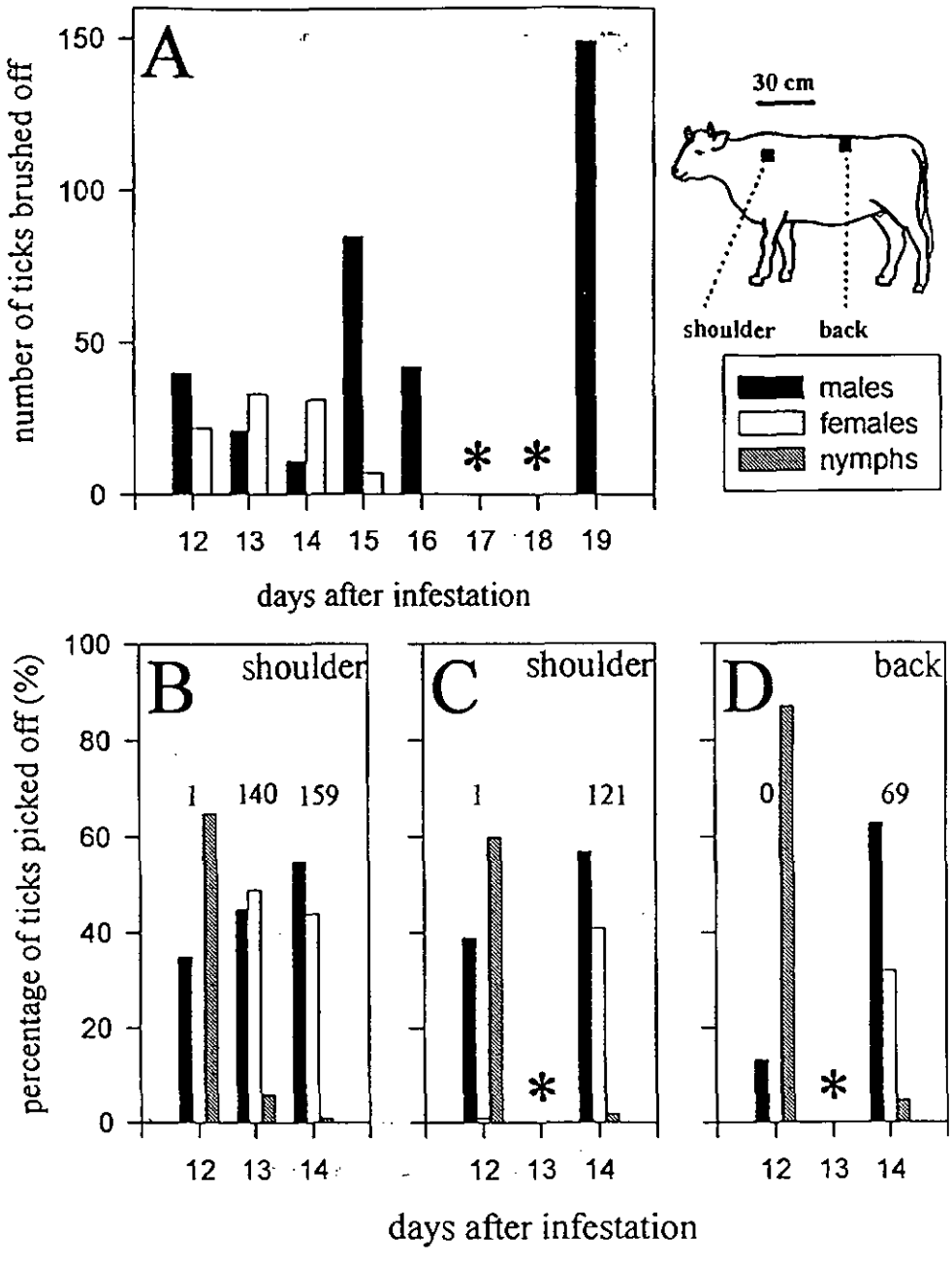


FIGURE 2. Density of *B. microplus* males and females and their mobility on the host. Samples were taken from the poulation of ticks that developed from single infestations of two young steers with *ca.* 10,000 larvae. Larvae were deposited in a 30 cm line from between the shoulders on day 0. **A**: Unattached (mobile) ticks collected from infestation 1 by brushing neck, shoulder, flank and back on one side of the host, alternating between sides from day to day. **B, C** and **D**: All ticks from an area of 55 cm^2 on the shoulder or back were removed with forceps (A: infestation 1, B and C: infestation 2). The location of the areas sampled is indicated in the drawing top right. Absolute number of females are indicated and asterixes indicate days without sampling.

Observations in a host-simulating arena and destructive treatment of females

To further reduce the presence of possible naturally occurring chemical and mechanical stimuli, female ticks were presented to males in a circular arena (40 mm dia.) consisting of a Baudruche® membrane (Joseph Long Inc., USA) 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%). This set-up is here referred to as "host-simulating" arena and is being used in this laboratory for studying the responses of *B. microplus* larvae to host derived chemicals (Kröber and Guerin personal communication, Guerin *et al.*, 1992).

In three separate experiments, one semi-engorged female *B. microplus* collected from the steer on day 19 was placed in the centre of the arena either 1) attached to the membrane ventrally with

double-sided adhesive tape, 2) lying loose or 3) the same female as in 2 but positioned ventral side up. In order to inhibit movement of the female the legs were cut off at the level of the genu two hrs before the experiment. A single male tick was released from a fine paintbrush directly on top of the female. In addition to recording the same events as above, the time the male spent in contact with the female was also recorded, *i.e.*, from the first moment all legs were in contact with her till the last leg lost contact. Males were allowed to contact the female during a maximum observation time of 180s. In order to characterise the movements of males over the body of the females, palpal tracks were recorded and related to defined areas of the female cuticle. In frame-by-frame video observations the position of the male's capitulum was traced on transparent plastic sheets from the moment of first contact to tip-over, or loss of contact with the female. Such tracks were also made for the observations in the feeding chamber mentioned above.

In an attempt to remove or destroy chemical information on the cuticle, individual semi-engorged female *B. microplus* collected from the steer on day 19 were immediately frozen at -20°C, kept there for 2 days and subsequently treated in one of the following ways after which they were stored again at -20°C: 1) control, 2) rinsed in 2ml hexane for 30 min, vortexed and dried under He, 3) extracted in chloroform:methanol 1:1 for 24 hrs, vortexed and dried under He, 4) as 3 and then rinsed 10 times for 2 min in 1ml chloroform and heated to 110°C for 12hrs. These females were presented to males as above, lying loose on the membrane and after being kept at 30°C for 4hrs.

Behavioural observations using glass beads as dummy females

A glass bead (*ca.* 5 mm dia., 3 mm high, 0.1-0.2 g), roughened with a wet-stone and flattened on one side to inhibit rolling, was placed in the centre of the host-simulating arena. Two such arenas were used simultaneously on the same warm plate, one bearing a bead treated with an extract of female ticks in solvent applied with a micro-pipette, the second treated with solvent alone as control. The extract was prepared by submerging freshly collected females (<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 equivalent/ µl and stored at -20°C. A single male tick, 2-14 days after moult, was released from a fine paintbrush onto the top of the bead. All males in a given experiment were tested on both the control and treated bead, half first on the control the other half first on the test. Different behaviours were quantified using THE OBSERVER 2.0 event recorder (Noldus Inf. Tech., Netherlands). The tick was recorded as either being on the bead, *i.e.*, from the first moment all legs were in contact with it 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 observations ended when the male 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 versus control). In addition, note was made of typical tip-over behaviour while on the bead (see results for definition).

RESULTS

Tick population development and mobility on the host

Under the rearing conditions mentioned above, ticks hardly migrate across the host from the area of infestation and most adults probably attach immediately where they moult or do not detach at all. Some males and females were found unattached on the host on day 12, 13 and 14 (Fig. 2A). However on day 15, males seem to be much more mobile than females and by day 16 all females are attached whereas an increased proportion of the male population is mobile. A large number of males is found actively walking on the host on day 19 when female drop-off is at its optimum. Results from several collections from the bovine host are shown in Fig. 2B,C and D. The number of ticks is less on the back of the steer further away from the area of infestation where development of nymphs to adults is also slightly delayed. On day 12 only a few females are present but by day 13 most females have moulted. The average sex ratio for days 13 and 14 is close to 1:1, though slightly in favour of males, and density of females for day 14 ranges from 1 to 3 per cm^2.

Observations on mate finding and mating behaviour in vivo and in vitro

The sequence of behavioural events in mating of *B. microplus* as observed *in vivo* and *in vitro* is summarised (Fig. 3A). The diagram includes detachment of the male as an event that precedes the searching phase. This is not what was actually observed as forcibly detached males were used in all our experiments. When male *B. microplus* were released on the ear of a rabbit, in the immediate vicinity of an attached conspecific tick, about half of the released males showed activity and started to walk, irrespective of the sex of the attached tick (Table 1). The other half did not move at all within the set period of 3 min or made movements resulting in males descending in the rabbit's fur. There is no correlation between activation of males and distance from the female within the range I tested. Most of the males that did walk, quickly located the female (70-90%).

TABLE 1. Behavioural responses of *B. microplus* males placed 3-12 mm from a male or female tick attached on rabbit ears *(in vivo)* or in a feeding chamber *(in vitro)*. Results are the number of males (n) observed walking, contacting the attached tick and tipping-over out of a total of N males. In addition, the part of the female body (as indicated in Fig. 4) where the first tip-over was attempted and the female's body lift response were scored.

attached tick	males				tip-over location		female
	total	walking	contact	tip-over	back	front/side	body lift
	N	n	n	n	n	n	n
			on rabbit ears (in vivo)				
female undistended	14	6	4	4	4	0	3
female semi-eng.	22	10	7	6	3	3	4
female gorging	18	8	7	7	2	5	0
male	10	5	5	3	1	2	-
			in feeding chamber (in vitro)				
female undistended	9	9	9	9	4	5	6

First contact was generally made with one of the front legs and immediately followed by a short exploration of the dorsal cuticle of the female. Depending on the size of the female and the thickness of the rabbit pelage, a male had to descend or mount to reach the female dorsum and did so with groping, scraping movements of the legs. Once fully on the female, he continued these movements while keeping his body in close contact with her. Since males (*ca.* 2 mm) are always smaller than females (*ca.* 3-10 mm) most legs were in contact with the female cuticle at this stage. The mouthparts engaged in movements up-down and backward-forward with respect to his body (flexing) while the legs at times made rapid vertical taps. Upon reaching an edge of the dorsum, males continued to slide along it, actively pushing forward. At some point during this process the female tick lifts her body at an angle to the host's skin facilitating male access to the ventral side of her body via the posterior end. The male, following the curvature of the body, would then tip-over and rapidly disappear under the female. This event in male behaviour is therefore termed 'tip-over'. Almost all males which had explored a female dorsum attempted this, and the first tip-over followed fairly rapidly after the male had mounted the female; only one male out of 17 observed took longer than 20s. However, the location of tip-over was not always chosen correctly for many males were engaged for from several seconds up to 1 min in efforts to pass via the side or the front of the female. This was physically impossible since at the front her capitulum was embedded in the host's skin and on the side her legs practically blocked the passage. Only on undistended females did all four males observed immediately find their way to the passage at the back of the idiosoma. Furthermore, the female does not always show the body lift response, in fact in none of the observations made on gorging females was she able or willing to lift her body.

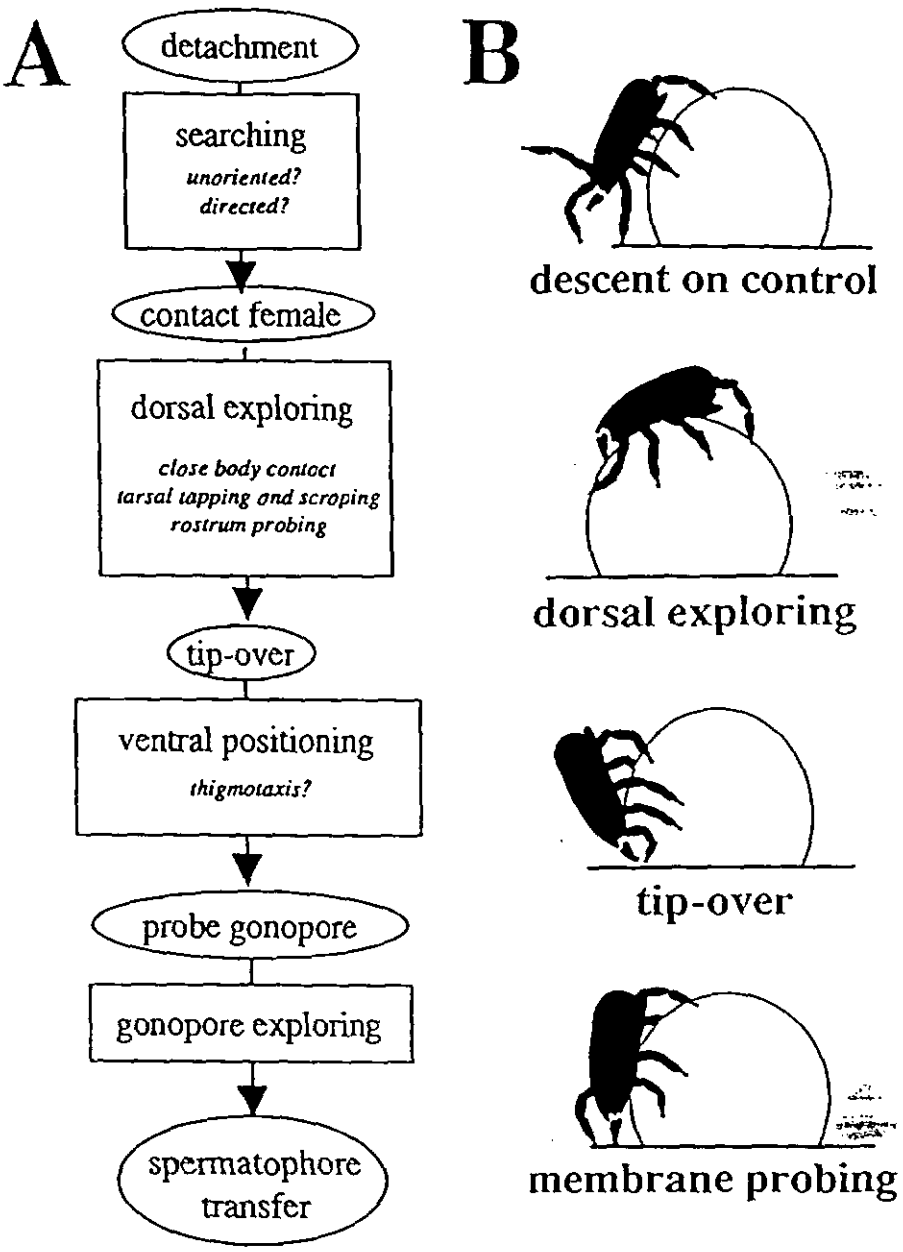


FIGURE 3. Schematic representation of male behaviour leading to copulation. A: Sequence of behavioural elements observed during *in vivo* and *in vitro* mating. Behavioural phases are characterised in squares whereas the events separating the phases are encircled. B: Typical male poses characterising behavioural elements observed on a glass bead

Part of the behaviour described here is not a specific response to females. In the observations of male responses to attached males, all males that walked located the attached male (table 1), all five spent more then 20 seconds in contact with him and three of them tried to tip-over. However, it is hard to compare male behaviour *vis-à-vis* males with that on females because the male body is so small that most of the legs of the mounting male are either in contact with the attached male's legs or the rabbit's pelage and the behaviour tends to be confused by entangled legs. Interestingly, at some point after testing several males, the two attached males that were used in these observations detached and walked off.

Males that were released in the feeding chamber, where females had been feeding *in vitro* for 2 days, all immediately walked and located one of the females (table 1). The

density of 17 females on the 12 cm^2 membrane was such that this high encounter rate was not a surprise. Mating proceeded as described above and could be observed easier than on the rabbit. It was confirmed in this *in vitro* situation that most males first contacted the female's body with the tarsus I (only one male made first contact with tarsus II). Which cuticle zone was first contacted did not seem to matter: 3 males first came into contact with the female scutum, 4 with the lateral zone and 2 with the festoon area (see Fig 4B). All males mounted, tipping-over within 20s with *ca.* 50% immediately reaching the venter via the back. Males that first tried tip-over on the side or front all eventually reached the venter by moving sideways while continuing to probe and push forward until a free passage to the venter was possible. Most males then continued to slide forward positioning themselves venter to venter with the female and became immobile, only making minor adjustments in the position of the legs which were clasped around the female body in between her coxa. The actual insertion of the chelicera into the gonopore could not be observed from above but, after folding the female forward, the mouthparts of the male could be seen, flexed at 90° to the body axis, and positioned on the gonopore.

Mating behaviour and orientation on detached females in a host-simulating arena

Female ticks that were removed from the host and placed in a host-simulating arena still induced males to investigate the dorsum and tip-over (Table 2). Moreover, placing males directly on the females did not seem to disturb normal courtship behaviour. Tip-over latencies on artificially attached, loose normal and loose inverted females were of the same order of magnitude as mentioned above and exploration of the cuticle seemed to involve the same scraping and probing movements of legs and mouthparts. In all three experiments some males left the female without tipping-over but on the artificially attached female more males had relatively short contact times, sometimes after fruitless attempts to tip-over. Tip-over was possible on females lying loose on the membrane, dorsal side up, but the female was moved around by the male under her. None of these males was able to complete ventral positioning and locate the gonopore and many males crawled back up on top of the female only to tip-over again, leading to long contact times. Interestingly, all males also readily tipped-over on inverted females. Some males actually contacted the area of the gonopore before tip-over but did not seem to be arrested by it at all. Movements of males over the female cuticle could be observed more precisely in these *in vitro* experiments. I did not observe any consistent pattern in the palpal tracks on any of the live females (Fig 4), *i.e.*, there was no obvious order in male orientation on artificially attached, loose normal or loose inverted females. As far as the palps are concerned, there was no evidence for contact with specific cuticular zones before tip-over nor was there any particular zone which was never contacted before tip-over. The tracks also give no evidence for any single cuticular zone guiding males to the correct location for tip-over (Fig. 4A,B). The first tip-over attempts of males on semi-engorged females lying loose on the membrane are located more or less randomly around the edge of the body (Fig. 4C).

Male behaviour on a dead female in the host-simulating arena was not considerably different (table 2) except for the fact that in this case 5 out of 10 males managed to locate the gonopore and became immobile under the female. Our attempts to remove the factor(s) inducing male arrestment by females and tip-over by various destructive treatments, were largely unsuccessful. After a 30 min hexane wash there was no apparent change in the colour or texture of the female tick. The chloroform:methanol extraction changed her colour to a deep red/orange which faded in subsequent chloroform rinses but all aspects of the cuticle appeared unchanged. Even after heating

at 110°C for 12 hrs the structure of the cuticle was completely preserved, including the numerous folds characteristic for female ticks, though it had collapsed due to evaporation of the contents and turned to a dark brown colour. None of these treatments completely removed arrestment but contact time was considerably reduced after heating the female to 110°C. Tip-over behaviour was not affected even after this harsh treatment and latencies remained short.

TABLE 2. Behavioural responses of *B. microplus* males when placed on females in a host-simulating arena. Females were semi-engorged and placed either dorsal side up, attached with double-sided adhesive tape (attached), or lying loose with dorsal side up (normal) or ventral side up (inverted) in the centre of a host-simulating arena. In an attempt to remove or destroy chemical information on the cuticle, such females were killed at -20°C and treated as follows: control = untreated, HEX. washed = rinsed in 2ml hexane for 30 min, vortexed and dried under He; CH:M extracted = extracted in chloroform:methanol 1:1 for 24 hrs, vortexed and dried under He; CH:M & heated = as previous, then rinsed 10x for 2 min in 1ml chloroform and heated to 110°C for 12hrs. Results are: the number of males (n) observed tipping-over out of a total of N males and the median latency of this response. In addition, the part of the female body (as indicated in Fig. 4) where the first tip-over was attempted and the contact time with the female were scored.

	males	tip-over		tip-over location		contact time		
	total		latency	back	front/side	<20s	20-180s	>180s
females	N	n	median (s)	n	n	n	n	n
				live females				
attached normal	13	10	9	5	5	3	6	4
loose normal	17	14	7	9	5	0	8	9
loose inverted	18	17	4	9	8	0	6	12
				dead females				
control	10	9	5	-	-	1	1	8
HEX washed	10	10	5	5	5	0	1	9
CH:M extracted	10	10	7	2	8	0	2	8
CH:M & heated	10	9	6	3	6	1	6	2

Arrestment and tip-over on glass dummies

Males were also arrested on glass beads treated with 3 female equivalents of a chloroform:methanol (1:1) extract of female *B. microplus* as is shown by the ratio between their contact times on extract-treated beads and solvent-treated controls (Fig. 5A). On test beads they engage in exploratory behaviour similar to that seen on live and dead females (see Fig 3B). Conversely, when placed on control beads, males generally leave within 20 seconds and very often raise their body and wave the first pair of legs in the air just before loosing contact with the bead. In addition to being arrested on test beads, some males descend toward the membrane while keeping their body in close contact with the surface of the bead. Such 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 is comparable to the tip-over observed on females. 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 resting on the membrane. This behaviour was termed 'membrane probing' and was occasionally observed 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.

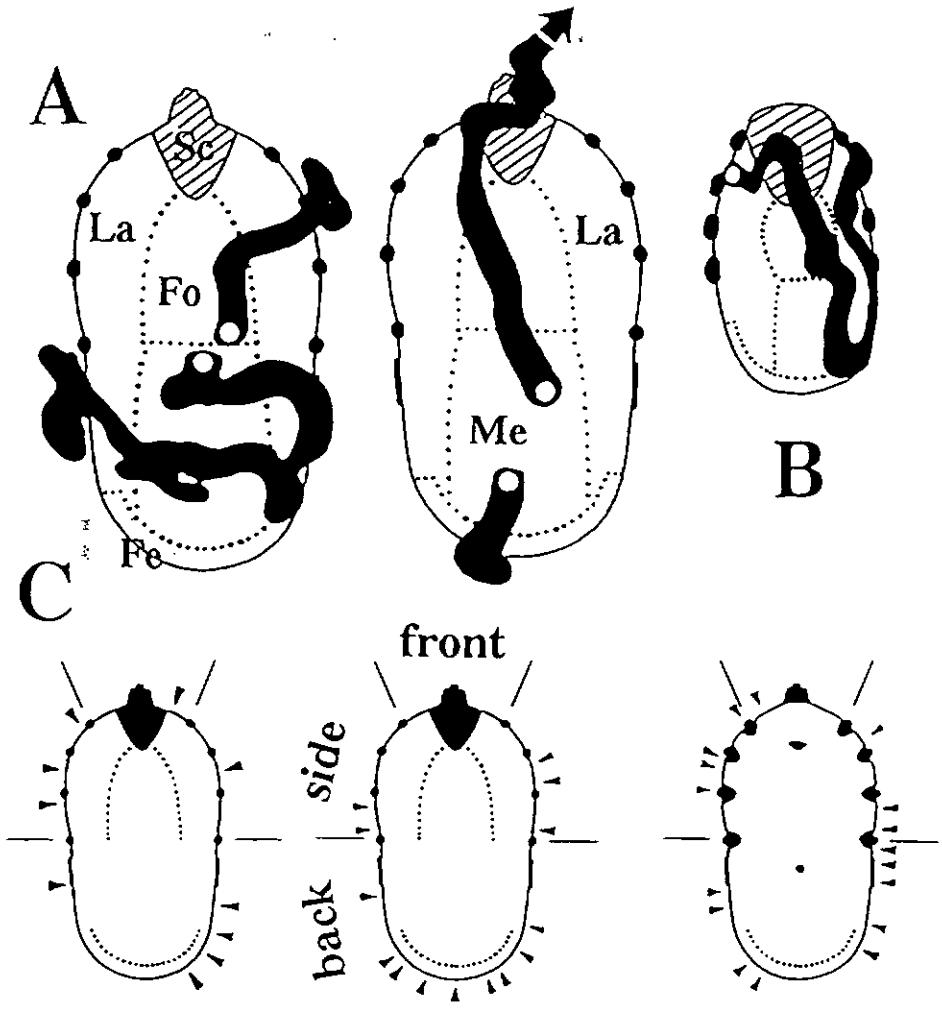


FIGURE 4. Orientation of male *B. microplus* movements relative to locations on the female body during dorsal exploration and tip-over in a host-simulating arena or artificial feeding chamber. **A:** Some examples of tracks made by the palps of males across the body surface of semi-engorged female ticks prior to male tip-over. Open circle marks first point of palpal contact after the male was placed on the female. An arrow marks the direction in which one male left the female without tipping-over. Cuticular zones referred to in the text are separated by dotted lines; Sc., scutum & capitulum; La., lateral zones; Fo., foveae dorsales zone; Me., medial zone; Fe., festoon zone **B:** One such track on a female in the artificial feeding chamber where the male contacted the female by himself. **C:** Location (arrow heads) of first tip-over of males on a semi-engorged female attached with double sided sticky tape (left), a loose female (middle) and this same female placed ventral side up (right). The locations used in table 1 and 2 are indicated by lines.

When both test and control bead were treated with solvent, males descended generally within 20 s and the logarithm of the arrestment ratio was normally distributed around the mean 0 (Fig. 5C). The influence of a certain variability in bead size was negligible; males took only slightly longer descending a bigger bead (Fig. 1B), but this was far from significant. Their seemed to be no influence of features around the arena on the orientation of males, *i.e.*, under these circumstances they were not attracted by stimuli associated with the experimenter (Fig. 5D). Temperature is apparently an important factor in male behaviour (Fig. 5A). Males were significantly arrested when temperatures are around those found on the host (35°C) as opposed to ambient temperature (22°C). Humidity, however, had no effect on arrestment but high relative humidity seemed to reduce the number of males showing tip-over behaviour. This is especially apparent at ambient temperature and 90% r.h. (cold & humid) when no tip-over was observed.

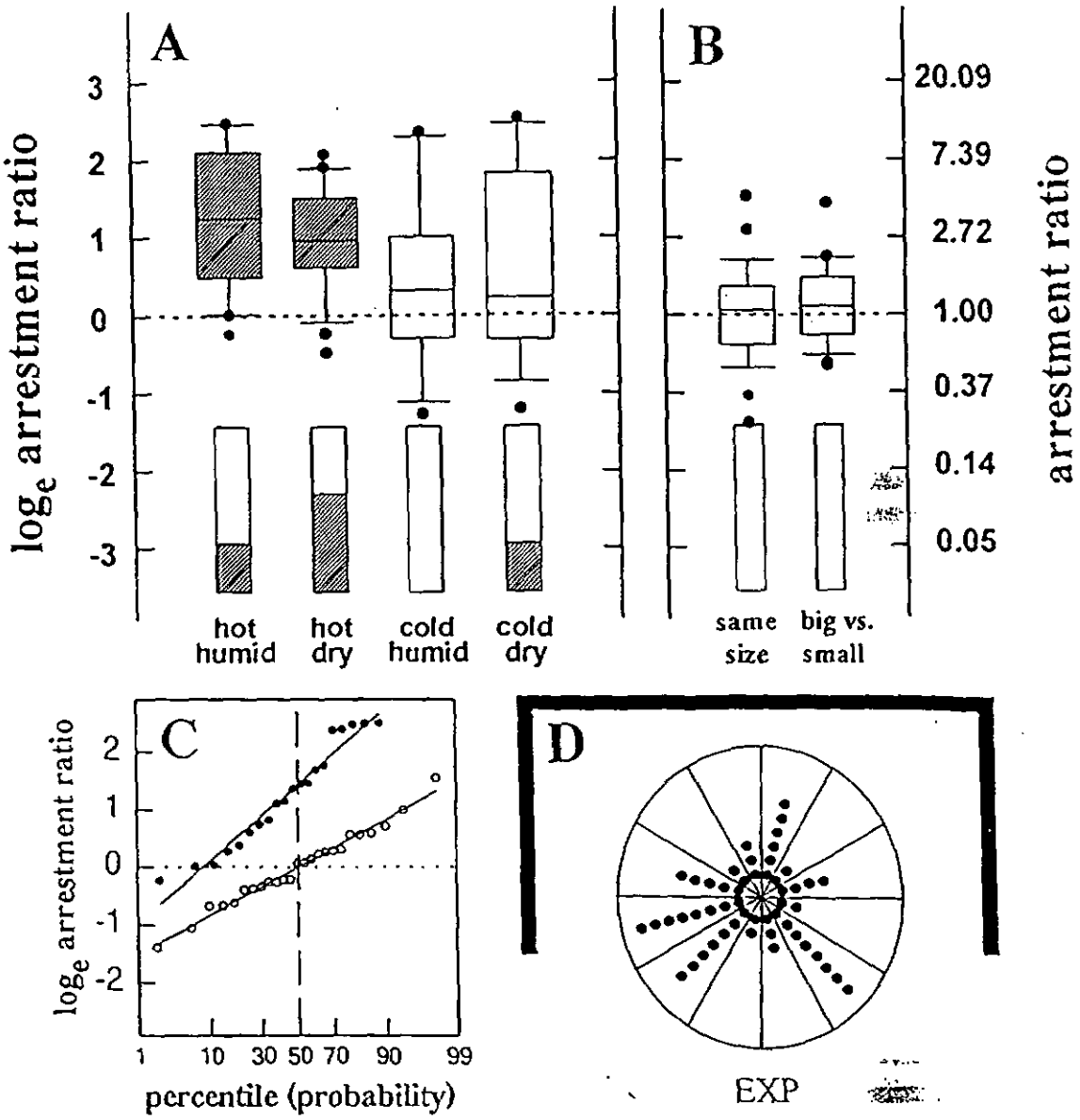


FIGURE 5. Arrestment ratio, tip-over and oriented responses of individual male *B. microplus* ($10 \leq n \leq 16$) placed in different test situations on glass beads in a host-simulating arena. Box plots show the distribution of the natural logarithm of the arrestment ratios, *i.e.*, the ratios between the contact time with 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 and B indicate the proportion of males exhibiting tip-over behaviour on the test bead. **A:** Influence of temperature and humidity in the arena on responses to 3 equivalents of a chloroform:methanol (1:1) extract of female ticks (test) as compared to solvent only (control), hot = 30°C, humid = 90% RH, cold = 22°C and dry = 40% RH. **B:** Effect of the difference in size of solvent treated beads, in "big vs. small" the test bead was twice the weight of the control bead. **C:** Percentile graph showing the distribution of the logarithms of the arrestment ratios in a control experiment (open circles, data from B 1:1) and with chloroform:methanol (1:1) extract of female ticks (closed circles, data from A: hot humid). The data are graphed against $p_i = (i - 0.5) / n$. When i is the rank of each datum in the dataset, the datum is said to be the p_i^{th} percentile (Cleveland, 1985). The dotted line marks $\log_e(\text{ratio}) = 0$, *i.e.*, no effect and the median is at the dashed line. **D:** Circular frequency distribution of the point where males crossed the edge of the arena after leaving a control bead. The experimenter (EXP) was sitting in front of the arena and light was shielded from the back and sides as indicated by the black border.

Arrestment was significant in unfed as well as fed males. Even males immediately after ecdysis, though very weak and slow, were arrested on extract treated beads but these data were not analysed for significance (Fig. 6). By contrast, newly moulted females are not arrested and do not show tip-over behaviour. The arrestment response in this bioassay is fairly constant with age of males (Fig. 7): consistent arrestment and tip-over behaviour can be observed in males from 3 till 17 days after moulting though an optimum might exist around day 5-9.

DISCUSSION

Sex ratio and density of female B. microplus on a young steer

On the back of a young steer, with a single infestation of *ca.* 10,000 larvae, I observed the appearance of newly moulted males after 12 days whereas females moult one day later. Under these same circumstances it has been established that fertilisation of females begins on day 15, about 4 days after male moult (Falk-Vairant *et al.*, 1994, Diaz et al., 1984a) when females have already been present for 3 days and many males have paired with them. The delay is probably related to the completion of spermatogenesis in males which is nevertheless two to three days faster in *B. microplus* than the *ca.* 6 days reported for other metastriate species (Oliver, 1982). Pairing with pharate or newly moulted females probably involves the first three phases of mating behaviour and can thus take place some days before and separated from copulation (Falk-Vairant *et al.*, 1994).

The sex ratio after moulting, during the days preceding copulation, is roughly 1:1. The 1.37 for *B. microplus* and 1.35 for *B. annulatus* reported by Davey *et al.* (1988) indicates that slightly more females were found. However, they collected ticks at a later stage when males are more difficult to notice relative to the much larger females and male mortality could have been higher. The density of females is fairly high, varying between 1 to 3 per cm^2. I did not find records on population densities on natural hosts in the Oriental region, where this species originates, but on dairy cows in tick infested pastures in Southern Queensland, Australia, Snowball (1957) observed peak densities of 0.5 female *B. microplus* per cm^2, in spite of regular arsenic spraying. Such densities are therefore not too far from reality so that the male searching strategy need not be very sophisticated as random searching would bring a male in the viscinity of female within a short time. The synchronised development observed here is a consequence of the single experimental infestation. Under natural conditions, newly moulted males encounter females at different stages of development.

Phases of mating behaviour

The mating behaviour of male *B. microplus* is described here as a four-phase process leading to spermatophore transfer. Defined events (detachment, contact with female, tip-over and probing the gonopore) initiate behavioural phases (searching, dorsal exploration, ventral positioning and gonopore exploring). Each phase will lead to occurrence of the next event or an alternative. While engaged in searching, a male will or will not contact a female. While exploring a female dorsum, a male will tip-over or leave the female. While engaged in ventral positioning, he will either locate the gonopore and probe it or not. While exploring the gonopore with his chelicera, he will produce a spermatophore and transfer it or not. If we assume a role of the female in the mating process we might combine dorsal exploration, ventral positioning and

gonopore exploration as courtship behaviour, incorporating the exchange of mechanical and chemical signals leading to copulation, *i.e.*, spermatophore transfer.

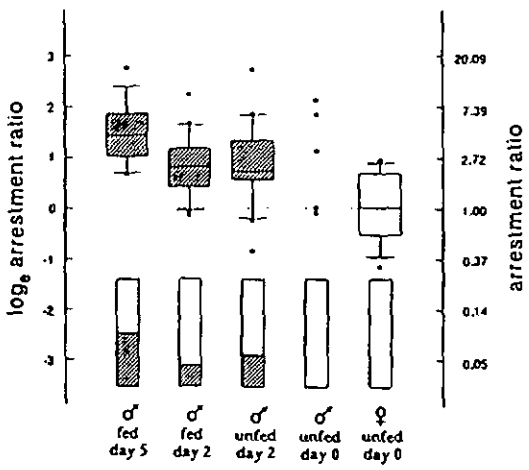


FIGURE 6. Behavioural responses of individual *B. microplus* ticks ($6 \leq n \leq 16$) to a glass bead treated with 3 equivalents of a chloroform:methanol (1:1) extract of female ticks (test) and solvent only (control). Five day-old (day 5), 2 day-old (day 2) and newly moulted (day 0) unfed or fed males were tested as well as newly moulted unfed females. For details on presentation of data see FIG 5. Individual data points rather then a box are shown for newly moulted males due to small numbers tested.

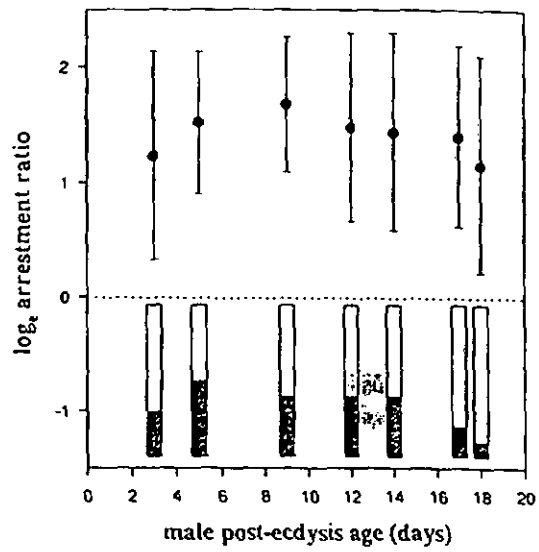


FIGURE 7. Behavioural responses of individual male *B. microplus* of different ages to glass beads treated with 3 equivalents of chloroform or chloroform:methanol (1:1) extract of female ticks. Data are mean arrestment ratios with standard error. The vertical bar diagrams indicate the proportion of males exhibiting tip-over behaviour on the test bead.

Detachment and searching: Male mobility

On days 12, 13 and 14, nymphs continue to moult on the back of the steer and the fact that adult ticks can be brushed off the host might be because such adults have not yet attached. However, on days 15 and 16 female mobility decreases whereas an increased number of males are active on the host. Since all nymphs have already moulted and this peak in activity coincides with the fertilisation of females (Falk-Vairant *et al.*, 1994) we can assume that a proportion of the male population has detached in order to search for a mate. After copulation, males and females remain paired and attached to the host (Falk-Vairant *et al.*, 1994). On day 19, large numbers of males are again observed walking on the host. It is likely that at that time many males find themselves exposed, after the female they were paired with has dropped from the host. This may then trigger a renewed search for a mate. Male *B. microplus* stay on the host for up to 40 days and like other metastriate ticks can copulate many times (Diaz, 1984b, Oliver, 1982).

I did not investigate which stimuli mediate male detachment. It is known that various stimuli such as heat or an airstream can stimulate males to detach and reattach elsewhere. Such a factor of disturbance is likely to have been involved in the case of the two attached males in the mating experiments on rabbit ears, who were repeatedly disturbed by contact with the males I placed in their viscinity. Upon inspecting males attached to rabbit ears in the absence of females, it was also often observed that one or two males were found unattached, walking either on the ear or the inside of the cotton bag. Although the unsuitability of the rabbit as a host might play a role, it seems that

males regularly detach and walk over the host. The presence of females might influence male detachment as is the case for *D. variabilis and D. andersoni* (Homsher and Sonenshine, 1976) and *A. hydrosauri* (Andrews and Bull, 1980).

Only *ca.* 50% of *B. microplus* males, which were forcibly detached and placed near a female, were activated within a period of 3 min. Since this was not observed *in vitro*, in the feeding chamber, where all males immediately walked off, it might have to do with the presence of arresting stimuli on the rabbit pelage which were not present in the feeding chamber. This is supported by the fact that a number of these males descended into the rabbit pelage and some actually attached. However, activated male *B. microplus* are capable of efficiently locating a female tick when at a distance of *ca.* 1 cm. Since males were positioned at random, not necessarily with their body-axis directed towards females, the observed high encounter rate cannot be explained by random movement. However males also located attached males with the same efficiency, indicating that, if a volatile attractant is involved, it is produced and emitted by both males and females. However, these results are by no means evidence for an attractant chemical. The distances over which this oriented movement was observed are rather small and rabbit pelage can be contaminated with non-volatile material from the attached ticks. In addition, vibrations produced by the feeding female, or small variations in temperature and humidity in her vicinity could provide cues to searching males.

Contact with the female and dorsal exploring

After locating and contacting the female, all males mount her and engage in a brief exploration touching the substrate with tarsi and palps and bringing the entire body in contact with her. It would seem that even when in contact with males this exploration takes place and I have no indication that it even leads to a rapid rejection of an attached male as a possible mating partner. This exploration phase does not involve any clearly oriented movements. The 180° turn observed in mating of *D. variabilis* (Sonenshine *et al.*, 1974) and in *A. hydrosauri* (Andrews and Bull, 1980) is not evident in the mating behaviour of *B. microplus*. The latter authors observed oriented movements on the dorsum leading to an allignment of male and female followed by a 180° turn bringing the male in the correct position for tip-over. In *A. limbatum* Andrews (1982) observed that the turning behaviour was not complete indicating that differences exist between species. I also did not observe a preference for palpating the area of the foveae dorsales which is reported by Khalil *et al.* (1983) for *H. dromedarii*.

The elements of exploration behaviour, tarsal scraping, flexing movements of the rostrum and close body contact can also be observed on host skin before attachment and must be associated with the investigation of any potentially "interesting" substrate. It can also be observed in males placed on a solvent treated glass bead (control) but it is rapidly abandoned, and males more typically walk with their body slightly above the substrate often waving their first pair of legs in the air before they leave the bead. By contrast, on glass beads treated with a chloroform:methanol (1:1) extract of female *B. microplus*, males engage in this behaviour for extended periods of time, some for more than 3 minutes, leading to long contact times quantified with the arrestment index.

Tip-over and ventral positioning

In almost all cases the dorsal exploration leads to the typical tip-over behaviour aimed at reaching the ventral side of the female. This behaviour, so essential for bringing a male into the position to explore the gonopore, also occurs when a female is placed

ventral side up, a situation in which such a move is not necessary. It indicates that this behaviour is a prerequisite of theirs and males cannot adapt to this unnatural situation. Males might have no way of recognising the ventral side of a female by means of chemical or other specific cues associated with it. However, the fact that this behaviour occurs irrespective of the female's lying position means that the stimulus (or stimuli) that induce(s) it must be present on the dorsal as well as the ventral cuticle. There is no evidence for the existence of special zones on the female cuticle that would serve as points of reference for male orientation towards her posterior end. I therefore assume that males orient at random and follow the cuticle till it slopes down, whereafter tip-over is performed. The curvature of the body, a mechanical stimulus that can be evaluated with the mouthparts relative to the body, would sufficiently explain the observed behaviour. On semi-engorged females such a curve exists along the whole body including just above the legs and mouthparts while in the undistended females the relatively flat cuticle near the coxa might prevent tip-over in these areas and concentrate male efforts on the posterior end of the body, the only zone with a clear curve.

Tip-over occurrs in all experimental situations I tested including on glass beads treated with an extract of female ticks. However, it does not occur on solvent treated glass beads. This demonstrates that it is not just the curvature of the surface that induces this behaviour but that a chemical factor is involved. The motivation to tip-over is large enough for some males to insist forcefully in ventral postioning, even though it is difficult for them to crawl under the bead which is substantially heavier than a female. Some succeed and actually displace the bead this way, others engage in sideway movements as was observed on live females attached to rabbit ears or in the feeding chamber when the passage to the venter was blocked by legs. These observations suggest that this is basically thigmotactic behaviour. A response to gravitational pull would not be plausible since females on the host can be positioned in any direction relative to the force of gravity. The results of the bioassays with either ambient or high humidity suggest that humidity is not essential or rather reduces tip-over. Whereas an lowered membrane temperature clearly reduces median arrestment, some males still tip-over when both humidity and temperature are at ambient, making a temperature gradient as a stimulus for guiding tip-over less plausible.

Female body lift

Male motivation to tip-over, while in contact with female cuticle is not dependant on behavioural feedback from females. It occurs in all situations tested here, including those with dead females and glass beads treated with extract form female ticks. The role of female body lift response in successful completion of copulation is unclear, as are the factors which induce this response, but it does not occur in all matings. It is possible that mechanical stimuli from male movement over the dorsum stimulates the female. The tapping and scraping movements of the legs of males on the female dorsum could play a role. On one occasion it was possible to induce body lift by stimulating the posterior region of a female attached to a rabbit ear with the small paintbrush used for placing males on the rabbit ear. Specific leg movements and/or chemical signals from the male, either olfactory or gustatory might be involved. The presence of a terminal pore on several seta along the edge of the female idiosoma has been observed (Vlimant, personal communication) indicating the presence of gustatory receptors. Since there is no indication of males recognising females specifically, maybe females can recognise males as has been suggested by Andrews (1982).

Probing and exploring the gonopore

The locating and probing of the gonopore by males was not studied here. During observations on the rabbit and in the artificial feeding chamber it was not possible to see the mouthparts after tip-over even if the female had lifted her body at an angle of almost 90° to the substrate. It is not clear how other authors have managed to observe this without interfering in the mechanical environment of the ticks by turning the female around. It was possible to infer gonopore probing and subsequent exploring from the gradual reduction of male movements under a female. Such immobility could be observed in matings on the rabbit ear as well as in the feeding chamber but I have not attempted to quantify this phenomenon. However, it was clear that none of the males on live semi-engorged females in the host-simulating arena reached this phase. By contrast, it was possible for males on a dead female in the same environment. This would suggest that live females can prevent males from reaching this phase and that death by freezing has removed this effect. Alternatively, males may locate the gonopore by mechanical argument.

I have also observed many matings where ventral positioning leads to attachment of males to the skin of the rabbit under the female rather than copulation. If females can refuse males access to the gonopore or if males respond with courtship behaviour to pharate females, stil enveloped in nymphal cuticle, they could find themselves engaged in ventral positioning without a possibility to probe the gonopore. It might be in such situations that males choose to attach under the female and guard her rather than leave and search for another mate. Alternatively, males that have not yet completed spermatogenesis might do the same. Results from bioassays with unfed males indicate that they do engage in courtship behaviour whereas they cannot inseminate females. The membrane probing behaviour observed on glass beads can also be an attempt by males to attach and feed while still keeping contact with a potential mate. However, this behaviour was not observed in any of the other experimental set-ups, *e.g.* lack of success in ventral positioning did not lead to membrane probing in experiments with a live females artificially attached to the membrane. Pre- and post-copulation associations between males and females have been described in many different insects species (Alcock, 1994)

Mating behaviour under differing experimental conditions: from steer to bioassay

The various experimental set-ups in which mating behaviour was observed allowed the development of a bioassay in which I could acurately quantify behavioural responses of male *B. microplus* to chemical stimuli as well as witness the effect of environmental factors on these responses. Observations on live females attached to laboratory rabbits are tedious, uncomfortable for the rabbit and now and again disturbed by the host's movements. The feeding chamber offers the advantage of a stable substrate and ease of manipulation, whilst mating follows its normal course in spite of the absence of various host stimuli. The host-simulating arena was designed to supply the following elements of the environment in which mating usually takes place: a relatively high temperature (35°C) a high humidity (>80%) and a membranous surface. Various odours and contact chemostimuli normally present on the host were absent as were mechanical stimuli such as hairs. Under all these circumstances, *in vivo* and *in vitro*, both live and dead females induced dorsal exploring, tip-over and ventral positioning. Attempts at removing activity from dead females with solvent extraction were largely unsuccessful and even heating such an extracted female to 110°C did not completely remove activity though arrestment was reduced.

In spite of the fact that I did not succeed in removing activity from females by solvent extraction some of the chemical stimuli responsible must have been extracted since males spend more time investigating glass beads treated with such an extract as compared to solvent treated control beads. In addition, the typical tip-over behaviour can be reproduced only on extract treated beads and not on controls. The fact that mating responses can be induced on glass beads with such an extract which is comprised of a mixture of chemical constituents from different zones of the female cuticle, supports the fact that male tip-over need not be guided by chemicals though it is clearly induced by them. In view of the difficulty of removing them, these chemical stimuli must be intimately associated with the cuticle of females and not too volatile or thermolabile.

Males of different ages, fed and unfed, are arrested and tip-over on extract-treated glass beads, but newly moulted unfed females do not respond this way. Since this is normally the only mobile stage for females they can be assumed not to show these male specific mating responses. Moreover, the fact that unfed males respond in this way to female extracts would explain the pairing before copulation as mentioned above. The results also demonstrate that the temperature gradient in the host-simulating arena is essential for normal mating responses to occur. Chilton and Andrews (1991)also found that the body temperature of their reptile host is an important factor in mating of *A. hydrosauri* and *A. limbatum*.

The mating behaviour of *B. microplus*, described here as a process that can be devided in four phases, is clearly mediated by chemical as well as mechanical factors. Males are capable of locating females on the host, pair with them and remain attached under them before and after copulation. Chemical stimuli play a crucial role during male exploration of the dorsal surface of female ticks and initiate tip-over behaviour that brings the male into a position to probe the female gonopore. Extracts of female ticks, when applied to glass dummies, induce arrestment and tip-over in male ticks.

Pergamon

J. Insect Physiol. Vol. 40, No. 2, pp. 143–154, 1994

0022-1910/94 $6.00 + 0.00

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

MARIEN DE BRUYNE,* PATRICK M. GUERIN*

Received 13 May 1993; revised 19 July 1993

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

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

Animals

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

Engorged nymphs or adult males were collected by carefully removing individuals from the host with forceps, and were transported to the laboratory in a humidified container. Engorged nymphs were kept in an incubator at 32°C and about 100% r.h. until they moulted, and adults were put on the ears of New Zealand White rabbits enclosed in cotton bags where they readily attached. Electrophysiological experiments were made with males that had moulted in the incubator, whereas behavioural experiments were done with males collected from the host before mating. When not on the rabbits, ticks were kept at room temperature (22–28°C) over water in closed containers to assure high relative humidity.

Chemicals and extracts

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

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

Electrophysiology

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

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

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

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

GC, GC-coupled electrophysiology and GC–MS

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

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

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

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

*All extractions terminated with 15 min sonication.

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

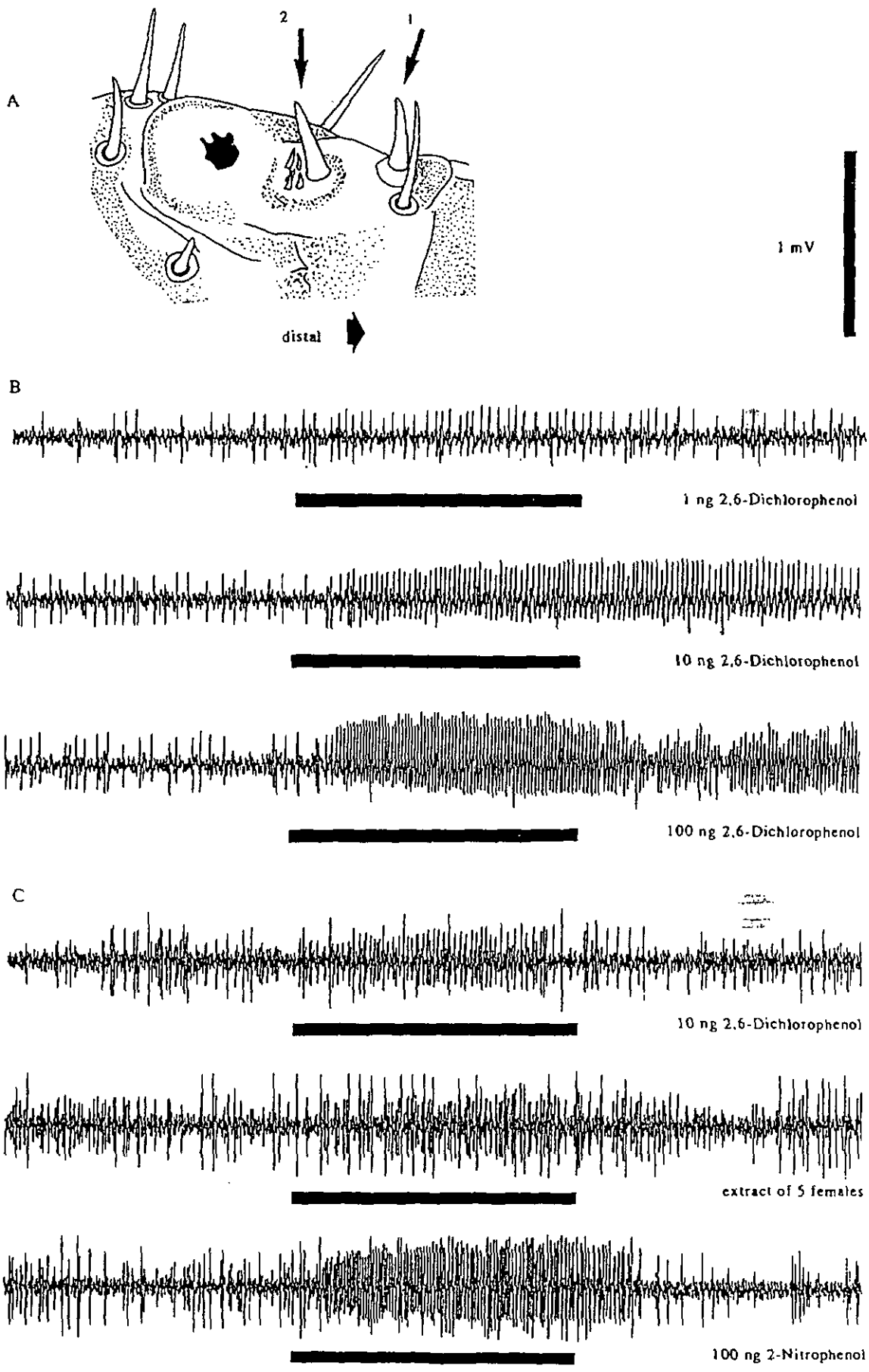


FIGURE 1. Characterization of olfactory receptors in two wall-pore single-walled sensilla on the dorsal side of tarsus I of male *B. microplus* ticks. (A) Dorsolateral abaxial view of Haller's organ and surrounding sensilla, showing the d I I (1) and d II I (2) sensilla. (B) and (C) Electrical signals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

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

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

Behavioural bioassay 1: dummy female on a membronous substrate

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

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

Behavioural bioassay 2: locomotion compensator and wind-borne odours

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

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

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

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

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

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

RESULTS

Electrophysiology of 2,6-dichlorophenol receptive sensilla

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

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

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

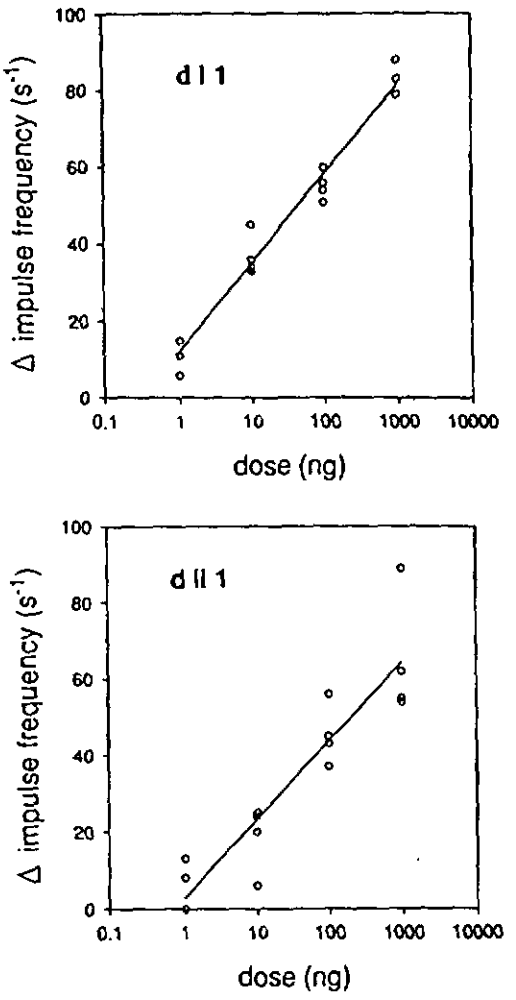


FIGURE 2. Dose–response relations for the combined activity of olfactory cells in two sensilla on the tarsus I of male *B. microplus* ticks (d I 1 and d II 1 of Fig. 1) in response to 2,6-dichlorophenol. Increase in impulse frequency was calculated by subtracting the number of impulses in the second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

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

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

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

FIGURE 3 (*Opposite.*)

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

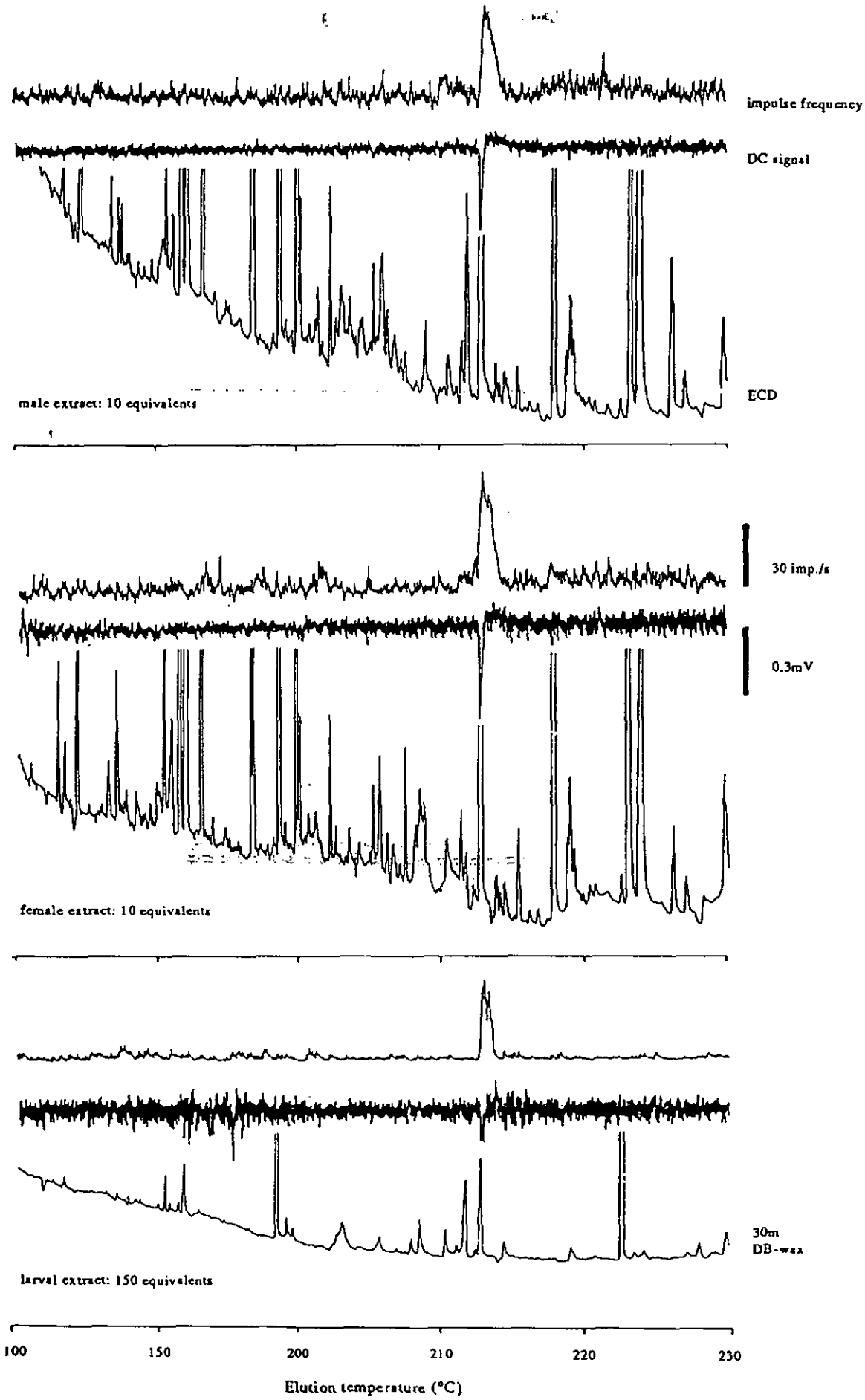


FIGURE 3. (*Caption opposite.*)

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

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

Behavioural bioassay on a dummy female

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

Walking behaviour in wind carrying 2,6-dichlorophenol

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

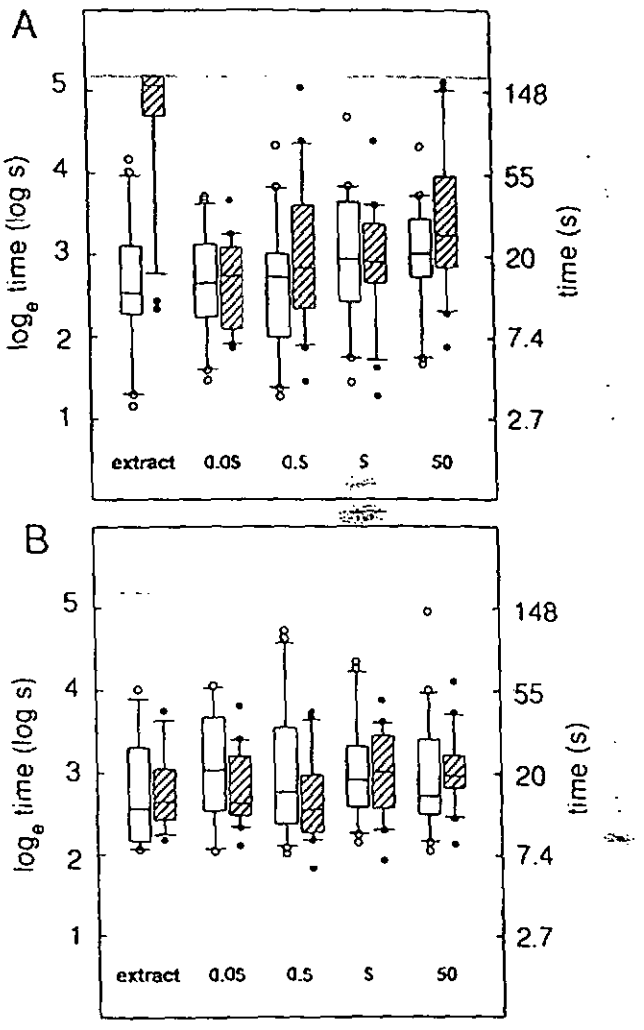


FIGURE 4. Box plots of the time spent by male *B. microplus* on a *ca* 0.5 mm glass bead (A) and the area around it (B) on a host-simulating arena. Open boxes are controls, hatched boxes are tests; the horizontal lines of each box represent from top to bottom the 90th, 75th, 50th, 25th and 10th percentiles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

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

DISCUSSION

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

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

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

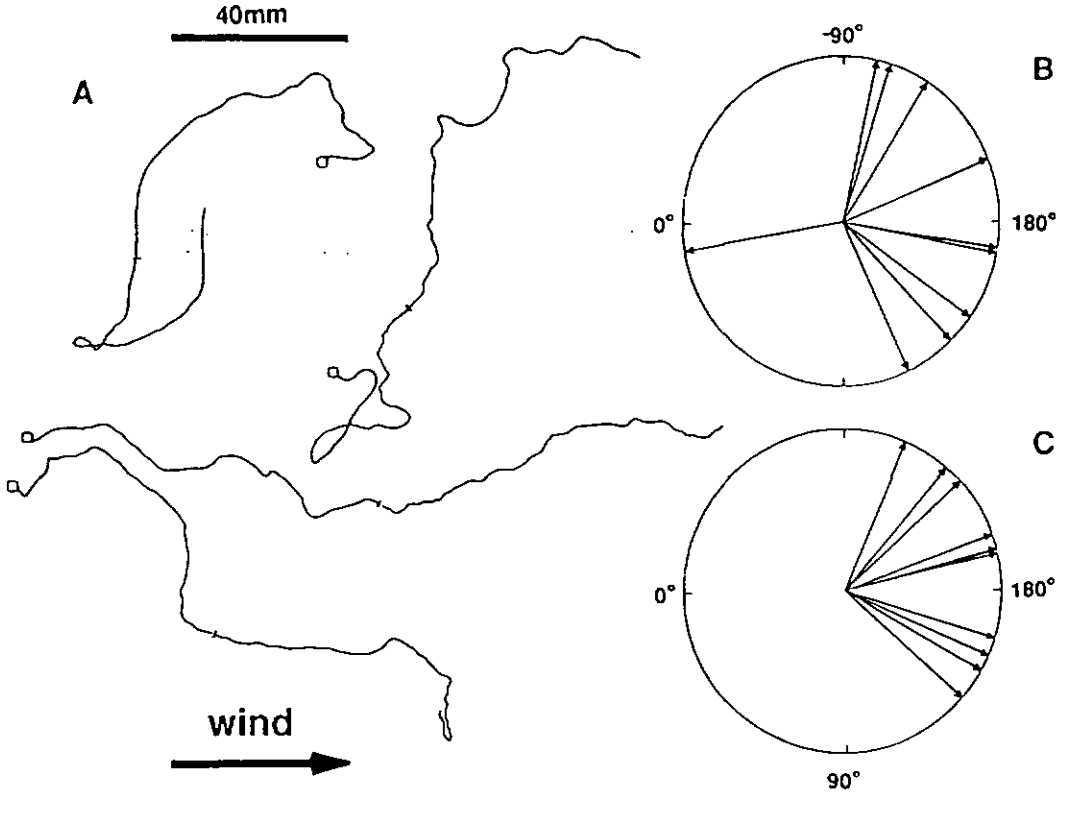


FIGURE 5. Walking behaviour of male *B. microplus* ticks on a locomotion compensator in a constant flow of air (15 cm/s. 90% r.h.). Displacement was recorded during 2 min with a resolution of 0.1 mm. The air over a 5 μg source of 2,6-dichlorophenol on filter paper under paraffin oil was introduced into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

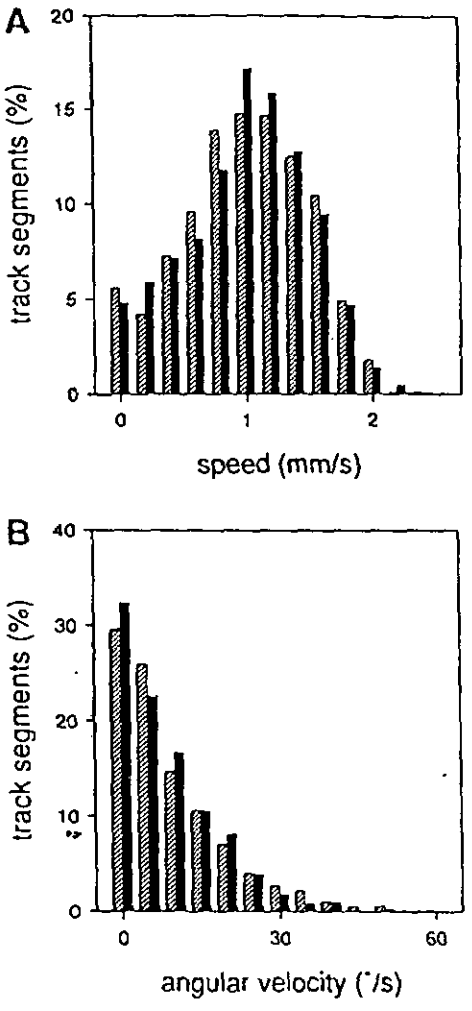


FIGURE 6. Frequency distributions of speed (A) and angular velocity (B) of 0.6 s segments of male *B. microplus* walking tracks on a locomotion compensator. Displacement of ticks was first recorded for 1 min to the conditioned air-stream (15 cm/s, 90% r.h.) with air from a blank stimulus bottle added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

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

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

We also could not demonstrate an oriented response on the part of walking male *B. microplus* to 2,6-DCP in an air-stream on the servosphere. Other tick species, Bruchid beetles and Triatomine bugs do show oriented responses to semiochemicals in the same experimental set-up (Guerin unpublished; Taneja unpublished). The air-stream can apparently be perceived by the ticks since they show an overall downwind walking behaviour, a phenomenon also noted for some of the other arthropods cited above. We therefore conclude that 2,6-DCP does not evoke anemotactic responses in male *B. microplus* ticks guiding them to the stimulus source. Some kind of anemotactic response however, is likely to be involved in the orientation of *A. hebroeum* and *A. variegotum* males to a combined source of CO_2 and 2,6-DCP in the field (Norval *et al.*, 1991) though the role of CO_2 in the blend could be decisive. The doses tested on the locomotion compensator were equivalent to those evoking strong responses in electrophysiology. Unoriented responses such as a change in walking speed or angular velocity (rate of turning) indicating kinetic orientation mechanisms (orthokinesis and klinokinesis, respectively) were also excluded by our experiments.

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

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

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

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

REFERENCES

Apps P. J., Viljoen H. W. and Pretorius V. (1988) Aggregation pheromones of the bont tick *Amblyamma hebraeum*: identification of candidates for bioassay. *Onderstepoort J. Vet. Res.* 55, 135–137.

Batschelet E. (1981) *Circular Statistics in Biology*. Academic Press, London.

Berger R. S. (1972) 2,6-Dichlorophenol, sex pheromone of the lone star tick. *Science* 177, 704–705.

Berger R. S., Dukes J. C. and Chow Y. S. (1971) Demonstration of a sex pheromone in three species of hard ticks. *J. med. Ent.* 8, 84–86.

Bull C. M. and Andrews R. H. (1984) Two different mating signals used by female reptile ticks. In *Acarology VI* (Eds Griffiths D. A. and Bowman C. E.), pp. 427–429. Ellis Horwood, Chichester.

Byrne K., Gore W. E., Pearce G. T. and Silverstein R. M. (1975) Porapak-Q collection of airborne organic compounds serving as models for insect pheromones. *J. chem. Ecol.* 1, 1–7.

Chow Y. S., Lu F. M., Peng C. T. and Cheng P. C. (1972) Isolation of lipids and sex pheromone from hard ticks. *Bull. Inst. Zool. Acad. Sin. (Taipei)* 11, 1–8.

Chow Y. S., Wang C. B. and Lin L. C. (1975) Identification of a sex-pheromone of the brown dog tick *Rhipicephalus sanguineus*. *Ann. ent. Soc. Am.* 68, 485–488.

Diehl P. A., Guerin P. M., Vlimant M. and Steullet P. (1991) Biosynthesis, production site, and emission rates of aggregation-attachment pheromone in males of two *Amblyomma* ticks. *J. chem. Ecol.* 17, 833–848.

Dinnik J. and Zumpt F. (1949) The integumentary sense organs of the larvae of Rhipicephalinae (Acarina). *Psyche (Camb.)* 56, 1–17.

Falk-Vairant J., Guerin P. M., de Bruyne M. and Rohrer M. (1994) Some observations on mating and fertilisation in the cattle tick *Boophilus microplus*. *Med. Vet. Ent.* 8. In press.

Gladney W. J., Grabbe R. R., Ernst S. E. and Oehler D. D. (1974) The gulf coast tick: evidence of a pheromone produced by males. *J. med. Ent.* 11, 303–306.

Gödde J. (1989) Vibrating glass stylets: tools for precise microsurgery on cuticular structures. *J. Neurosci. Meth.* 29, 77–83.

Graf J.-F. (1975) Ecologie et éthologie d'*Ixades ricinus* L. en Suisse (Ixodoidea; Ixodidae), cinquième note: mise en évidence d'une phéromone sexuelle chez *Ixades ricinus*. *Acarologia* 17, 436–441.

Guerin P. M., Steullet P., Kröber T., Diehl P. A., Vlimant M., de Bruyne M., Cordas T., Falk-Vairant J., Kuhnert F. and Lösel P. M. (1992) The chemical ecology of ticks at the host vector interface. In *Proc. 1st Int. Conf. Tick-borne Pathogens at the Host-vector Interface: an Agenda for Research* (Eds Munderloh U. G. and Kurtii T. J.), pp. 314–323. Minnesota University, Saint Paul, MN.

Haggart D. A. and Davis E. E. (1981) Neurons sensitive to 2,6-dichlorophenol on the tarsi of the tick *Amblyamma americanum* (Acari, Ixodidae). *J. Med. Ent.* 18, 187–193.

Hess E. and Vlimant M. (1986) Leg sense organs of ticks. In *Morphology, Physiology, and Behavioural Biology of Ticks* (Eds Sauer J. R. and Hair J. A.), pp. 361–390. Ellis Horwood, Chichester.

Kellum D. and Berger R. S. (1977) Relationship of the occurence and function of 2,6-dichlorophenol in two species of *Amblyamma*. *J. med. Ent.* 13, 701–705.

Khalil G. M., Nada S. A. and Sonenshine D. E. (1981) Sex pheromone regulation of the mating behaviour in the camel tick *Hyalomma dromedarii* (Ixodoidea: Ixodidae). *J. Parasit.* 67, 70–76.

Kramer E. (1976) The orientation of walking honeybees in odour fields with small concentration gradients. *Physiol. Ent.* 1, 27–37.

Leahy M. G. and Booth K. S. (1983) Attraction of metastriate ticks (Acari: Ixodidae) to sex pheromone 2,6-dichlorophenol and to substituted phenols. *J. med. Ent.* 1, 104–105.

Leonovich S. A. (1981) Occurence of a sex pheromone in the Ixodidtick *Hyalomma asiaticum* (Ixodidae). *Parasitologiye (Leningr.)* 15, 159–156.

Londt J. G. H. and Arthur D. R. (1975) The structure and parasitic life cycle of *Boophilus microplus* (Canestrini, 1888) in South Africa (Acarina: Ixodidae). *J. Ent. Soc. S. Afr.* 38, 321–340.

McDowell P. G. and Waladde S. M. (1986) 2,6-Dichlorophenol in the tick *Rhipicephalus appendiculatus* Neumann: a reappraisal. *J. chem. Ecol.* 12, 69–82.

Noldus L. P. J. J. (1991) The observer: a software system for collection and analysis of observational data. *Behav. Res. Meth. Instrum. Comput.* 23, 415–429.

Norval R. A. I., Peter T., Yunker C. E., Sonenshine D. E. and Burridge M. J. (1991) Responses of the ticks *Amblyomma hebraeum* and *A. variegatum* to known or potential components of the aggregation-attachment pheromone. I. Long-range attraction. *Exp. Appl. Acarol.* 13, 11–18.

Schöni R., Hess E., Blum W. and Ramstein K. (1984) The aggregation-attachement pheromone of the tropical bont tick *Amblyomma variegatum* Fabricius (Acari: Ixodidae): isolation, identification and action of its components. *J. Insect Physiol.* 30, 613–618.

Schulze P. (1942) Die Rückensinnesfelder (Foveae dorsales) der Zecken. *Z. Morph. Oekol. Tiere* 39, 1–20.

Smith J. J. B., Mitchell B. K., Rolseth B. M., Whitehead A. T. and Albert P. J. (1990) SAPID tools: microcomputer programs

for analysis of multi-unit nerve recordings. *Chem. Senses* 15, 253–270.

Sonenshine D. E. (1985) Pheromones and other semiochemicals of the Acari. *A. Rev. Ent.* 30, 1–28.

Sonenshine D. E., Silverstein R. M., Plummer E. C., West J. R. and McCullough T. (1976) 2,6-Dichlorophenol, the sex pheromone of the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles and the American dog tick, *Dermacentor variabilis* (Say). *J. chem. Ecol.* 2, 201–209.

Sonenshine D. E., Gainsburg D. M., Rosenthal M. D. and Silverstein R. M. (1981) The sex pheromone glands of *Dermacentor variabilis* (Say) and *Dermacentor andersoni* Stiles, sex pheromone stored in neutral lipid. *J. chem. Ecol.* 7, 345–357.

Sonenshine D. E., Silverstein R. M. and Rechav Y. H. (1982) Tick pheromone mechanisms. In *Physiology of Ticks* (Eds Obenchain F. D. and Galun R. L.), pp. 439–468. Pergamon, Oxford.

Sonenshine D. E., Silverstein R. M. and West J. R. (1984) Occurrence of the sex attractant pheromone, 2,6-dichlorophenol, in relation to age and feeding in the American dog tick, *Dermacentor variabilis* (Say) (Acari, Ixodidae). *J. chem. Ecol.* 10, 95–100.

Waladde S. M. (1982) Tip recording from ixodid tick olfactory sensilla: responses to tick related odours. *J. comp. Physiol.* 148, 411–418.

Wood W. F., Leahy M. G., Galun R., Prestwich G. D., Meinwald J., Purnell R. E. and Payne R. C. (1975) Phenols as pheromones of ixodid ticks: a general phenomenon? *J. chem. Ecol.* 1, 501–509.

Yunker C. E., Peter T., Norval R. A. I., Sonenshine D. E., Burridge M. J. and Butler J. F. (1992) Olfactory responses of adult *Amblyomma hebraeum* and *A. variegatum* (Acari: Ixodidae) to attractant chemicals in laboratory tests. *Exp. Appl. Acarol.* 13, 295–301.

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

Cholesteryl Esters and other Contact Chemostimuli in the Mating Behaviour of the Cattle Tick *Boophilus microplus*.

MARIEN DE BRUYNE, PATRICK M. GUERIN

manuscript intended for submission to Arch. Insect Biochem. Physiol.

INTRODUCTION

Ticks (Acari: Ixodida) are obligate blood-sucking ectoparasites of vertebrates. All life-stages feed but females take a big blood meal that is used to produce a large quantity of eggs. Fertilisation of eggs is a necessity and mating is necessary for engorgement by females (Pappas and Oliver, 1972). Copulation takes place on the host in metastriate ticks and mating behaviour is relatively similar in different species (Feldman-Muhsam, 1986, Sonenshine, 1985). Males detach and search for females. Upon contact, the male mounts the attached female and investigates her dorsum, probing the surface with his tarsi and mouthparts. He then proceeds to the ventral side, aided by the female who lifts her body, locates the gonopore and inserts his chelicerae. After some time, a spermatophore is produced and transferred to the gonopore.

Volatile pheromones have been shown to stimulate aggregation on the host in several *Amblyomma* species (Gladney *et al.*, 1974b) and have recently been suggested for *Hyalomma truncatum* (Dongus and Gothe, 1995). Such aggregation-attachment pheromones are produced by feeding male ticks to attract other conspecifics to a host and induce attachment. They generally consist of a mixture of phenols and short-chain fatty acids (Schöni *et al.*, 1984, Apps *et al.*, 1988). On the host, 2,6-dichlorophenol has repeatedly been suggested as a volatile sex pheromone in many tick species which induces males to detach and move towards females (Sonenshine, 1985).

A potent non-volatile pheromone mediates mating behaviour once the male contacts the female (Hamilton and Sonenshine, 1988) and cholesteryl oleate and other cholesteryl esters have been implied 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 these mating responses, whereas a second sex pheromone containing fatty acids mediates the probing of the gonopore (genital sex pheromone, Allan *et al.*, 1988). Cholesteryl esters have also been reported to induce mating responses in *Rhipicephalus appendiculatus* (Hamilton *et al.*, 1994). Other non-volatile compounds (assembly pheromones) are involved in off-the-host aggregation in several argasid and ixodid tick species (Leahy *et al.*, 1973, Háková *et al.*, 1980, Petney and Bull, 1981). Guanine, an excretory product of ticks, has been identified as an arrestant for *Argas persicus* and *Rhipicephalus sanguineus* (Otieno *et*

al., 1985) and other purines are thought to play a role in this behaviour as well (Dusbábek *et al.*, 1991b).

Boophilus microplus (Canestrini) is a major pest of cattle throughout the tropical and subtropical regions causing extensive losses to the industry by weakening cattle as well as transmitting babesiosis and anaplasmosis. This is a one-host species, *i.e.*, whereas most tick species drop from the host to moult, this species goes through all life-stages on the same bovine host. In spite of its obvious importance very little is known about the mating behaviour and chemical ecology of *B. microplus*. It has been shown that 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 in the night of the third day after male moult when females are well attached 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 gound to start oviposition (2-4 days after copulation). The males can live on the host for up to 40 days after moulting and are able to fertilise several females (Thompson, *et al.*, 1980).

Ticks bear gustatory terminal pore sensilla on the palps and legs (see appendix II). The fourth segment of the palps in Ixodid ticks is a modified movable organ that can be folded back and inwards. On the apical surface of this palpal organ, *Amblyomma americanum* bears 10 sensilla of two types. Four are Tp A type with a double lumen and electron dense sensillum liquid around the dendrites (upto 4) in the inner lumen and six areTp B with a single lumen and its dendrites (7-12) enclosed in a cuticular sheath, one of which is not innervated by a tubular body at the base (Foelix and Chu Wang, 1972). Ivanov and Leonovich (1983) describe the same for *Hyalomma asiaticum* and some other Ixodid ticks. However *B. microplus* adults have only nine such terminal pore sensilla, six Tp B and three Tp A (FIG 8B), one of which is placed a little off the apical surface of the palp (Waladde, 1978). All are innervated by several dendrites, the cell bodies of which lie deep down in the palpal organ. On the tarsus of the first pair of legs *B. microplus* carries twelve more gustatory sensilla (Hess and Vlimant, 1986) one pair of Tp B is located distally just behind the claws, ten other Tp sensilla on tarsus I are of the A type: one pair distally below the Tp B, two pairs placed ventrally and more proximal and two dorsally behind Haller's organ.

Little is known about the physiology of these sensilla, though Balashov *et al.* (1976) noticed electrical differences between Tp A and B sensilla of the palpal organ of *H. asiaticum*, and reported responses to mechanical stimuli and NaCl solutions. Palpal chemoreceptors are suggested to perceive assembly pheromones in Argassid ticks (Leahy *et al.*, 1975a). Masking experiments suggest that terminal pore sensilla on the tip of the tarsi of the first pair of legs perceive a contact sex pheromone in *Dermacentor* (Phillips and Sonenshine, 1993). Other gustatory sensilla are present on the chelicerae (Waladde and Rice, 1977) and it has been shown in *D. variabilis* that they respond to 20-hydroxyecdysone which has been proposed as another component of the genital sex pheromone (Taylor *et al.*, 1991).

Wall-pore olfactory sensilla are exclusively located on the tarsi of the first pair of legs especially in Haller's organ (Hess and Vlimant, 1986). Their olfactory function has been demonstrated in several species, most recently in *Amblyomma variegatum* (Steullet and Guerin, 1994a&b). Two wall-pore sensilla on the tarsi of *B. microplus* males house receptors for 2,6-dichlorophenol, and this compound 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.

Here we present evidence for behaviourally and electrophysiologically active components isolated from *B. microplus* ticks which mediate mating in this species. Males are arrested *in vitro* on a substrate treated with these extracts and behavioural elements strongly resemble mating behaviour. One active fraction is identified as containing cholesteryl esters but other components are required to inducing the full mating behaviour.

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 %RH. 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 *ca.* 100 %RH until they moulted, and males were put on the ears of New Zealand White rabbits enclosed in cotton bags where they readily attached. Males were removed from the rabbit 20-60 min before bioassays and kept at *ca.* 30°C over water in a closed container to assure high relative humidity. Males were kept in an incubator at 18°C on humid tissue paper for 1-4 days before electrophysiological recordings.

Behavioural Bioassay

A glass bead (*ca.* 5 mm dia., 3 mm high, 0.1-0.2 g), roughened with a wet-stone and flattened on one side to inhibit rolling, was placed in the centre of a round arena (40 mm dia.) consisting of a Baudruche® membrane (Joseph Long Inc., USA) 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 5x or 21x with a Canon CI-20P colour CCD video camera attached to a Zeiss 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 first on the control the other half first on the test. Different behaviours were quantified using THE OBSERVER 2.0 event recorder (Noldus Inf. Tech., Netherlands). The tick was recorded as either being on the bead, *i.e.*, from the first moment all legs were in contact with it 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 was ended when it 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 versus control). 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, cholestanol (dihydrocholesterol), ecdysone (2,3,14,22,25-pentahydroxycholest-7-ene-6-one), caffeine (1,3,7-trimethyl-2,6-dioxopurine) and MSTFA (N-Methyl-N-trimethylsilyl-trifluoroacetamide) were obtained form Sigma, USA. Fatty acid methyl esters and 2,6-dichlorophenol were obtained from Supelco, USA. Palmitoleic acid was purchased from Larodan

AG, Germany. All other fatty acids, cholest-4-en-3-one and lecithine (1,2-dioleoyl-sn-glycero-3-phosphocholine) were from Fluka, Switzerland. Cholesterol and all solvents (analytical grade) were from Merck, USA. The 9,10,16-trihydroxypalmitic acid (aleuritic acid) was kindly supplied to us by Dr S. Schulz, University of Hamburg, Germany.

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 equivalent/µl and stored at -20°C. Tick washes were obtained by extracting them for only 30 min at room tempereature (*ca.* 22°C), using either hexane, chloroform:methanol or methanol:water (1:1). A bovine hair extract was obtained by repeatedly washing hair shaved off *ca.* 0.3 m^3 of the flank of a Simmental steer in dichloromethane. The filtered extract was concentrated and contained *ca.* 10 µg/l of low volatile mass (Kröber, unpublished).

Thin layer chromatography and solid phase extraction

Comparative chemical analysis and preparative chromatography was carried out on 20 x 20 cm thin layer chromatography (TLC) plates with 0.25 mm layers of silica gel 60 including a 2.5 cm concentration zone (Merck, 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 dia. 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: I) hexane to 17 cm, toluene to 17 cm and twice hexane:diethyl ether:acetic acid (70:30:1) to 12 cm or II) chloroform:methanol:water (69:27:4) to 12cm. 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 was 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 3). The silica gel was subsequently eluted over glass wool in a pasteure pipette with 2 ml chloroform for each fraction, then dried under nitrogen and redissolved in a smaller volume of chlorofrom.

Preparative solid phase extraction (SPE) was done on 500 mg Silica gel in a glass Chromabond® column (Machery-Nagel, 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 *ca.* 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 methanol (F11), and methanol:water (F12) fractions were dried under nitrogen and redissolved in chloroform. These fractions were tested as A (F1+2+3+4), B (F5+6+7) and C (F8+9+10+11+12) in the behavioural bioassays.

Isolation 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 ug 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, USA, 0.25 µm film thickness, 0.25 mm ID) in a Carlo Erba HRGC 5160 gas chromatograph (GC) with H_2 as carrier gas at 1.5 ml/min (0.5 m/s) temperature programmed from 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 for 10 min. Identification was by comparing retention times with standards and 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 being flame-sealed.

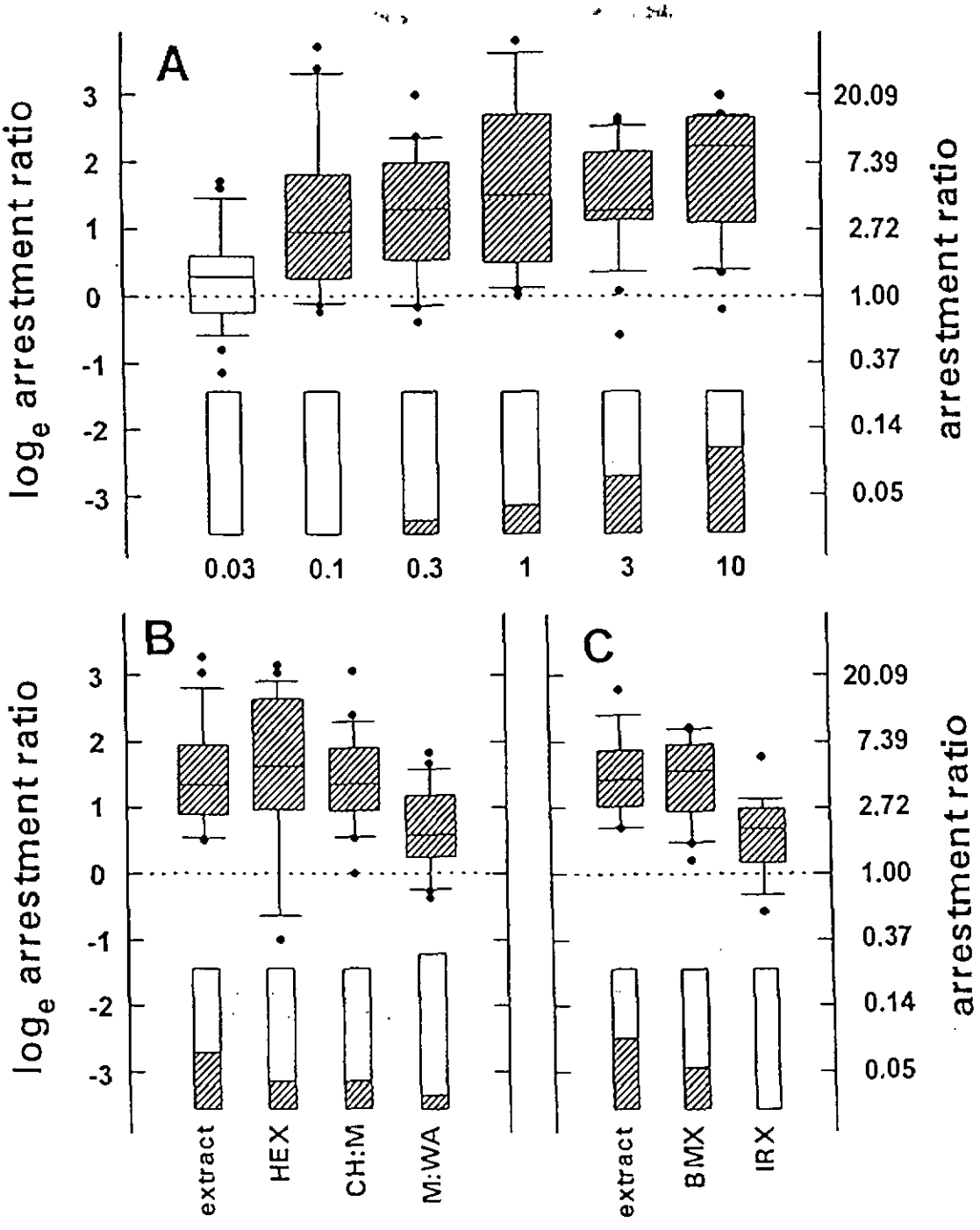


FIGURE 1. Behavioural responses of individual male *B. microplus* ticks ($16 \leq n \leq 29$) to a glass bead treated with extracts of female ticks (test) or solvent only (control). Box plots show the distribution of the natural logarithm of the arrestment ratios, *i.e.*, the ratios between the time 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 indicate 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 such an extract 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 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.

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 an Hewlett Packard 5890 series II gas chromatograph. The sample (1 ul) was injected on-column via a 1 m precolumn into a 15 m DB-5HT nonpolar capillary column (J&W scientific, USA, 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 combistereo microscope (1000x, working distance 11-mm, M3Z, Wild, Switzerland). A glass reference electrode filled with 0.05% polyvinylpyrrolydon 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 dia.), dipped in the stimulus solution and filled from the back with electrolite. 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 (1000x) with a universal AC/DC amplifier (UN-03, Syntech, The Netherlands) and recorded via a DAS 16 analogue to digital card (Metrabite Corp., USA) 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 (electrolite 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 a chloroform:methanol (1:1) extract of female ticks they are arrested 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. Conversly, when placed on control beads, males generally leave within 20 seconds and very often raise their body and wave the first pair of legs in the air just before loosing contact with the bead. Arrestment can be observed at several doses of this extract but not below 0.1 female equivalent per bead (FIG 1A). At elevated 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 scored as 'tip-over' (FIG 1). Other males stop all locomotion and pierce the membrane with their mouthparts often attaching perpendicular to the membrane, with six legs on the bead while the first pair rests on the membrane. This behaviour was termed 'membrane probing' and not analysed further, though it should be noted that it occasionally occured 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. The number of males showing tip-over on the test bead was dose

dependant and no males tipped-over on beads treated with less than 0.3 tick equivalents.

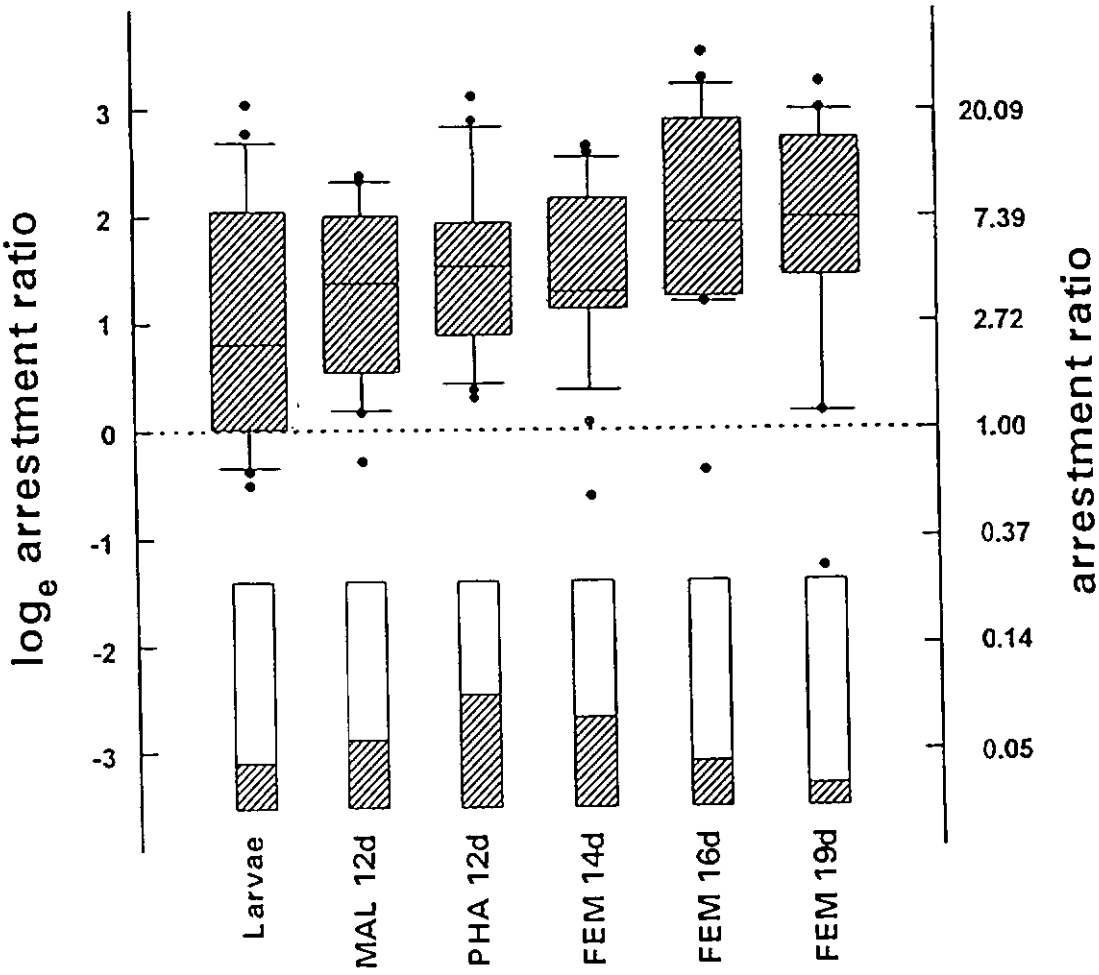


FIGURE 2. Behavioural responses of individual male *B. microplus* ticks to glass beads treated with extracts of different life-stages and different ages (days after infestation) tested at 3 tick equivalents; MAL, male, PHA, pharate female, FEM, female. Unfed larvae were tested at 300 equivalents. For details on presentation of data see FIG 1.

Males were also significantly arrested on beads treated with 30min washes of female ticks in chloroform:methanol (1:1) or hexane at room temperature but less males tipped over (FIG 1B). The more polar methanol:water (1:1) wash was less active. Comparing an extract made of recently moulted female *B. microplus* (day 12) to an equivalent extract of nymphal exuviae, males react similarly on both, except that they tip-over less on the exuvial extract (FIG 1C.). 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 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 2). Strongest tip-over was observed on extract of newly moulted females. Equivalent responses can also be obtained to an extract of newly moulted males and even to 300 equivalents of unfed larvae. However, the bovine hair extract did not induce arrestment on the bead at 6, 20, 60 and 200 ug though some males showed tip-over on the two higher doses (results not shown)

Males are arrested by an apolar fraction but tip-over is lost

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 3A). The three darkest spots show up as bright 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 conditons was performed and ten fractions were scraped off the plate corresponding to some of these spots. After recombining all fractions the original activity at 10 tick quivalents was only partly recovered: male arrestment was considerably less and no tip-over could be scored for any of the fractions (FIG 3B). The limited arrestment was entirely associated with F8. TLC separation of extracts of female and male *B. microplus* is very similar except that spot intensities are reduced for the male, but interestingly the pattern is different for the larval extract (FIG 4). Most notably, the two spots corresponding to F6 and F7 in figure 3A are absent from the *B. microplus* larval extract and that of *I. ricinus* exuviae, wheras bovine hair extract separates similarly to the female *B. microplus* extract. Ecdysone, remaining at the origin, and cholestanol both show up bright orange under UV (366 nm) like the material at the origin in female, male and larval extracts but not so for that of the *Ixodes* and bovine hair extract.

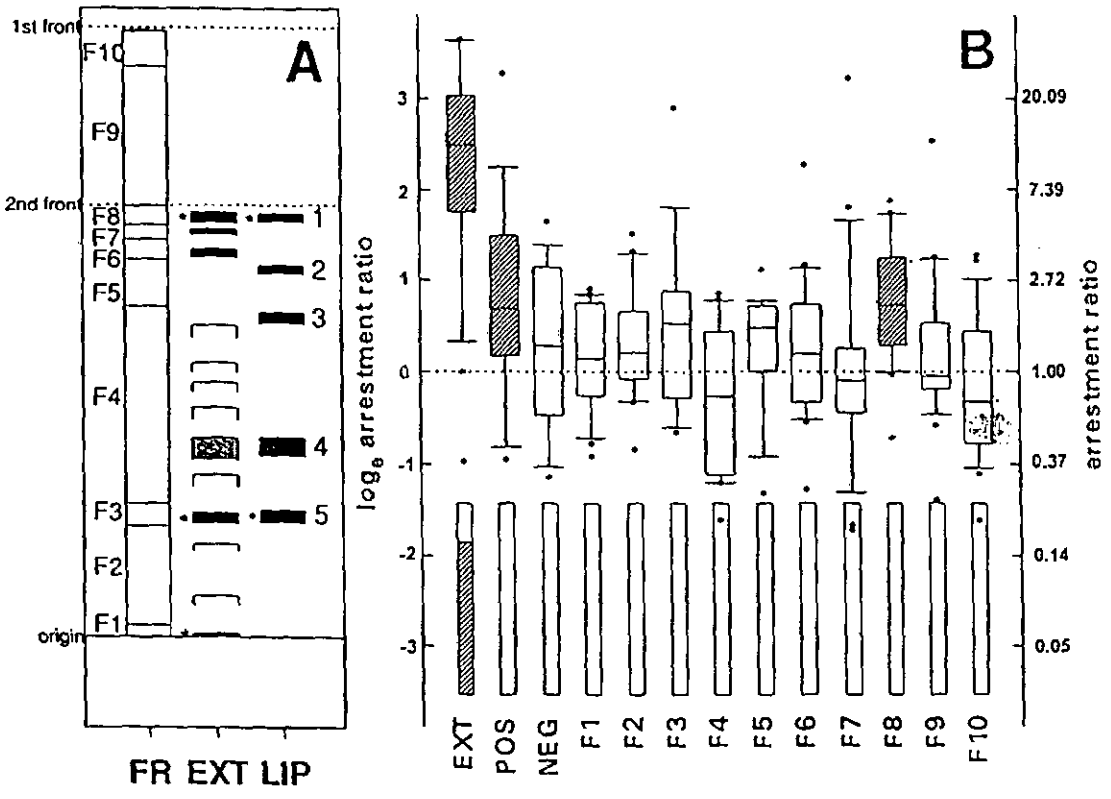


FIGURE 3. Preparative thin layer chromatography (TLC) and bioassay of the fractions of a chloroform extract of female *B. microplus*. A: TLC separation on 0.25mm silica gel of extract (EXT) and lipid standards (LIP). FR indicates fractions of the silica gel scraped off the plate, solvent extracted, and 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 (366nm) are open, the asterisk marks those spots that turn up orange in UV and the grey shading indicates intensity after charring. Standards are 1. cholesterol, 2. oleic acid, 3. triolein, 4 methyl oleate, 5 cholesteryl oleate. B: Behavioural responses of individual male *B. microplus* ticks to a glass bead treated with the female extract and its TLC fractions at 10 equivalents. 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 FIG 1.

A second group of more polar fractions restores total activity.

After separation of 150 female equivalents of a chloroform:methanol (1:1) extract on a small SPE column, recombination of all fractions restores the full activity (FIG 5). Some arrestment is associated with the early eluting, apolar, A set of fractions as might be expected from the behavioural responses to the apolar TLC fraction (compare FIG 3 and FIG 5). However, considerable arrestment is also obtained with the more polar C fractions, whereas the fractions in set B are not active. Of the different combinations, most activity is found after combining A with C. The tip-over on the bead appears to be associated with C and particularly with CA. Analytical TLC using the more polar solvent system II (FIG 6) revealed the presence of several components in the material remaining at the origin with solvent system I (FIG 5A), whereas all spots above cholesterol elute close to the solvent front.

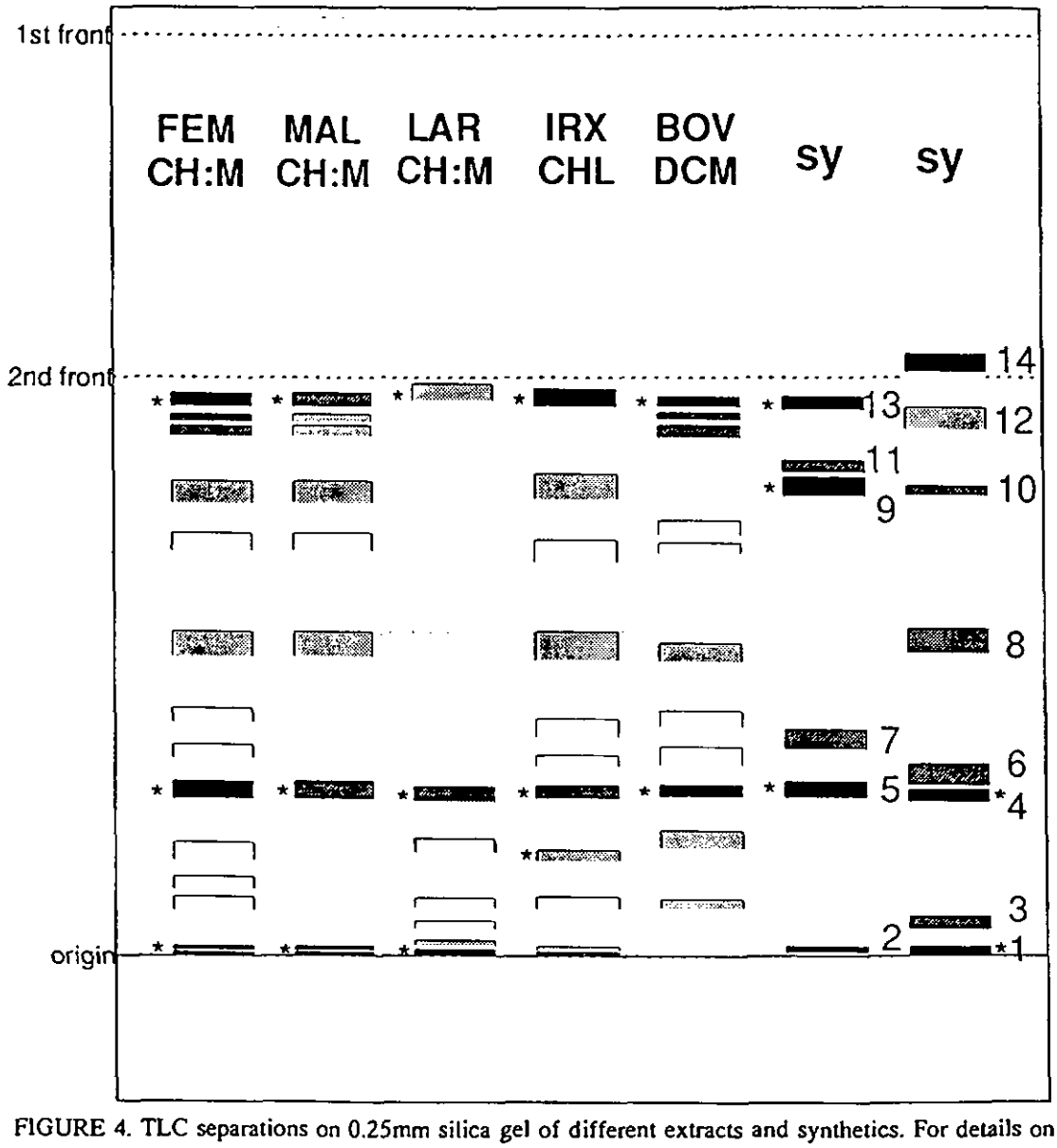


FIGURE 4. TLC separations on 0.25mm silica gel of different extracts and synthetics. For details on solvent system I employed and presentation of data see FIG 3A. FEM, females, MAL, males, LAR, unfed larvae, IRX, *Ixodes ricinus* exuviae, BOV, bovine hair, CHL, chloroform, CH:M, chloroform:methanol (1:1), DCM, dichloromethane. Synthetics (sy) are 1. ecdysone, 2. 9,10,16-trihydroxy-palmitic acid, 3. monoolein, 4. cholestanol, 5. cholesterol, 6. diolein, 7. cholest-4-en-3-one, 8. oleic acid, 9. cholesteryl acetate, 10. triolein, 11. methyl oleate, 12. oleoyl oleate, 13. cholesteryl oleate and 14. squalene.

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. Synthetic mixtures of these products tested in behavioural bioassays with male ticks are indicated on the right.

cholesteryl esters		fatty acid methyl ester (FID peak area) pmol/tick						synthetic mixtures nmol		
trivial name	code	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*	*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
totals		1182	*99*	536	*99*	19	*100*	100	99	97

—, not detected or not present, *,tentative identification of this fatty acid

TABLE 2 Arrestment of male *B. microplus* on a glass bead treated with cholesteryl esters and mixtures*. Data are medians of the arrestment ratios, i.e., the ratio between time spent on the test versus control bead for individual males ($13 \leq n \leq 20$).

cholesteryl esters		median arrestment ratio dose (nmol/bead)			
trivial name	code	1	10	100	1000
		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.*	—
		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
		mixtures			
saturated moieties	mix I	*n.s.*	*n.s.*	1.75	—
unsaturated moieties	mix II	*n.s.*	*n.s.*	*n.s.*	—
combination	mix III	*n.s.*	1.73	1.28	—

*, mixtures as described in table 1, —, not tested, *n.s.*, not significant (Wilcoxon's paired ranks test $p>0.05$)

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 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 capilary 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. The pharate female extract is quite comparable with the female *B. microplus* extract. We could not confirm the presence of methyl cerotate (FAME 26:0) by comparing with the synthetic analogue but it was inferred from its retention time as an additional peak in a regular series from 14:0 to 24:0. The identification of other 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 absence of the longer chain fatty acids.

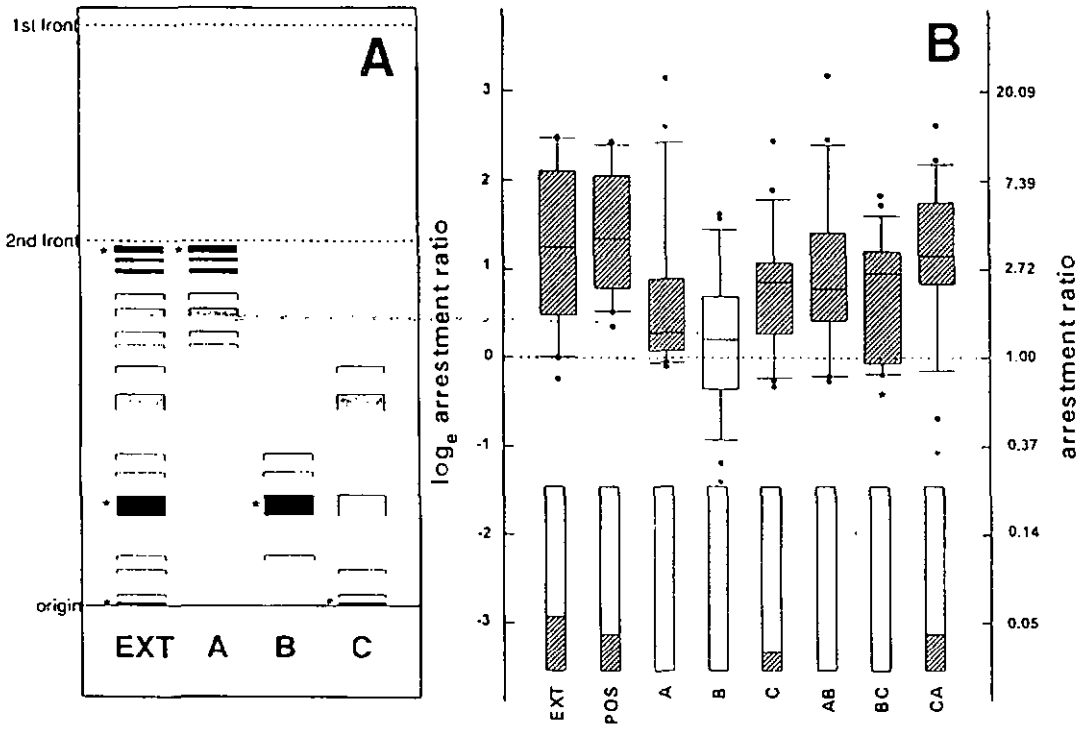


FIGURE 5. Preparative solid phase extraction (SPE) on a small silica gel column of a chloroform:methanol (1:1) extract of female *B. microplus* and bioassay of fractions. SPE eleution was with 4 ml hexane:chloroform (75:25), 3 ml chloroform, 3 ml chloroform:methanol (1:1), 2 ml methanol and 2 ml methanol:water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

When the most important cholesteryl esters were tested individually, none of them arrests male ticks at doses comparable to those present in the extract (TABLE 2). Activity was observed only at very high doses (1000 nmol) of individual products. Of the three different mixtures tested, only the most complete, including both saturated and unsaturated fatty acid esters, was active at the physiologically relevant level of 10 nmol, corresponding to *ca.* 10 tick equivalents.

Cholesterol, free fatty acids and 2,6-dichlorophenol inactive at natural levels

GC-MS analysis of the dichloromethane:methanol fraction form an SPE column indicated the presence of cholesterol and free fatty acids in a chloroform:methanol (1:1) extract of female *B. microplus* at *ca.* 3 nmol cholesterol and *ca.* 0.5 nmol of total free fatty acids per female. A small peak at the retention time of cholesterol was also observed in the SPE fraction F8 used for the bioassays as were free fatty acids. This could be expected from the TLC analysis (FIG 5A).

Cholesterol induces arrestment in male *B. microplus* at doses just above the level present in 10 female equivalents (TABLE 3). A synthetic mixture 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 (FIG 7), nor did it influence tip-over. In addition, no effect was observed for 2,6-dichlorophenol when added to this complex mixture at naturally occurring amounts (*i.e.*, 1.5 ng for 3 equivalents, see de Bruyne & Guerin, 1994).

TABLE 3 Arrestment of male *B. microplus* on a glass bead treated with cholesterol and fatty acid mixture isolated from a chloroform:methanol extract of females. Data are medians of the arrestment ratios ratios, *i.e.*, the ratio between time spent on the test versus control bead for individual *B. microplus* males ($16 \leq n \leq 20$).

synthetic product		median arrestment ratio dose (nmol/bead)			
trivial name	code	1	10	100	1000
cholesterol	*CHol*	*n.s.*	*n.s.*	1.72	2.19
fatty acid mix	*FA*	*n.s.*	*n.s.*	*n.s.*	—

—, not tested, *n.s.*, not significant (Wilcoxon's paired ranks test $p>0.05$)

Electrophysiological responses of gustatory sensilla on male palps: response to the more polar SPE fractions

With the tip recording method used here we could not obtain noticeable electrical contact with palpal sensilla 3, 4 and 9 (FIG.8B). Sensillum 6 was difficult to reach and therefore 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 which consistently responded with a tonic spike train of 30 - 60 spikes/s to the control (FIG.8C). 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 equivalent/µl of SPE fractions F8, F9 and F11.

Fraction F10 was active but to a lesser extent, whereas some inhibition occurred with fraction F5 (FIG.9). Responses to different doses of fraction F8 were obtained (0.001, 0.003, 0.01, 0.03 and 0.1 equivalents/ul) and the response to F9 resembles that to 0.01 equivalent of F8 (FIG.8C). Clearly a receptor or receptors other than the one responding to KCl is/are involved in the perception of these fractions. However, responses are complicated by superposition of spikes, the similarity of spike sizes within single recordings and variability of spike size between recordings (FIG.8) from the same sensillum. A clear response was also obtained to 0.01 and 0.1 equivalents/µl of the unseparated extract but not to 0.1 ug/µl of bovine hair extract.

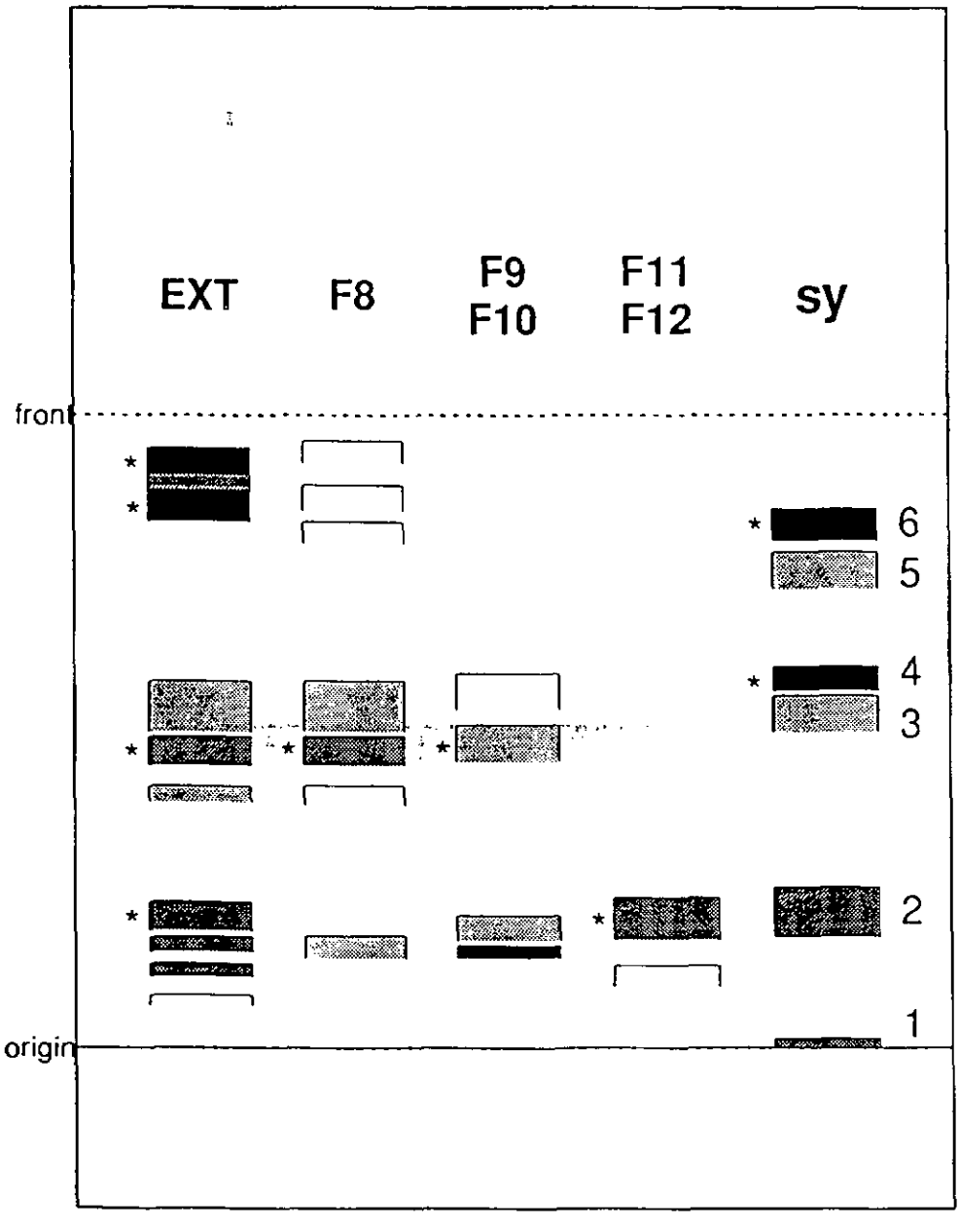


FIGURE 6. TLC separation on 0.25mm silica gel of a chloroform:methanol (1:1) extract (EXT), the polar SPE fractions (that make up C the set in FIG 5A) and some synthetics (sy) with solvent system II (chloroform:methanol:water 69:27:4 to 12cm). Spots that could only be observed under UV light (366nm)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

DISCUSSION

Male behavioural responses to chemical stimuli on the cuticle of females

The mating behaviour 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 distincly different from that on an untreated bead. Responses are obtained to doses of extracts near naturally occurring levels. This response is obtained to extracts of whole ticks and of exuviae. Several elements are present in the behaviour observed on the glass beads which also occur during mating on the host such as the strong drive to tip-over and crawl under the bead.

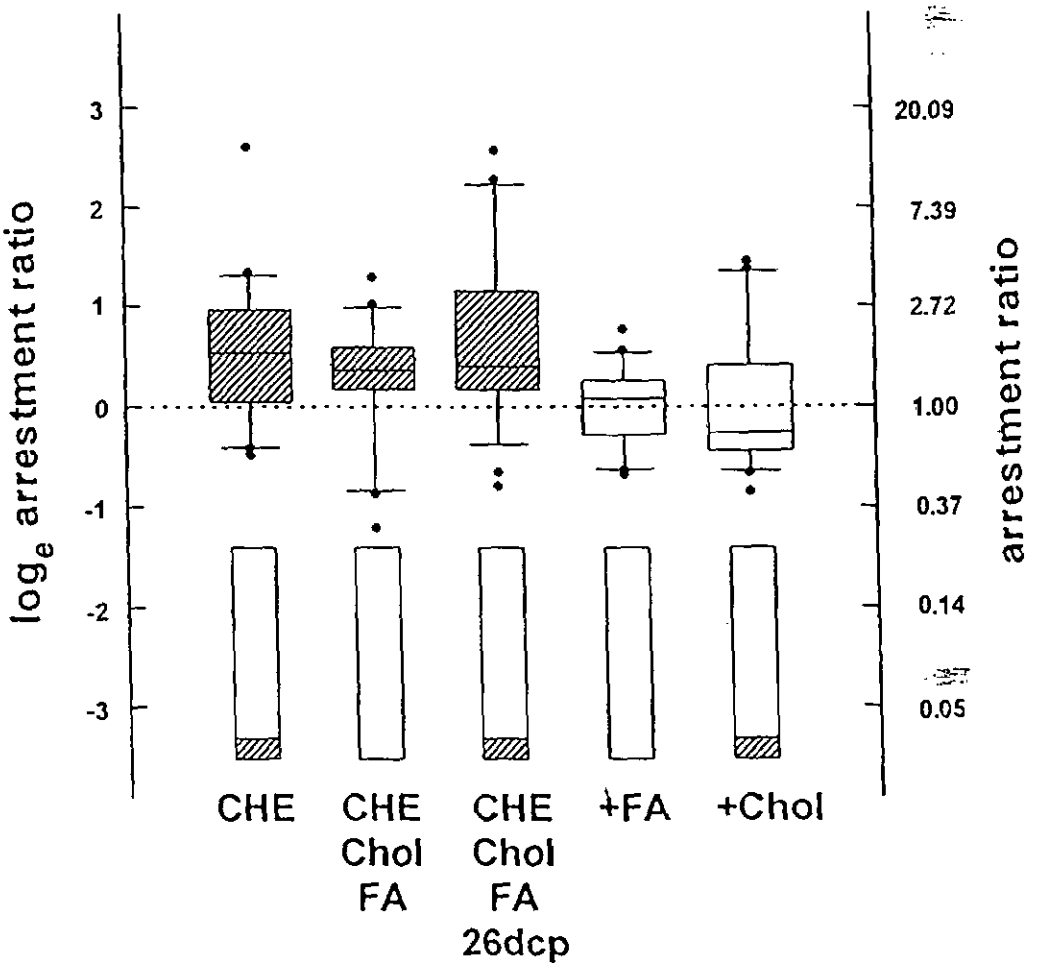


FIGURE 7. Behavioural responses of individual male *B. microplus* ticks ($16 \leq n \leq 29$) to a glass bead treated with different combinations of synthetic products identified in extracts of female *B. microplus*, tested at doses comparable to those found in 3 tick equivalents; CHE, cholesteryl ester mix, FA, fatty acid mix, Chol, cholesterol, 26DCP, 2,6-dichlorophenol. In the case of +FA both test and control were treated with CHE but FA was added on the test bead. Similarly cholesterol was added to CHE and FA in +Chol. For details on presentation of data see FIG 1.

The most quantifiable effect is above all an arrestment. Males spend more time investigating the treated bead. The arrestment ratio, the parameter we have analysed in bioassays, is defined related to location of an individual male tick, *i.e.*, on the bead or on the surrounding membrane-arena and measured in time (duration of contact). The second effect, quantified in the parameter tip-over, is defined in behavioural terms and

its incidence simply 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 doing so. This could explain the slight increase in arrestment with extract dose. However, other behaviours cause arrestment without, or before tip-over. In several experiments there is arrestment without tip-over and arrestment increases in response to extracts of older females but less males show tip-over. We observed many males probing and sampling the bead's surface for a long time, either stationary or while moving around. Conversely, some males only show a brief attempt to crawl under the bead which does not contribute much to total arrestment.

Since arrestment and tip-over involve distinct behaviours, these can then also be mediated by different cues. This is supported by the fact that the tip-over response disappears after TLC fractionation of the female extract, whereas arrestment is still significant. The primary effect of cholesteryl esters, isolated and identified by TLC, is therefore on other motor pattems causing arrestment. A second chemical cue is then necessary to induce a complete response, *i.e.,* increasing arrestment and including tip-over.

The tipping over is more typical of what occurs during mating. More males show this response at higher doses of the female extract. It can and probably will be only performed on an appropriately shaped object. Hamilton and Sonenshine (1995) have shown that two *Dermacentor* species can differentiate between different sizes of plastic beads treated with female extracts. In our experiments, we regularly observed male ticks that had just left a bead, to mount it again after recontacting it. This was observed with control as well as test beads, indicating that untreated beads also represent a mechanical stimulus sufficient for mounting. Since tip-over behaviour was never observed in controls we conclude that it is triggered by a chemical cue even though mechanical ones probably play an important role in its control.

Role of cholesteryl esters and cholesterol in arrestment

We isolated and identified several cholesteryl esters from tick extracts which mediate arrestment of male *B. microplus* on glass beads. In addition, substantial amounts of cholesterol were found in extracts of female *B. microplus*. The cholesteryl esters contained various fatty acid moieties, predominantly saturated long-chained ones but also unsaturated. Cholesterol and cholesteryl esters have been shown to be very abundant components of cuticle lipids of female *B. microplus* (Cherry, 1969). The 0.8-0.9 ug of cholesterol per tick reported by this author corresponds well with our results (3 nmol ≡ 1 ug) and cholesteryl ester levels are only a factor 3 lower in our analysis (0.8 ug, as calculated from formula weights, compared to 2.6 ug).

Like insects, ticks are presumed not to be able to synthesise the steroid ring structure and rely on the cholesterol content of the blood meal (Maroun and Kamal, 1976). The cholesterol on the cuticle, free or esterified, is thought to be derived from feeding. It could be equally possible that the fatty acid profile of the esters on the cuticle of *B. microplus* is a direct reflection of diet. The excretion of certain plant steroids in human skin for instance has been shown to be directly related to diet (Bhattacharyya *et al.*, 1983). However cholesterol and cholesteryl esters on the cuticle of *B. microplus* might also be, at least partly, contaminants from bovine skin secretions. The relatively high proportion of saturated straight chain fatty acids found

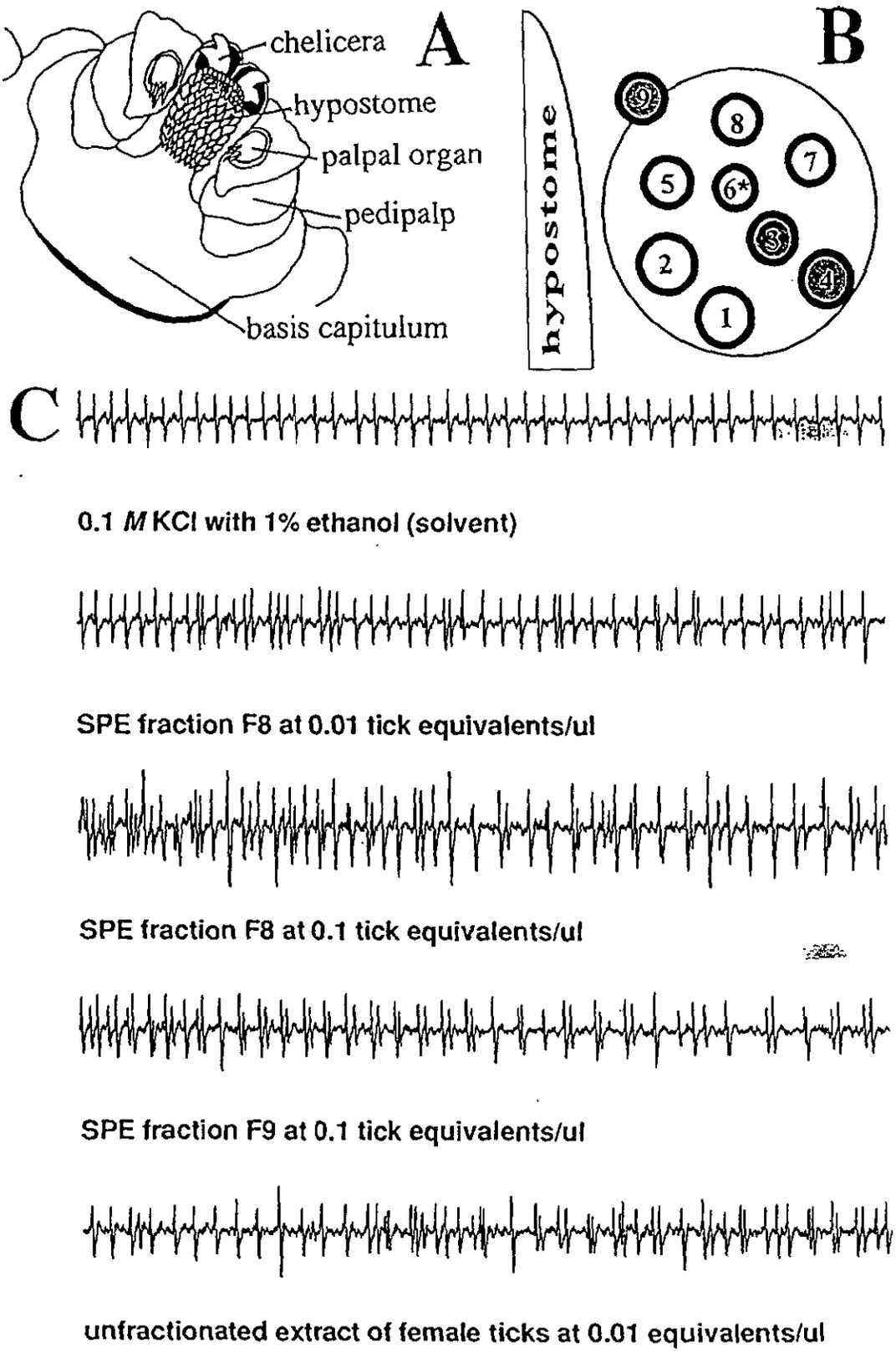


FIGURE 8. Electrophysiological responses from contact chemoreceptors in terminal pore sensilla on the palps of male *B. microlpus* in response to stimulation with an extract of female ticks or fractions thereof (SPE, solid phase extraction). A: Ventral view of the mouthparts showing the palps and the 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 (0.5s) obtained 0.1s after bringing a glass electrode containing the stimulus dissolved in 0.1*M* KCl and 1% ethanol, into contact with the tip of sensillum 7.

here on the female cuticle is 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. This in turn compares well with the chromatogram that Chow *et al.* (1972) present for fatty acid methyl esters of *B. microplus*. Downing and Lindholm (1982) report the presence of two classes of wax esters that elute between triglycerides and cholesteryl esters in their TLC analysis of surface lipids of the cow, which make up more than 50% of the total. Since their TLC system can be compared to ours we may conclude that these are the components present in our TLC fractions F6 and F7 of female extract. The absence of this material from *B. microplus* larval and *I. ricinus* exuvial extracts could stem from the fact that both *B. microplus* larvae and *I. ricinus* nymphs have not been in contact with a bovine host. The former are reared on mice and the latters entire cuticle is synthesised from material present in the egg. The cholesteryl ester profile of *B. microplus* larvae also differs from that of pharate and adult females.

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. Cholesterol and free fatty acids appear to have no effect on their own nor in combination with cholesteryl esters. Cholesterol is however the only product which shows activity on its own at a relatively low dose compared to levels occurring on the cuticle of females.

Whether ticks are capable of differentiating between fatty acid moieties of cholesteryl esters at the sensory level remains unknown. We have not been able to make satisfactory recordings from the sensilla on the tarsi that are suggested by Phillips and Sonenshine (1993) to play a role in perception of cholesteryl esters in *Dermacentor*. However, such recordings have been made successfully in this laboratory from one of the two paired Tp sensilla on the tip of the tarsus I of *Ixodes ricinus* (Guerin *et al.*, 1992). These authors also demonstrated the crucial role in mating behaviour of receptors on the first and second pair of tarsi for *B. microplus* with masking experiments.

A second chemical signal mediating tip-over

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. Both methods use solid-liquid chromatography on silica gel, hence detrimental effects can only come 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.

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. This could point to a more complex composition of the adequate stimuli for these receptors than just a single compound or class of compounds.

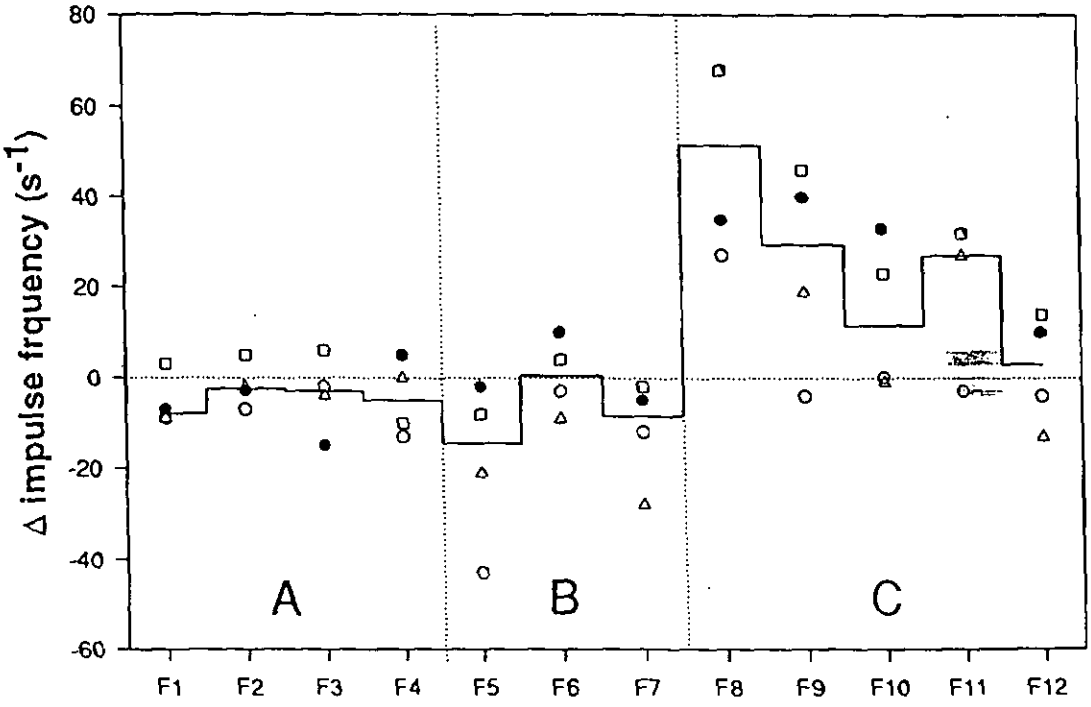


FIGURE 9. Electrophysiological responses of sensillum 2 (closed symbols) and 7 (open symbols) on the palpal organ of five male *B. microlpus* to twelve fractions obtained after separation of a female extract on a small silica column (SPE, solid phase extraction). The letters A, B and C indicate the combined fraction sets tested in the behavioural bioassay (see FIG 5). Responses are calculated by substracting the total number of impulses in controls (0.1*M* KCl + 1% ethanol) from the total number of impulses recorded in response to stimulation with the individual fractions (0.1 female equivalents/µl in 0.1*M* 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 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. On test beads, males cling to the substrate, bringing the palps into close contact with it and make vigorous probing movements with the mouthparts. Therefore, responses to the more polar fractions of the female extract eluting from the small silica column in two of the nine gustatory sensilla on the palpal organ gives 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. On extract-treated beads the capitulum was often flexed at 90° to the substrate. This might also indicate the involvement of the chelicerae and even efforts to penetrate the surface. 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 in an effort to compensate for loss of stimulation. Electrophysiological investigations of the palpal organ's mechano-gustatory sensilla have been rare so little information is available on the relevant chemical stimuli ticks are able to perceive via these sensilla. A more elaborate description of their sensitivity and specificity to various chemicals is needed.

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 occur between species (Sonenshine *et al*, 1991). The second signal isolated here offers the possibility of more species specific signals. The lack of tip-over and reduced arrestment to the cuticle extract of *I. ricinus* suggests that at least in this species this signal is either absent or different since cholesteryl esters do appear in the TLC separation. However, it is known that *B. microplus* intermates with both *B. annulatus* (Graham *et al.*, 1972) and *B. decoloratus* (Spicket 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 be that it is particularly prominent in females shortly after moulting.

In tick species previously investigated a role for a second more polar signal in addition to the isolated cholesteryl esters in mating behaviour 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 mating cannot proceed normally. However, use of delipidised females carries the inherent danger that, while the solvent rinse they employ is effective in removing the cholesteryl ester fraction responsible for arrestment, it may not succeed in removing additional products acting synergistically. The mating behaviour can than only be restored when cholesteryl esters are added to them. Alternatively, it could be that mating behaviour of *B. microplus* is different 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). At least, 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).

Concluding, we can postulate that mating in this one-host tick species involves cholesteryl esters and other chemical signals present on the cuticle of females and that these other products are most likely perceived by gustatory sensilla on the palps of male ticks.

Acknowledgements—We are indebted to the Hasselblad, Roche and Sandoz Foundations as well as to the Swiss National Science Foundation (Grant Nos. 3.609-0.87, 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 palpal sensilla and Dr. A. Rawyler for an introducion to the transmethylation method. This paper is part of the Ph.D. thesis of Marien de Bruyne submitted to the University of Neuchâtel.

General Discussion

1. The ghost of *Bombyx mori*: Concepts in pheromone research

Research on pheromones has always been dominated by the particularities of sex pheromones in Lepidopteran insects. Since the isolation by Butenandt in the 1950's of a sex attractant from the silk moth *Bombyx mori* (Karlson, 1995), up to 397 species of moths have been investigated and found to produce a multitude of different long chain alcohols, aldehydes and esters, or mixtures of these (Arn *et al.*, 1992) from a gland at the tip of the female abdomen. In many of these species, definite proof has been given that males are attracted to these compounds in the field over fairly long distances and many other aspects of the courtship behaviour are regulated by the same pheromone (Bartell and Shorey, 1969).The flight behaviour involved in moth upwind orientation has been thoroughly investigated and chemically mediated anemotaxis is now commonly accepted as a basic strategy in insect orientation, though the mechanisms involved are still disputed (Kennedy, 1983). The idea that chemicals in the environment influence behaviour, and hence ecology, of animal species led to the growth of a new field called chemical ecology. The discovery, that specialised olfactory hairs on the antenna of *Bombyx mori* and other moth species, contain sensory cells that respond very specifically and to very low concentrations of components of these pheromone blends (Kaissling and Priesner, 1970, Hansson, 1995), sending their information to enlarged olfactory glomeruli in the brain that process it separately from other odours (Christensen and Hildebrand, 1987), has strengthened the general notion of pheromone specificity. With their huge plumose antenna, designed to capture odour molecules sent off by calling females hidden somewhere in the landscape, lepidopteran males are the ultimate example of specificity and sensitivity of a pheromone system. But are they really a good example of chemical communication in arthropods as a whole, or are they an exception?

The amount of data available on pheromones in moths far outnumbers that available for any other arthropod taxon. However, in other insect taxa similar systems seem to exist. Sexual dimorphism has also been found in the olfactory system of the cockroach both centrally and on the periphery (Boeckh *et al.*, 1987). Positive anemotactic responses have been demonstrated in this primitive walking insect, mediated by a female produced pheromone, periplanone B; a compound which is nevertheless of a quite different nature than the moth pheromones (Bell, 1981).

I will try to argue here that chemical communication in *B. microplus* and ticks in general has not reached the level of sophistication seen in insects and moths in particular. It seems to me that evidence for the existence of specific chemical signals in ticks, evoking specific behavioural responses in conspecifics, is limited. Their is very little evidence for production of any such signal produced exclusively by females. The only putative sex pheromone 2,6-dichlorophenol — here referred to as 2,6-DCP — is said to be produced by the foveal glands which occur in all life stages of ticks and the product is here isolated from larvae, males, pharates, unfed and fed females of *B. microplus*. Evidence from most other tick species suggests that it is much the same (TABLE 1). Furthermore, there is no evidence for specialised "pheromone receptors". The olfactory hairs which house 2,6-DCP and 2-nitrophenol receptors also house receptors for host-odour components (Steullet, 1993) while the gustatory hairs that are supposed to perceive cholesteryl esters and guanine have not yet been investigated.

Moreover, female ticks bear the same sensilla, and in the case of 2,6-DCP it has been shown that they also have the same sensitivity (Haggart and Davis, 1981). The lack of a behavioural response to 2,6-DCP in female ticks has only been shown once (Kellum and Berger, 1977) whereas female *A. variegatum* are attracted to this product in the field (Norval *et al.*, 1991). In the case of contact stimuli it was never tested whether cholesteryl esters evoke the same behaviour from females as it does from males, whereas an aggregation response to tick excreta and its main constituent guanine is commonly shown by all life-stages off the host.

Of the products isolated from ticks and reported to play a behavioural role, 2-nitrophenol was not isolated from extracts of female *B. microplus* while 2,6-DCP was found but no evidence was obtained here for a behavioural role. Cholesteryl oleate was isolated from extracts of female ticks but only found active in our bioassay at doses much higher than physiological levels, whereas guanine was tested in the bioassay but found completely inactive at several doses (see appendix IV). However, a very potent and as yet unidentified non-volatile chemical signal is described here for this species.

2. Evolution of chemical signals and chemosensory systems in ticks

An interesting correlation exists between the recently published phylogeny (Black and Piesman, 1994, see general introduction FIG 2), which separates the Amblyomminae in two distinct groups, and reports of male or female produced chemical signals in this subfamily. The three species where males are known to produce chemicals that attract females to the host and stimulate pairing are all from group 1, whereas *A. americanum* where it is males that migrate to females on the host belong to group 2. If we observe the evidence for the existence of pheromones that could have a function in uniting the sexes and compare it with what little is known about evolution of the different tick taxa we notice that firstly, some non-volatile aggregation stimuli active off the host seem to exist in Argasid and Prostriate ticks which are largely nidicolous without a need for attracting conspecifics over long distances. Prostriate ticks do not posses foveal glands and *I. ricinus* probably does not contain 2,6-DCP (Diehl and Grenacher, personal communication). Secondly, in almost all groups of metastriate ticks some free living species which infest mobile hosts have been found to produce 2,6-DCP with quantities being particularly high in those species that actively hunt for their hosts such as *Amblyomma* and *Hyalomma* species. Thirdly, in *Amblyomma* group 1 containing *A. variegatum, hebraeum* and *maculatum* which infest mobile hosts in absence of association with a burrow, male emitted pheromones are associated with attracting partners to the same host.

From the limited studies made on the olfactory system of members of the Ixodidae, Steullet (1993) concluded that a remarkable homology existed between receptors in terms of their structure, location, number and physiological responses. The general conservative nature of tick evolution appears in their sensory systems as well. Moreover, it seems that at least some of the compounds reported as pheromone components, notably 2-nitrophenol, nonanoic acid, 2-methylpropanoic acid, 4-methylphenol and benzaldehyde are present in host odours as well (Steullet and Guerin, 1994a&b). A methyl salicylate receptor was also found in a field of olfactory sensilla on the tarsus of a predatory mite that uses this compound to locate its spider mite prey on leaves of plants (de Bruyne et al., 1991). *B. microplus* possesses a 2-nitrophenol receptor which in this species has probably no pheromonal function. When compared to members of the genus *Amblyomma*, evolutionary more primitive, *B. microplus* bears less olfactory sensilla on its tarsus. This reduced tarsal sensory

system, however, does not lead to a reduction in number of receptor cells and is probably simply related to a smaller body size (Hess and Vlimant, 1986). The presence of a receptor sensitive to 2,6-DCP in *I. ricinus* suggests it was present in ticks before the capability to synthesise 2,6-DCP evolved. Even *Sarcoptes scabei*, a parasitic mite on humans, can apparently perceive this product (Arlian and Vyszenski-Moher, 1996). It might be that the receptors that respond to this compound are not tuned to 2,6-DCP specifically but that halogenated phenols stimulate it just as fluorinated derivatives of aliphatic pheromone compounds do in moths (Prestwich, 1993). The fluorinated and brominated anologs also stimulate this receptor in *B. microplus*. All this demonstrates the probably ancient origins of the limited number of receptors of ticks, as well as the broad range of contexts in which the compounds they perceive play a role. In addition, it shows the adaptation of this limited but multi-functional olfactory system to the specific needs of different species of ticks and maybe even of the Acari as a whole.

3. The tick mating system

Pheromone communication implies a certain level of social interaction between individuals. Sociality is a continuum, with the so-called social insects at one extreme of the spectrum. However, natural selection is thought to augment the prevalence of genes that give each individual member of a species the best chances for survival and reproduction. It is even suggested to imagine an animal as a machine designed by and made to preserve and propagate the individual genes it carries (Dawkins, 1976, Thornhill and Alcock, 1983). The strategy that each individual in the population uses to achieve a certain goal *e.g.* the insemination of a female is an evolutionary stable strategy (ESS). *i.e.*, the strategy that (once fixated in the population) cannot be invaded by a mutant showing any alternative strategy. The best strategy for a given individual is strongly dependant upon the strategy played by his competitors. I will try briefly to answer some questions on the evolution of tick pheromones with this idea of intraspecific conflict in mind (Parker, 1978).

While insemination of females off the host, *i.e.*, before feeding, is common in Argasidae and most Prostriates, metastriate ticks have lost this ability. A blood meal is needed to produce sperm but with it they have gained the possibility of renewing their stock of sperm, unlike Prostriata which can copulate with only a limited number of females (Yuval *et al.*, 1990). In a mating system where a mechanism of last male sperm precedence exists such as has been demonstrated in *I. dammini* (Yuval and Spielman, 1990), the number of females a male inseminates is less important than being the last male to inseminate one particular female. Whether last male sperm precedence occurs has not been investigated for *B. microplus* but the mate guarding observed here would suggest that this is the case. A male stays with a female from copulation until she drops from the host, to ensure that only his genes are propagated.

I have observed males mating with females, already guarded by another male, tipping over and during ventral positioning succeeding to slide in between the paired couple. I do not know whether 'guarding' males are capable of preventing spermatophore transfer by the second arriving male but this newly arrived male would certainly not find the space to attach under the female and would be obliged to leave, while the attached male would still be best positioned to inseminate her again. Maybe that is why multiple inseminations by the same male occur. If this is true it would also mean that older females, right till the moment of drop-off, remain attractive for males. This seems to be supported by the data presented here. Males can then either guard a female from first insemination till drop-off or try to copulate with already paired

females, not wasting time guarding her, and still be the last to mate with her. What a male does will depend on the density of females on the host, the state of most females (pharate, paired or just before drop-off), and the operational sex ratio at a particular moment in time.

The sex ratio of 1:1 observed here under experimental conditions of single infestations will be close to the primary sex ratio. Due to the longer life-span of males on the host and the fact that females drop-off after completing their bloodmeal, the operational sex ratio in the field will be more biased towards males. If single massive infestations occur in the field male-male competition for females will be higher after some time. The early emergence of males observed here has also been desribed in numerous species of insects and it has been argued that it improves male reproductive success because early-emerging individuals would have access to more free females than late emergers (Wiklund and Fagerström, 1977) After moulting, males should search for mates early, probably before spermatogenesis is completed and pair with pharate females to be the first to copulate with her and subsequently guard her to make sure to be also the last. Such a strategy is also seen in phytophagous mites (Cone, 1979, Enders, 1989). For the male tick, the position attached under the female also offers a sheltered place to feed and produce the next spermatophore. After she has dropped off he still has the possibility to inseminate more females but under conditions of a more male biased sex ratio and with most females already paired. If multiple infestations occur in the field development of males and females will be less synchronised and newly moulted males will find both established pairs with females ready for drop-off as well as pharate females.

4. No need for a volatile mating signal

Finding a host is likely to be much more difficult for a non-nidicolous tick than finding a mate once on the host, especially since the host is usually highly mobile and mates invariably sessile. The important provision is that that host is infested with conspecific ticks. The combination between a sex attractant and an attractant for the food source is known from the pheromone system of bark beetles (Mustaparta et al., 1979; Dickens, 1979) where the compounds used are very similar to and probably derived from the host. An effect of host plant volatiles on sex pheromone responses has also been demonstrated for some species of moths (van der Pers *et al.*, 1980). If the principle role of tick produced volatiles is to attract other ticks to the host rather than function as signals on the host, an argument can be made for the absence of such signals in *B. microplus*. Since it is larvae that search hosts there would be a considerable delay before the benefits of such a strategy, *i.e.*, recruitment of adult mates, could be reaped. It could be that all strategies for attracting ticks to occupied hosts have been dropped by this species in favour of fast development. In his review of tick toxins which cause tick paralysis in hosts Gothe (1984) suggested that these toxins may have as practical significance the increased production of CO_2 and possibly other host odours by influencing the neural control of breathing. Such toxins are described in many species of ticks from different genera but not from *Boophilus*.

Male *A. hydrosauri* attached to reptiles detach and search for a mate only when a female has located the same host, has fed for some time and starts emitting a volatile detachment signal (Bull, 1986). Waiting for a chemical signal to detach would not be an evolutionary stable strategy for *B. microplus*. A male detaching spontaneously and early, to search for a female at random, would be at an advantage because his presence

on the host is by definition an indication that some females will also be present, since they all derive from the same batch of larvae which infested the host.

With the high densities of females we observed on the host it can be argued that a chemical signal for guiding males to a female is not of much use either. Since a host has a limited surface area and ticks tend to be concentrated in certain body regions, the number of possible encounters/cm^2 is such that males responding to an attractant would have only a marginal advantage over random walking males (De Vita *et al.*, 1982). As long as males strike a balance between searches and feeding periods a strategy of spontaneous detachment and random searches could ensure an optimum speed in development since, when spermatogenesis is completed, males can copulate immediately with the female they paired with.

The sensilla that I recorded from house *ca.* 25% of all olfactory receptor neurons of this tick (appendix II). If we take only the wall-pore single walled sensilla, from which most responses to aromatic and phenolic components of tick extracts have been recorded, they represent 40%. Therefore there is still a possibility that other volatiles perceived by other olfactory receptors play a role in the mating behaviour of this tick.

TABLE 1: Maximum quantities of 2,6-DCP (ng) isolated from different metastriate species. The state of the females used in the analysis is given as either newly moulted and unfed (moult), unfed after several days or fed. Most studies have focused on fed females, less on males, and almost none include nymphs and larvae.

Species	male	fem.	ny.	larva	females state			reference
					moult	unfed	fed	
A. maculatum	30	65			—	++	++	Kellum & Berger, 1971
A .variegatum		16					++	Wood *et al.*, 1975
	61						++	Lusby *et al.*, 1991
	150	*105*					++	Price *et al.*, 1994
A. hebraeum	*53*						++	Lusby *et al.*, 1991
	90	*76*					++	Price *et al.*, 1994
A .americanum	++	5				++		Berger, 1971
	1	60			—	++	++	Kellum & Berger, 1971
		2					++	Wood *et al.*, 1975
Hae. leporispalustris		++					++	Berger, 1983
Hy. truncatum		2					++	Wood *et al.*, 1975
Hy. dromedarii	—	35					++	Silverstein *et al.*, 1983
Hy. anato. excavatum		20					++	Silverstein *et al.*, 1983
Hy. marginatum		++					++	Rechav and Silverstein unpub
D. variabilis		2					++	Sonenshine *et al.*, 1976
	0.35	1.5			—	++	++	Sonenshine *et al.*, 1984
D. andersoni		2					++	Sonenshine *et al.*, 1976
D. albipictus		++					++	Berger, 1983
R. sanguineus		2-3					++	Chow *et al.*, 1975
R. appendiculatus		—					++	Wood *et al.*, 1975
	2	2-12		++		++	++	McDowell & Waladde, 1986
R. pulchellus		—					—	Wood *et al.*, 1975
R. simus		—					—	Wood *et al.*, 1975
R. compositus		—					—	Wood *et al.*, 1975
B. microplus	0.3	0.6	0.3	0.02	++	++	++	de Bruyne and Guerin, 1994

fem. female, ny. nymph, ++ presence not quantified, — not detected

5. An alternative role for 2,6-dichlorophenol

If 2,6-DCP does not play a role in the behaviour of males of this species it seems likely that some other function for the production of this extraordinary compound can be found. Investigations that examine the role of pheromones almost invariably focus on single functions and the possibility that a natural product may serve more than one function is usually not considered. From an evolutionary point of view this realisation is extremely important. It may be that on the production as well as the perception of a particular compound other selective pressures play a much more important role than

those revolving around chemical communication. 2,6-DCP is a toxic, strong smelling compound with antiseptic and antioxidant properties and its role in tick biology may well have to do with one of these characteristics. Simple phenols are present in the defensive secretions of beetles, hemiptera and millipedes and 4-hydroxybenzoic acid is used by *Dytiscus* as a cleaning agent to keep its cuticle free of micro-organisms (Harborne, 1988). On the interface between the host and the environment, with relatively high temperatures and humidity, and in contact with an open wound, protection from micro-organisms is probably a primary concern of ticks. In addition, feeding females can become prey for oxpeckers and other birds that feed on ectoparasites of Bovidae (Stutterheim *et al.*, 1988). A brominated ether is known as a feeding deterrent from sea hares, (Gastropoda, Kinnel *et al.*, 1979).

Many examples of the adaptation of defensive and antimicrobial secretions to pheromonal use are known in insects (Blum, 1996). The fact that 2,6-DCP is produced by ticks and can be perceived by them has probably led to behavioural adaptations and to their additional use as sex pheromones in those species which apparently had a need for such a signal. It may well be that in *B. microplus* this need is not there and that behavioural responses have been lost or never evolved.

6. Chemical and mechanical signals in mating behaviour

Males in the host-simulated arena were regularly observed to contact and re-mount control beads, sometimes up to 5 times. This indicates that contacting a glass substrate in this environment can be an appropriate stimulus for mounting it and that previous experience with the same substrate does not deter males from doing so. The term "mounting" sex pheromone used by Hamilton *et al.* (1988) is misleading. Firstly, because male ticks mount other objects probably as a result of a general tendency to move upward and/or investigate certain substrates. This cannot readily be associated with reproduction at all. Secondly, it distracts the attention from the tip-over behaviour which is much more typical of mating. Thirdly, the complete set of specific motor patterns mediated by such a pheromone is as yet not properly described. Rather than implying a certain behaviour in the definition it would be more appropriate to describe the chemical signals present in the cuticle of *B. microplus* as a "contact" sex pheromone. It has been adequately demonstrated here that non volatile components are sufficient and that at least part of the chemicals involved are perceived by contact chemoreceptors. Furthermore, the more polar components mediating tip-over are associated with the cuticle of *B. microplus* and not with *I. ricinus* and induce behavioural responses in males of this species and not in females. Therefore, the term sex pheromone is appropriate even if its components are also present on the cuticle of other life-stages.

Cholesterol esters are then included as one component of this pheromone. For *B. microplus* these compounds merely induce primary arrestment. The arrestment is short-lived and if no further appropriate stimuli are encountered the treated surface is abandoned. The male response is probably a first step in the process of mating, *i.e.*, a cholesteryl ester-treated curved hard surface merits a closer investigation by males (but not by females).

Some of the elements of the dorsal exploration phase observed on females occur during attachment to hosts and some males in our bioassays with extract-treated beads probed the membrane and attached. In preliminary experiments I applied female extract to the membrane of the host-simulating arena at a concentration of 10 eq./cm^2 which is equal to 10 eq. on the glass bead. Obviously in this situation males could not tip-over

but otherwise the behaviour in the first 30s was very similar to that on the bead. Especially strongly expressed were the flexing movements of the mouthparts and in 12 out of 20 cases males immediately penetrated the membrane and spent up to 2 min either attached, crawling or walking around on the treated surface (dia 2 cm) of the membrane. On solvent treated membranes (control) males left the treated zone generally within 20 s but a few males (2 out of 20) also probed and penetrated the membrane. The same surface area treated with a steer hair extract at 20 $\mu g/cm^2$ also arrested males and led to 7 out of 20 attaching to the membrane. Clearly, both extracts contain chemicals which arrest males and even stimulate them to probe and penetrate the substrate. The behavioural repertoire of ticks is probably very limited and a general tendency to probe and penetrate a substrate impregnated with the appropriate stimuli could be sufficient for males to locate the gonopore. They could do this by concentrating their efforts on indented parts of surface especially when they have reached a dead end while sliding along the surface of a female tick. The gonopore of *B. microplus* is roughly located where a male tick following tip-over can no longer progress between the female and the host skin. I observed several males spending a lot of time probing the folds in the cuticle of semi-engorged females and reports exist for *Ixodes* species where this leads to attachment to females (Moorhouse and Heath, 1975). The behavioural responses of males during mating are then not very specific and most of it may be explained parsimonously by simple motor patterns controlled by mechanical and chemical stimuli.

7. Contacting the right female or choosing the right male

In many species of insects males are known to attempt mating with other males, and male *Drosophila* even mimic the behaviour of unreceptive females to get rid of these attacks (Chapman, 1969). There is not much evidence for the specificity of male mating responses in *B. microplus*. Males are arrested and engage in tip-over behaviour on attached unfertilised females and on glass beads treated with extracts of such females, but also on extracts of fertilised engorged females, males, and even larvae of this species. It would seem therefore that males cannot recognise potential mates by chemical cues alone. Observations of male behaviour *vis-à-vis* attached males on rabbit ears tend to confirm this. Sex pheromones are generally conceived to be of use because they focus male searching and copulation attempts on females, members of their own species only, and often permit males to recognise when such females are most receptive, i.e., when their chances of propagating their genes are optimal. The contact pheromone isolated here does not seem to serve that purpose.

Males and females are often considered to have different interests. Since females contribute more of their time and energy to production of offspring, i.e., in ticks they produce the larger of the two gametes and supply all the food for the development of the embryo, their interest is limited to one copulation since they obtain enough spermatids to fertilise all eggs. Conversely, male interests are best served by many copulations with different females. This has led to the idea of females being more choosy. They want to make sure that the only male they mate with is the right one. The issue of female mate selection is controversial and complex (Thornhill and Alcock, 1983, Thornhill and Gwynne, 1995), but if females can really deny males access to their venter or their gonopore it would be interesting to study mating, and the use of chemical signals in that process, from that perspective. Male sex pheromones are known from insects and most of them operate at short range, causing female reactions that increase the likelihood that she will mate with the male (Shorey, 1973). In this

respect, the role of chemical or mechanical contact stimuli in the mating process which induce the female body lift response in *B. microplus* merits more attention.

8. Future prospects

This is the first extensive study on chemical signals in the mating behaviour of *B. microplus*. The behaviour and ecology of this important ixodid pest is still poorly understood. Because of its small size and one-host life cycle on large bovine hosts it is not an easy model for laboratory studies. However, it is its life-style, so markedly different from other tick species which makes it an interesting subject. Several concepts about mating behaviour, the involvement of the host and other environmental factors, as well as the evolution of sensory systems with corresponding behavioural responses need to be tested and compared to related species with different habits. The ideas developed in this discussion can only be working hypotheses but the nature of the sensory input as well as the behavioural output in mating behaviour of *B. microplus* has been established clearly here. Chemical stimuli play an important role and this tick, though equipped with a limited number of chemosensory receptors, and displaying simple motor patterns, has nevertheless been extremely successful in establishing itself around the world. Because the system appears to be simple, it challenges us to investigate the relation between (chemo)sensory input and behavioural output in more detail. It would be interesting for instance, to see how far the glass bead treated with the appropriate chemical stimuli would really mimic a mating partner for male ticks after modifying its mechanical characteristics. Would the addition of a small hole at a point where males are blocked after tip-over induce them to transfer a spermatophore?

Properly quantifying the elements of male behaviour would require knowledge of exact doses of compounds in relation to their effect on behaviour. This is even more important if synergistic or additive effects between components of a blend need to be evaluated. Only when the nature of the chemicals involved is known, can it be determined whether the interaction between cholesteryl esters and the polar fractions of female extracts is a true synergism or merely an additive effect. It is therefore essential to isolate and identify the compound or compounds in the polar fractions. There may be an intricate combination of stimuli involved as is suggested by results in appendix III.

It is unfortunate that more detail on systematics, ecology and behaviour of this species is lacking. It is possible that reproductive isolation is not achieved between members of the genus *Boophilus* as is suggested by experiments on interspecific mating (Graham *et al.*, 1972, Spicket and Malan, 1978). If *B. microplus* developed allopatrically there may have been no need to develop species recognition strategies. It would be interesting therefore to compare the role of chemical stimuli in mating behaviour in Boophilids with members of the genus *Rhipicephalus* and *Hyalomma* which occur sympatrically with *Boophilus* species on the same hosts. It could well be that the role of mechanical differences (size, shape) and different predilection sites on the host keep the species apart.

The combination of analytical, electrophysiological and behavioural techniques has made it possible to shed some light on the sensory physiology of mating behaviour in *B. microplus*. It has become apparent that contact chemostimuli play a crucial role in the different elements of male behavioural responses leading to copulation. We can therefore truly talk about the "taste" of tick sex, even though we, seeing through the sensory window nature has bestowed on us, cannot really imagine what it tastes like.

REFERENCES

Alcock J. (1994) Postinsemination associations between males and females in insects: The mate-guarding hypothesis. *Annu. Rev. Entomol.* **39**, 1-21.

Allan S.A., Phillips J.S. and Sonenshine D.E. (1989) The role of genital sex pheromones in mating behavior of metastriate ticks. In *Host regulated developmental mechanisms in vector arthropods* (eds. Borovsky D. and Spielman A.), pp. 302-311. University of Florida, Vero Beach.

Allan S.A., Phillips J.S. and Sonenshine D.E. (1991) Role of genital sex pheromones in *Amblyomma americanum* and *A. maculatum* (Acari: Ixodidae). *Exp Appl Acarol* **11**,9-21.

Allan S.A., Phillips J.S., Taylor D. and Sonenshine D.E. (1988) Genital sex pheromones of ixodid ticks: evidence for the role of fatty acids from the anterior reproductive tract in mating of Dermacentor variabilis and Dermacentor andersoni. *J. Insect Physiol.* **34**, 315-323.

Altner H. (1977) Insektensensillen: Bau- und Funktionsprinzipien. *Verh. Dtsch. Zool. Ges.* 139-153.

Altner H. and Prillinger L. (1980) Ultrastructure of invertebrate chemo-, thermo- and hygroreceptors and its functional significance. *Int. Rev. Cytol.* **67**, 69-139.

Andrews R.H. (1982) Mating behaviour and reproductive isolation of three species of reptile tick. *Anim. Behav.* **30**, 514-524.

Andrews R.H. and Bull C.M. (1980) Mating behaviour in the australian reptile tick *Aponoma hydrosauri*. *Anim. Behav.* **28**, 1280-1286.

Andrews R.H. and Bull C.M. (1982a) An attractant pheromone with common properties in three reptile ticks. *Experientio* **38**, 99-100.

Andrews R.H. and Bull C.M. (1982b) A comparrison of the mating behaviour between populations of the reptile ticks *Aponomma hydrosauri* and *Amblyomma albolimabatum*. *Aust. J. Zool.* **30**, 635-642.

Andrews R.H., Bull C.M. and Orbach J. (1986) The mode of transmission of the excitant sex pheromone in two Australian reptile ticks. *J. Parasitol.* **72**, 330-333.

Apps P.J., Viljoen H.W. and Pretorius V. (1988) Aggregation pheromones of the bont tick *Amblyomma hebroeum* : identification of candidates for bioassay. *Onderstepoort J. Vet. Res.* **55**, 135-137.

Arlian L.G. and Vyszenski-Moher D.L. (1996) Responses of *Sarcoptes scabei* (Acari: Sarcoptidae) to nitrogenous waste and phenolic compounds. *J. Med. Entomol.* **33**, 236-243.

Arn H., Tóth M. and Priesner E. (1992) *List of sex pheromones of lepidoptera and related attractants*. International Organisation for Biological Control, Montfavet.

Arthur D.R. (1960) *Ticks, a monograph of the Ixodoidea. Part V, the genera Dermacentor, Anocentor, Cosmiomma, Boophilus, Margaropus*. University Press, Cambridge.

Balashov Y.S., Ivanov V.P. and Ignatiev A.M. (1976) Fine structure and function of the palpal receptor organ in ixodid ticks (Acarina,Ixodoidea). *Zool. Zh.* **55**, 1308-1317.

Balashov Y.S. and Leonovich S.A. (1976) Morphological peculiarities of Haller's organ in ixodid ticks of the tribe Amblyommatini (Acarina, Ixodidae). *Entomol. Obozr.* **55**, 946-952.

Bartell R.J. and Shorey H.H. (1969) Pheromone concentrations requiered to elicit successive steps in the mating sequence of males of the light-brown apple moth Epiphyas postvittana. *Ann. Entomol. Soc. Am.* **62**, 1206-1207.

Batschelet E. (1981) *Circular statistics in biology*. Academic press, London, N.Y..

Bell W.J. (1981) Pheromones and behaviour. In *The american cockroach* (eds. Bell W.J. and Adiyodi K.G.), pp. 371-397. Chapman & Hall, London.

Belozerov V.N. (1991) evolution of life-cycles in ticks (Ixodidae) due to climate seasonality. In *Modern acarology* (eds. Dusbábek F. and Bukva V.), pp. 135-139. Academia, Prague.

Berger R.S. (1972) 2,6-Dichlorophenol, sex pheromone of the lone star tick. *Science* **177**, 704-705.

Berger R.S. (1974) Incorporation of chloride from Na36Cl into 2,6-dichlorophenol in Lone star and Gulf coast ticks. *Ann. Entomol. Soc. Am.* **67**, 961-963.

Berger R.S., Dukes J.C. and Chow Y.S. (1971) Demonstration of a sex pheromone in three species of hard ticks. *J. Med. Entomol.* **8**, 84-86.

Bhattacharyya A.K., Connor W.E. and Lin D.S. (1983) The origin of plant sterols in the skin surface lipids in humans: from diet to plasma to skin. *J. Invest. Dermatol.* **80**, 294-296.

Binnington K.C. (1972) The distribution and morphology of probable photoreceptors in eight species of ticks (Ixodoidea). *Z. Parasitenk.* **40**, 321-332.

Binnington K.C. (1975) Secretory coxal gland, active during apolysis in Ixodid and Argasid ticks (Acarina). *Int. J. Insect Morphol. Embryol.* **4**, 183-192.

REFERENCES

Binnington K.C. (1987) Histology and ultrastructure of the acarine synganglion. In *Arthropod brain. Its evolution,development,structure,and functions* (ed. Gupta A.P.), pp. 95-109. John Wiley & Sons, New York,Chichester,Brisbane,Toronto,Singapore.

Black W.C. and Piesman J. (1994) Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proc. Natl. Acad. Sci. USA* 91, 10034-10038.

Blum M.S. (1996) Semiochemical parsimony in the Arthropoda. *Annu. Rev. Entomol.* 41, 353-374.

Boeckh J., Ernst K.-D. and Selsam P. (1987) Neurophysiology and neuroanatomy of the olfactory pathway in the cockroach. In *Olfaction and taste IX* (eds. Roper S.D. and Atema J.), pp. 39-43. Annals New York Academy of Sciences, New York.

Boeckh J., Kaissling K.-E. and Schneider D. (1965) Insect olfactory receptors. *Cold Spring Harbar Symp. Quant. Biol.* 30, 263-280.

Brownell P. and Farley R.D. (1979) Detection of vibrations in sand by tarsal sense organs of the nocturnal scorpion, *Paruroctonus mesaensis*. *J. Camp. Physial.* 131, 23-30.

Bull C.M: (1986) Ticks on reptiles, a difference of scale. In *Morphalagy, physiology and behavioral bialogy of ticks* (eds. Sauer J.R. and Hair J.A.), pp. 391-405. Ellis Horwood Ltd., Chicester.

Bull C.M. and Andrews R.H. (1984) Two different mating signals used by female reptile ticks. In *Acarology VI* (eds. Griffiths D.A. and Bowman C.E.), pp. 427-429. Ellis Horwood, Chichester.

Byrne K.J., Gore W.E., Pearce G.T. and Silverstein R.M. (1975) Porapak-Q collection of airborne organic compounds serving as models for insect pheromones. *J. Chem. Ecol.* 1, 1-7.

Carroll J.F. and Pickens L.G. (1987) Spectral sensitivity to light of two species of ticks (Acarina:Ixodidae). *Ann. Entomol. Soc. Am.* 80, 256-262.

Chapman R.F. (1969) *The insects, structure and function*. English University Press, London.

Chapman R.F. (1995) Chemosensory regulation of feeding. In *Regulatory mechanisms af feeding* (eds Chapman R F and de Boer G), pp 101-136, Chapman & Hall, New York.

Cherry L.M. (1969) Cholesterol in the cuticular wax of *Boophilus microplus*. *Nature* 222,777.

Chilton N.B. and Andrews R.H. (1988) Mating behaviour and parapatry in two species of Australian reptile tick. *Oecalagia (Berl)* 75,146-152.

Chow Y.S. (1979) Electro-olfactory potential of Ixodes tick to 2,6 dichlorophenol. In *Proceedings of the 4th International Congres of Acarology* (ed. Piffl E.), pp. 501-505. Akadémiai Kiadó, Budapest.

Chow Y.S., Lu F.M., Peng C.T. and Cheng P.C. (1972) Isolation of lipids and sex pheromone from hard ticks. *Bull. Inst. Zool. Acad. Sin. (Taipei)* 11, 1-8.

Chow Y.S., Wang C.B. and Lin L.C. (1975) Identification of a sex-pheromone of the female brown dog tick *Rhipicephalus sanguineus*. *Ann. Entomol. Soc. Am.* 68, 485-488.

Christensen T.A. and Hildebrand J.G. (1987) Functions, organization, and physiology of the olfactory pathways in the lepidopteran brain. In *Arthropod brain: its evolution, development, structure, and functions* (ed. Gupta A.P.), pp. 457-483. John Wiley & Sons,inc.,

Cleveland W.S. (1985) *The elements of graphing data*. Wadsworth, Monterey CA.

Cone W.W. (1979) Pheromones of the Tetranychidae. In *Recent advances in Acarology, Vol II* (ed. Rodriguez J.G.), pp. 309-317. Academic press, New York.

Davey R.B. (1987) Effect of age of *Boophilus microplus* larvae (Acari:Ixodidae) on attachment to cattle. *J. Med. Entomol.* 24, 118-120.

Davey R.B. and Cooksey L.M. (1988) Sex ratios of *Boaphilus* ticks (Acari: Ixodidae) reaching adulthood on cattle. *J. Med. Entamol.* 25,82-84.

Dawkins R. (1976) *The selfish gene*. Oxford University press, Oxford.

de Bruyne M., Dicke M. and Tjallingii W.F. (1991) Receptor cell responses in the anterior tarsi of *Phytoseiulus persimilis A.-H.* to volatile kairomone components. *Exp. Appl. Acarol.* 13, 53-58.

de Bruyne M. and Guerin P.M. (1994) Isolation of 2,6-dichlorophenol from the cattle tick *Boophilus microplus*: Receptor cell responses but no evidence for a behavioural response. *J. Insect Physiol.* 40, 143-154.

De Vita J., Kelly D. and Payne S. (1982) Arthropod encounter rate: a null model based on random motion. *Am. Nat.* 119, 499-510.

Diaz G., De la vega R. and Arozarena V. (1984a) Conducta sexual de *Baophilus microplus* (Ixodoidea). II. Numero de copulas del macho e influencia del apareamiento sobre la capacidad reproductiva de la hembra. *Revta. Cub. Cienc. Vet.* 15,63-68.

Diaz G., De la vega R., Fernandez C. and Arozarena V (1984a) Conducta sexual de *Baophilus microplus* (Ixodoidea: Ixodidae). I. Tiempo de precopula del macho. *Revta. Cub. Cienc. Vet.* 15,59-62.

REFERENCES

Dicke M. and Sabelis M.W. (1988) Infochemical terminology: Based on cost-benefit analysis rather than origin of compounds? *Func. Ecol.* 2, 131-139.

Dickens J.C. (1979) Electrophysiological investigations on bark beetles. *Bull. Soc. Entomol. Suisse* 52, 203-216.

Diehl P.A., Aeschlimann A. and Obenchain F.D. (1982) Tick reproduction: oogenesis and oviposition. In *Physiology of ticks* (eds. Obenchain F.D. and Galun R.), pp 278-350, Pergamon Press, Oxford.

Diehl P.A., Guerin P.M., Vlimant M. and Steullet P. (1991) Biosynthesis, production site, and emission rates of aggregation-attachment pheromone in males of two *Amblyomma* ticks. *J. Chem. Ecol.* 17, 833-847.

Dinnik J. and Zumpt F. (1949) The integumentary sense organs of the larvae of Rhipicephalinae (Acarina). *Psyche (Camb.)* 56, 1-17.

Dongus H. and Gothe R. (1995) Evidence for pheromones in unfed and fed male ticks of *Hyalomma truncatum* (Ixodidae). *Appl. Parasitol.* 36, 252-263.

Donzé G. and Guerin P.M. (1994) Behavioral attributes and parental care of *Varroa* mites parasitizing honeybee brood. *Behav. Ecol. Sociobiol.* 34, 305-319.

Downing D.T. and Lindholm J.S. (1982) Skin surface lipids of the cow. *Comp. Biochem. Physiol. B Comp. Biochem.* 78B, 327-330.

Drew M. and Samuel W.M. (1989) Instar development and disengagement rate of engorged female winter ticks, *Dermacentor albipictus* (Acari: Ixodidae) following single- and trickle-exposure of moose (*Alces alces*). *Exp. Appl. Acarol.* 6, 189-196.

Dusbábek F., Jegorov A. and Simek P. (1991a) Artificial assembly pheromone of argasid ticks (Ixodoidea: Argasidae). In *Modern Acarology: Proc.VIIth.Int.Congr.Acarol.,Vol.I* (eds. Dusbábek F. and Bukva V.), pp. 59-68. Czechoslovak Academy of Science, Prague.

Dusbábek F., Simek P., Jegorov A. and Triska J. (1991b) Identification of xanthine and hypoxanthine as components of assembly pheromone in excreta of argasid ticks. *Exp. Appl. Acarol.* 11, 307-316.

Elizarov Y.A. (1963) Insect and tick chemoreception: electrophysiologic study of the chemoreception of Ixodidae. *Vestn. Mosk. Univ. Ser. VI Biol. Pocvoved.* 16, 16-24.

Enders M. (1989) *Intrasexueller Konkurenzkampf von maenchen der Gemeinen Spinnmilbe Tetranichus urticae KOCH.* Thesis; Ludwig-Maximilians Univ., Munich, Germany. (UnPub)

Estrada-Peña A., Estrada-Peña R. and Peiró J.M. (1992) Differentiation of *Rhipicephalus* ticks (Acari: Ixodidae) by gas chromatography of cuticular hydrocarbons. *J. Parasitol.* 78, 982-993.

Falk-Vairant J., Guerin P.M., de Bruyne M. and Rohrer M. (1994) Some observations on mating and fertilization in the cattle tick *Boophilus microplus*. *Med. Vet. Entomol.* 8, 101-103.

Feldman-Muhsam B. (1986) Observations on the mating behaviour of ticks. In *Morphology, physiology ond behaviour of ticks* (eds. Sauer J.R. and Hair J.A.), pp. 217-232. Ellis Horwood Ltd., Chicester.

Feldman-Muhsam B. and Borut S. (1983) On the spermatophore of ixodid ticks. *J. Insect Physiol.* 29, 449-457.

Feldman-Muhsam B. and Shechter R. (1970) Some notes on the genus *Boophilus* (Ixodidae), with special reference to species found in Israel. *J. Med. Entomol.* 7, 677-686.

Foelix R.F. (1985) Mechano- and chemoreceptive sensilla. In *Neurobiology of Arachnids* (ed. Barth F.G.), pp. 118-137. Springer, Berlin.

Foelix R.F. and Axtell R.C. (1971) Fine structure of tarsal sensilla in the tick Amblyomma americanum. *Z. Zellforsch.* 114, 22-37.

Foelix R.F. and Axtell R.C. (1972) Ultrastructure of Haller's organ in the tick Amblyomma americanum L. *Z. Zellforsch.* 124, 275-292.

Foelix R.F. and Chu Wang I.W. (1972) Fine structural analysis of palpal receptors in the tick *Amblyomma americanum* (L.). *Z. Zellforsch.* 129, 548-560.

George J.E. (1981) The influence of aggregation pheromones on the behavior of Argas cooleyi and Ornithodoros concanensis (Acari: Ixodoidea: Argasidae). *J. Med. Entomol.* 18, 129-133.

Gladney W.J. (1971) Mate-seeking by female Amblyomma maculatum (Acarina: Ixodidae) on a bovine. *Nature* 232, 401-402.

Gladney W.J., Ernst S.E. and Grabbe R.R. (1974a) The aggregation response of the gulf coast tick on cattle. *Ann. Entomol. Soc. Am.* 67, 750-752.

Gladney W.J., Grabbe R.R., Ernst S.E. and Oehler D.D. (1974b) The gulf coast tick: Evidence of a pheromone produced by males. *J. Med. Entomol.* 11, 303-306.

REFERENCES

Gothe R. (1984) Tick paralyses: Reasons for appearing during Ixodid and Argassid feeding. In *Current topics in vector research. Vol.II* (ed. Harris K.F.), pp. 199-224. Praeger Scientific, New York.

Gothe R. (1987) Tick pheromones. *Onderstepoort J. Vet. Res.* 54, 439-441.

Gothe R., Weck P. and Kraiss A. (1984) Zur pheromonal induzierten kommunikation von *Argas* (Persicargas) walkerae und biologisch-chemischen bekämpfung durch kombinierten einsatz eines pheromons und Pheromon-Analogons mit flurmethrin. *Zentralb. Veterinärmed. Reihe B* 31, 161-179.

Göddc J. (1989) Vibrating glass stylets: tools for precise microsurgery on cuticular structures. *J. Neurosci. Methods* 29, 77-83.

Graf J.-F. (1975) Ecologie et éthologie d'*Ixodes ricinus* L. en Suisse (Ixodoidea; Ixodidae), cinquième note: mise en évidence d'une phéromone sexuelle chez *Ixodes ricinus*. *Acarologia* 17, 436-441.

Graham O.H., Price M.A. and Trevino J.L. (1972) Cross-mating experiments with *Boophilus annulatus* and *B. microplus* (Acarina: Ixodidae). *J. Med. Entomol.* 9,531-537.

Grenacher S. and Guerin P.M. (1994) Inadvertent introduction of squalene, cholesterol, and other skin products into a sample. *J. Chem. Ecol.* 20, 3017-3025.

Guerin P.M., Steullet P., Kröber T., Grenacher S., Diehl P.A., Vlimant M., de Bruyne M., Cordas T., Falk-Vairant J., Kuhnert F. and Lösel P.M. (1992) The chemical ecology of ticks at the host vector interface. In *Proceedings of the first International Conference on Tick-borne Pathogens at the Host-Vector Interface: an Agenda for Research* (eds. Munderloh U.G. and Kurtii T.J.), pp. 314-323. Minnesota University, Saint Paul, Minnesota.

Haggart D.A. and Davis E.E. (1979) Electrophysiological responses of two types of ammonia-sensitive receptors on the first tarsi of ticks. In *Recent advances in Acarology* (ed. Rodriguez J.G.), pp. 421-425. Academic Press, New York.

Haggart D.A. and Davis E.E. (1980) Ammonia-sensitive neurones on the first tarsi of the tick, *Rhipicephalus sanguineus*. *J. Insect Physiol.* 26, 517-523.

Haggart D.A. and Davis E.E. (1981) Neurons sensitive to 2,6-dichlorophenol on the tarsi of the tick *Amblyomma americanum* (Acari,Ixodidae). *J. Med. Entomol.* 18, 187-193.

Haller G. (1881) Vorläufige Bemerkungen über das Gehörorgan der Ixodiden. *Zool. Anz.* 4, 165-167.

Hamdy B.H. (1972) Biochemical and physiological studies of certain ticks (Ixodoidea). Nitrogenous excretory products of *Argas (Persicargas) arboreus* Kaiser, Hoogstraal & Kohls, and of other argasid and ixodid species. *J. Med. Entomol.* 9, 346-350.

Hamdy B.H. and Sidrak W. (1982) Guanine biosynthesis in the ticks (Acari) *Dermacentor andersoni* (Ixodidae) and *Argas (Persicargas) arboreus* (Argassidae): Fate of labelled guanine precursors. *J. Med. Entomol.* 19, 569-572.

Hamilton J.G.C., Papadopoulos E., Harrison S.J., Lloyd C.M. and Walker A.R. (1994) Evidence for a mounting sex pheromone in the brown ear tick *Rhipicephalus appendiculatus*, Neuman 1901 (Acari: Ixodidae). *Exp. Appl. Acarol.* 18, 331-338.

Hamilton J.G.C. and Sonenshine D.E. (1988) Evidence for occurrence of mounting sex pheromone on body surface of female *Dermacentor variabilis* (Say) and *Dermacentor andersoni* (Stiles) (Acari: Ixodidae). *J. Chem. Ecol.* 14, 401-410.

Hamilton J.G.C. and Sonenshine D.E. (1995) The effect of female body size on male mounting behaviour in *Dermacentor variabilis* and *D. andersoni*. *Med. Vet. Entomol.* 9, 219-223.

Hamilton J.G.C., Sonenshine D.E. and Lusby W.R. (1989) Cholesteryl oleate: mounting sex pheromone of the hard tick Dermacentor variabilis (Say) (Acari:Ixodidae). *J. Insect Physiol.* 35, 873-879.

Hansson B.S. (1995) Olfaction in lepidoptera. *Experientia* 51, 1003-1027.

Harborne J.B. (1988) *Introduction to ecological chemistry*. Academic Press, London.

Hassanali A., Nyandat E., Obenchain F.D., Otieno D.A. and Galun R. (1989) Humudity effects on response of Argas persicus (Oken) to guanine, an assembly pheromone of ticks. *J. Chem. Ecol.* 15, 791-798.

Hájková Z., Bouchalová J. and Leahy M.G. (1980) A pre-attachment aggregation pheromone in the adult metastriata tick Hyalomma dromedarii Koch (Acarina, Ixodidae). *Folia Parasital. (Prague)* 27, 367-372.

Hájková Z. and Leahy M.G. (1982) Pheromone-regulated aggregation in larvae, nymphs and adults of Ixodes ricinus (L.) (Acarina,Ixodidae). *Folia Parasitol. (Prague)* 29, 61-69.

Hess E. and De Castro J.J. (1986) Field tests of the response of female *Amblyomma variegatum* (Acari:Ixodidae) to the synthetic aggregation-attachment pheromone and its components. *Exp. Appl. Acarol.* 2, 249-255.

Hess E. and Loftus R. (1984) Warm and cold receptors of two sensilla on the foreleg tarsi of the tropical bont tick *Amblyomma variegatum*. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 155, 187-195.

Hess E. and Vlimant M. (1984) The distal tarsal slit sense organ (DTSSO), a new type of mechanoreceptor on the walking legs of the ixodid tick *Amblyomma variegatum* Fabricius 1794 (Ixodidae:Metastriata). In *Acarology VI Vol.1* (eds. Griffiths D.A. and Bowman C.E.), pp. 253-260. Ellis Horwood, Chichester.

Hess E. and Vlimant M. (1986) Leg sense organs of ticks. In *Morphology, physiology, and behavioural Biology of Ticks* (eds. Sauer J.R. and Hair J.A.), pp. 361-390. Ellis Horwood, Chichester.

Hindle E. and Merriman G. (1912) The sensory perception of Argas persicus (Oken). *Parasitology* 5, 203-216.

Homsber P.J. and Sonenshine D.E. (1976) The effect of females on spermatogenesis and early mate seeking behaviour in two species of *Dermacentor* ticks. *Acarologia* 17,226-233.

Hoogstraal H. and Aeschlimann A. (1982) Tick-host specifity. *Mitt. Schweiz. Entomol. Ges.* 55, 5-32.

Hoogstraal H. and Kim K.C. (1985) Tick and mammal coevolution, with emphasis on *Haemaphysalis*. In *Coevolution of parasitic arthropods and mammals* (ed. Kim K.C.), pp. 505-568. John Wiley and Sons, New York.

Howard R.W. and Blomquist G.J. (1982) Chemical ecology and biochemistry of insect hydrocarbons. *Annu. Rev. Entomol.* 27, 149.

Ishii S. and Kuwahara Y. (1968) Aggragation of the german cockroach, *Blattella germanica* nymphs. *Experientia* 24, 88-89.

Ivanov V.P. and Leonovich S.A. (1983) Sensory organs. In *An Atlas of Ixodid Tick Ultrastructure* (ed. Balashov Y.S.), pp. 191-220. Entomological Society of America,

Jorgensen W.K. (1984) *The ultrastructure of the sense organ of tarsus 1, the palps and chelicerae of the larval cattle tick Boophilus microplus (Canestrini)(Ixodidae), including a study of the ultrastructure and function of the cattle tick podium.* Thesis; Univ.Queensland, St.Lucia, Australia. (UnPub)

Kaissling K.-E. and Priesner E. (1970) Die Riechschwelle des Seidenspinners. *Naturwissenschaften* 57, 23-28.

Kaltenrieder M. (1990) Scototaxis and target perception in the camel tick *Hyalomma dromedarii*. *Exp. Appl. Acaral.* 9,267-278.

Kaltenrieder M., Labhart T. and Hess E. (1989) Spectral sensitivity, absolute threshold, and visual field of two tick species, *Hyalomma dromedarii* and *Amblyomma variegatum*. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 165, 155-164.

Karlson P. (1995) Obituary: Adolf Butenandt (1903-1995). *Nature* 373, 660.

Karlson P. and Lüscher M. (1959) 'Pheromones': a new term for a class of biologically active substances. *Nature* 183, 55-56.

Keirans J.E. (1992) Systematics of the Ixodida (Argassidae, Ixodidae, Nuttalliellidae): An overview and some problems. In *Tick vector biology, Medical and veterinary aspects* (eds. Fivaz B., Petney T. and Horak I.), pp. 1-21. Springer, Berlin.

Kellum D. and Berger R.S. (1977) Relationship of the occurence and function of 2,6-dichlorophenol in two species of *Amblyomma*. *J. Med. Entamol.* 13, 701-705.

Kennedy J.S. (1983) Zigzagging and casting as a programmed response to wind-borne odour: a review. *Physiol. Entomol.* 8, 109-120.

Khalil G.M., Nada S.A. and Sonenshine D.E. (1981) Sex pheromone regulation of the mating behaviour in the camel tick *Hyalomma dromedarii* (Ixodoidea: Ixodidae). *J. Parasitol.* 67, 70-76.

Khalil G.M., Sonenshine D.E., Sallam O. and Homsher P.J. (1983) Mating regulation and reproductive isolation in the camel ticks, *Hyalamma dromedarii* and *Hyalomma anatalicum excavatum*. *J. Med. Entomol.* 20, 136-145.

Kinnel, R.B., Dieter, R.K., Meinwald, J., Van Engen, D., Clardy, J., Eisner, T., Stallard, M.O. and Fenical, W. (1979) Brasilenyne and cis-dihydrorhodophytin: antifeedant medium-ring haloethers from a sea hare (*Aplysio brasiliana*). *Proc. Natl. Acad. Sci. USA* 76,3576-3579.

Kitaoka S. (1961) Physiological and ecological studies on some ticks V. Nitrogen and iron excretion during the blood-sucking process in the tick. *Q. Nat. Inst. Animal Health* 1, 96-104.

REFERENCES

Klompen J.S.H., Black W.C., Keirans J.E. and Oliver J.H.,Jr. (1996) Evolution of ticks. *Annu. Rev. Entomol.* **41**, 141-161.

Kramer E. (1976) The orientation of walking honeybees in odour fields with small concentration gradients. *Physiol. Entomol.* **1**, 27-37.

Krijgsman B.J. (1937) Reizphysiologische untersuchungen an blutsaugenden arthropoden im zusammenhang mit ihrer nahrungswahl. III. Teil: Versuche mit zecken. *Arch. Neerl. Zool.* 2, 401-413.

Kuhnert F. (1995) *Untersuchungen zur in vitro Zucht von Schildzecken (Acari: Ixodidae).* Cuvillier Verlag, Göttingen.

Kuhnert F., Diehl P.A. and Guerin P.M. (1995) The life-cycle of the bont tick *Amblyomma hebraeum in vitro. Int. J. Parasitol.* **25**, 887-896.

Lahille, F. (1905) Contributions à l'étude des Ixodidés de la République Argentine. *Anales de Minist. de Agri. Sección de Zaotecnia etc. (Buenos Aires)* 2, 107-109

Leahy M.G. (1979) Pheromones of argasid ticks. In *Recent advances in Acarology. Vol. II* (ed. Rodriguez J.G.), pp. 297-308. Academic Press, New York.

Leahy M.G. and Booth K.S. (1978) Perception of a sex pheromone, 2,6-dichlorophenol, in hard ticks. In *Tick-borne diseases and their vectors* (ed. Wilde J.K.H.), pp. 88-94. University of Edinburgh, Edinburgh.

Leahy M.G. and Booth K.S. (1983) Attraction of metastriate ticks (Acari: Ixodidae) to sex pheromone 2,6-dichlorophenol and to substituted phenols. *J. Med. Entomol.* **1**, 104-105.

Leahy M.G., Galun R. and Van de Hey R. (1973) Assembly pheromone(s) in the soft tick Argas persicus (Oken). *Noture* **246**, 515-516.

Leahy M.G., Karuhize G., Mango C. and Galun R. (1975a) An assembly pheromone and its perception in the tick Ornithodorus moubata (Murray) (Acaridae: Argassidae). *J. Med. Entomol.* 12, 284-287.

Leahy M.G., Kovacic A., Mannion C. and Schulze L. (1983) Pheromone-induced aggregation of Ixodid ticks before host contact. *Experientia* 39, 859-860.

Leahy M.G., Sternberg S., Mango C. and Galun R. (1975b) Lack of species specifity in the assembly pheromones of soft ticks (Acari: Argasidae). *J. Med. Entomol.* **12**, 413-414.

Lees A.D. (1948) The sensory physiology of the sheep tick, Ixodes ricinus. *J. Exp. Biol.* 25, 145-207.

Leonovich S.A. (1981) Occurence of a sex pheromone in the Ixodid tick *Hyalommo asiaticum* (Ixodidae). *Parasitologiye (Leningr.)* **15**, 159-156.

Leonovich S.A. (1986) The orientational behaviour of the tick Hyalomma asiaticum. *Parasitologiye (Leningr.)* **20**, 431-439.

Levinson H.Z., Levinson A.R. and Müller K. (1991) Functional adaptation of two nitrogenous waste products in evoking attraction and aggregation of flour mites (*Acarus sira* L.). *Anz. Schädlingskd. Pflonzenschutz, Umweltschutz* 64, 55-60.

Lindholm J.S., McCromick J.M., Colton S.W. and Downing D.T. (1980) Variation of skin surface lipid composition among mammals. *Comp. Biochem. Physiol. B Comp. Biochem.* 69, 75-79.

Londt J.G.H. and Arthur D.R. (1975) The structure and parasitic life cycle of *Boophilus microplus* (Canestrini, 1888) in South Africa (Acarina: Ixodidae). *J. Entomol. Soc. South. Afr.* **38**, 321-340.

Lusby W.R., Sonenshine D.E., Yunker C.E., Norval R.A.I. and Burridge M.J. (1991) Comparison of known and suspected pheromonal constituents in males of the African ticks, Amblyomma hebraeum Koch and Amblyomma variegatum (Fabricius). *Exp. Appl. Acarol.* 13, 143-152.

Maroun N.A. and Kamal K.A. (1976) Biochemical and physiological studies of certain ticks (Ixodoidea) Absence of sterol biosynthesis in *Dermocentor ondersani* Stiles (Acarina: Ixodiadae). *J. Med. Entomol.* 13, 219-220.

McCamish M. and Cannell G.R. (1980) *Boophilus microplus*, the australian cattle tick: egg wax, cuticle wax, and a strange cholesterol economy. In *Recent developments in mass spectroscopy in biochemistry and medicine* (eds. Frigerio A. and McCamish M.), pp. 143-151. Elsevier, Amsterdam.

McDowell P.G. and Waladde S.M. (1986) 2,6-dichlorophenol in the tick *Rhipicephalus appendiculatus* Neumann: a reappraisal. *J. Chem. Ecol.* **12**, 69-82.

Moorhouse D.E. and Heath A.C.G. (1975) Parasitism of female ticks by males of the genus *Ixodes. J. Med. Entomol.* 5, 571-572.

Morel P.C. (1969) *Contribution a la connaissance de la distribution des tiques (Acariens, Ixodidae et Amblyommidae) en Afrique ethiopienne continentale.* Thesis, Univ.Paris, Paris, France. (UnPub)

Mount G.A., Haile D.G., Davey R.B. and Cooksey L.M. (1991) Computer simulation of *Boophilus* cattle tick (Acari: Ixodidae) population dynamics. *J. Med. Entomol.* 28, 223-238.

Mumcuoglu Y., Galun R. and Ikan R. (1986) The aggregation response of human body louse (*Pediculus humanus*) (Insecta:Anoplura) to its excretory products. *Insect Sci. Appl.* 7, 629-632.

Mustaparta H., Angst M.E. and Lanier G.N. (1979) Specialization of olfactory cells to insect- and host-produced volatiles in the bark beetle Ips pini (say). *J. Chem. Ecol.* 5, 109-123.

Newton W.H., Price M.A., Graham O.H. and Trevino J.L. (1972) Chromosome patterns in Mexican *Boophilus annulatus* and *B. microplus*. *Ann. Entomol. Soc. Am.* 65,500-512.

Nolan J. (1985) Mechanisms of resistance to chemicals in arthropod parasites of veterinary importance. *Vet. Parasitol.* 18, 155-166.

Noldus L.P.J.J. (1991) The Observer: A software system for collection and analysis of observational data. *Behav. Res. Meth. Instrum. Comput.* 23, 415-429.

Norval R.A.I., Andrew H.R. and Yunker C.E. (1989) Pheromone-mediation of Host-Selection in Bont Ticks (*Amblyomma hebraeum* Koch). *Science* 243, 364-365.

Norval R.A.I., Peter T., Yunker C.E., Sonenshine D.E. and Burridge M.J. (1991) Responses of the ticks *Amblyomma hebraeum* and *A. variegatum* to known or potential components of the aggregation-attachment pheromone. I. Long-range attraction. *Exp. Appl. Acarol.* 13, 11-18.

Norval R.A.I. and Short N.J. (1984) Interspecific competition between *Boophilus decoloratus* and *Boaphilus microplus* in Southern Africa. In *Acarology VI, Vol. 2* (eds. Griffiths D.A. and Bowman C.E.), pp. 1242-1246. Ellis Horwood Ltd., Chichester.

Nuñez J.L., Muñoz-Cobeñas M.E. and Moltedo H.L. (1985) *Boophilus microplus, the common cattle tick*. Springer, Berlin.

Oliver J.H.,Jr. (1974) Symposium on the reproduction of arthropods of medical and veterinary importance. *J. Med. Entomol.* 11, 26-34.

Oliver J.H.,Jr. (1982) Tick reproduction: sperm development and cytogenetics. In *Tick physiology* (eds. Obenchain F.D. and Galun R.), pp. 245-275. Pergamon Press, Oxford New York Toronto Sydney Paris Frankfurt.

Oliver J.H.,Jr. (1983) Chromosomes, genetic variance and reproductive strategies among mites and ticks. *Bull. Entomol. Soc. Am.* 9-17.

Oliver J.H.,Jr. (1989) Biology and systematics of ticks (Acari: Ixodida). *Annu. Rev. Ecol. Syst.* 20, 397-430.

Otieno D.A., Hassanali A., Obenchain F.D., Sternberg A. and Galun R. (1985) Identification of guanine as an assembly pheromone of ticks. *Insect Sci. Appl.* 6, 667-670.

Pappas P.J. and Oliver J.H.,Jr. (1972) Reproduction in ticks (Acari: Ixodoidea). 2. Analysis of the stimulus for rapid and complete feeding of female *Dermacentor variabilis* (Say). *J. Med. Entomol.* 9, 47-50.

Parker G.A. (1978) Searching for mates. In *Behavioral ecology: an evolutionary approach* (eds. Krebs J R, Davies N B), pp 214-244, Blackwell, Oxford.

Petney T.N. and Bull C.M. (1981) A non-specific aggregation pheromone in two Australian reptile ticks. *Anim. Behav.* 29, 181-185.

Phillips J.S. and Sonenshine D.E. (1993) Role of the male claw sensilla in the perception of female mounting sex pheromone in *Dermacentor variabilis, Dermacentor andersoni* and *Amblyomma americanum*. *Exp. Appl. Acarol.* 17, 631-653.

Poffet J.-M. (1988) *L'utilisation des infrarouges dans l'orientation de la tique Amblyomma variegatum (Fabricius 1794)*. Dissertation; Univ.Neuchâtel, Neuchâtel, Switzerland. (UnPub)

Pomerantzev B.I. (1950) *Fauna of the U.S.S.R. Arachnida Vol. IV, No.2. Ixodid Ticks (Ixodidae)*. American Institute of Biological Sciences, Washington, USA.

Prestwich G.D. (1993) Chemical studies of pheromone receptors in insects. *Arch. Insect Biochem. Physiol.* 22, 75-86.

Price Jr,T.L., Sonenshine D.E., Norval R.A.I., Yunker C.E. and Burridge M.J. (1994) Pheromonal composition of two species of African *Amblyomma* ticks: similarities, differences and possible species specific components. *Exp. Appl. Acarol.* 18, 37-50.

Rawlins S.C. and Mansingh A. (1987) A review of ticks and screwworms affecting livestock in the Carribbean. *Insect Sci. Appl.* 8, 259-267.

Rechav Y. (1978) Specificity in assembly pheromones of the tick Amblyomma hebraeum (Acarina: Ixodidae). *J. Med. Entomol.* 15, 81-83.

Rechav Y. (1983) Evidence for the existance of a sex pheromone in *Hyalomma marginatum rufipes* (Acari: Ixodidae). *J. Med. Entomol.* 20, 342.

REFERENCES

Rechav Y., Parolis H., Whitehead G.B. and Knight M.M. (1977) Evidence for an assembly pheromone(s) produced by males of the bont tick Amblyomma hebraeum (Acarina, Ixodidae). *J. Med. Entomol.* **14**, 71-78.

Rechav Y., Whitehead G.B. and Knight M.M. (1976) Aggregation response of nymphs to pheromone(s) produced by males of the tick Amblyomma hebraeum (Koch). *Nature* **259**, 563-564.

Rechav Y.H., Terry S., Knight M.M. and Cross R.H.M. (1977) Chemoreceptor organs used in detection of pheromone(s) of the tick Amblyomma hebraeum (Acarina, Ixodidae). *J. Med. Entomol.* 14, 395-400.

Rickli M., Diehl P.A. and Guerin P.M. (1994) Cuticle alkanes of honeybee larvae mediate arrestment of bee parasite *Varroa jacobsoni*. *J. Chem. Ecol.* **20**, 2437-2453.

Robert D. (1985) *Perception de la gravité et rôle de l'organe à fente tarsal (DTSSO) chez Amblyomma variegatum (Fabricius 1794).* Dissertation; Univ.Neuchâtel, Neuchâtel, Switzerland. (UnPub)

Roberts F.H.S. (1965) The taxonomic status of the species of the genera *Rhipicepahlus* Koch and *Boophilus* Curtice (Acarina: Ixodidae) occurring in Australia. *Aust. J. Zool.* **13**, 491-523.

Sahli R., Germond J.E. and Diehl P.A. (1985) *Ornithodoros moubata:* Spermateleosis and secretory activity of the sperm. *Exp. Parasitol.* 60,383-395.

Schlein Y. and Gunders A.E. (1981) Pheromone of Ornithodoros spp. (Argasidae) in the coxal fluid of female ticks. *Parasitology* **82**, 467-571.

Schofield C.J. and Patterson J.W. (1977) Assembly pheromone of *Triatoma infestans* and *Rhodnius prolixus* nymphs. *J. Med. Entomol.* **13**, 727-734.

Schöni R., Hess E., Blum W. and Ramstein K. (1984) The aggregation-attachement pheromone of the tropical bont tick *Amblyomma variegatum* Fabricius (Acari:Ixodidae): isolation, identification and action of its components. *J. Insect Physiol.* **30**, 613-618.

Schulze P. (1942) Die Rückensinnesfelder (Foveae dorsales) der Zecken. *Z. Morphol. Oekol. Tiere* 39, 1-20.

Schulze P. and Schröder E. (1949) Zur Sinnesphysiologie der Lederzecken (Argasidea). *Biol. Zentralbl.* 68, 321-333.

Shepherd J., Oliver J.H.,Jr. and Hall J.D. (1982) A polypeptide from male accessory glands which triggers maturation of tick spermatozoa. *Int. J. Invertebrate Reproduction Develop.* 5,129-137.

Shorey H.H. (1973) Behavioral responses to insect pheromones. *Annu. Rev. Entomol.* 18, 349-380.

Silverstein R.M., West J.R., Sonenshine D.E. and Khalil G.M. (1983) Occurrence of 2,6-dichlorophenol in hard ticks, *Hyalomma dromedarii* and *Hyalomma anatolicum excavatum*, and its role in mating. *J. Chem. Ecol.* 9, 1543-1550.

Sinitsina E.E. (1974) Electrophysiological reactions of Haller's organ neurons in the tick Hyalomma asiaticum P.Sch. and E.Sch. to odor. *Parasitologiye (Leningr.)* 8, 223-226.

Smith J.J.B., Mitchell B.K., Rolseth B.M., Whitehead A.T. and Albert P.J. (1990) SAPID tools: microcomputer programs for analysis of multi-unit nerve recordings. *Chem. Senses* **15**, 253-270.

Snowball G.J. (1957) Ecological observations on the cattle tick *Boophilus microplus* (Canestrini). *Aust. J. Agric. Res.* 8,394-413.

Sonenshine D.E. (1985) Pheromones and other semiochemicals of the Acari. *Annu. Rev. Entomol.* **30**, 1-28.

Sonenshine D.E. (1993) *Biology of Ticks Volume 2*. Oxford University Press, New York, Oxford.

Sonenshine D.E., Gainsburg D.M., Rosenthal M.D. and Silverstein R.M. (1981) The sex pheromone glands of *Dermacentor variabilis* (say) and *Dermacentor andersoni* Stiles, sex pheromone stored in neutral lipid. *J. Chem. Ecol.* 7, 345-357.

Sonenshine D.E., Hamilton J.G.C., Phillips J.S. and Lusby W.R. (1991) Mounting sex pheromone: its role in regulation of mate recognition in the Ixodidae. In *Modern Acarology: Proc.VIIth.Int.Congr.Acarol* (eds. Dusbábek F. and Bukva V.), pp. 69-78. Czechoslovak Academy of Science, Prague.

Sonenshine D.E., Khalil G.M., Homsher P.J. and Mason S.T. (1982) *Dermacentor variabilis* and *Dermacentor andersoni*: Genital Sex Pheromones. *Exp. Parasitol.* **54**, 317-330.

Sonenshine D.E., Silverstein R.M., Layton E.C. and Homsher P.J. (1974) Evidence for the existence of a sex pheromone in 2 species of Ixodid ticks (Metastigmata: Ixodidae). *J. Med. Entomol.* **11**, 307-315.

Sonenshine D.E., Silverstein R.M., Plummer E.C., West J.R. and McCullough T. (1976) 2,6-dichlorophenol, the sex pheromone of the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles and the American dog tick, *Dermacentor variabilis* (Say). *J. Chem. Ecol.* 2, 201-209.

Sonenshine D.E., Silverstein R.M. and Rechav Y.H. (1982) Tick pheromone mechanisms. In *Physiology of ticks* (eds. Obenchain F.D. and Galun R.L.), pp. 439-468. Pergamon, Oxford.

Sonenshine D.E., Silverstein R.M. and West J.R. (1984) Occurence of the sex attractant pheromone, 2,6-dichlorophenol, in relation to age and feeding in the American dog tick, *Dermacentor variabilis* (Say) (Acari, Ixodidae). *J. Chem. Ecol.* **10**, 95-100.

Spickett A.M. and Malan J.R. (1978) Genetic incompatibility between *Boophilus decoloratus* Kock,1844) and *Boophilus microplus* (Canestrini,1888) and hybrid sterility of australian and south african *Boophilus miocroplus* (Acarina: Ixodidae). *Onderstepoort J. Vet. Res.* 45, 149-153.

Stämpfli-Pauchard N. (1989) *Etude de certains facteurs intervenant lors de la recherche de l'hote, chez la tique Amblyomma variegatum Fabricius 1794 (Acarina: Ixodidae).* thesis; Univ. Neuchâtel, Neuchâtel, Switzerland. (UnPub)

Steullet P. (1993) *Perceptian of vertebrate volatiles in the tropical bont tick,Amblyomma variegatum Fabricius.* thesis; Univ. Neuchâtel; Neuchâtel, Switzerland. (UnPub)

Steullet P. and Guerin P.M. (1994) Identification of vertebrate volatiles stimulating olfactory receptors on tarsus I of the tick *Amblyomma variegatum* fabricius (Ixodidae). I. Receptors within the Haller's organ capsule. *J. Comp. Physial. A Sens. Neural Behav. Physiol.* 174, 27-38.

Steullet P. and Guerin P.M. (1994) Identification of vertebrate volatiles stimulating olfactory receptors on tarsus I of the tick *Amblyomma variegatum* fabricius (Ixodidae). II. Receptors outside the Haller's organ capsule. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 174, 39-47.

Stone B.F. (1963) Parthenogenesis in the Cattle Tick, *Boophilus microplus. Nature* 200, 1233 only.

Stutterheim I.M., Bezuidenhout J.D. and Elliot E.G.R. (1988) Comparative feeding behaviour and food preferences of oxpeckers (*Buphagus erythrorhynchus* and *B. africanus*) in captivity. *Onderstepoort J. Vet. Res.* 55, 173-179.

Taylor D., Phillips J.S., Allan S.A. and Sonenshine D.E. (1987) Absence of assembly pheromones in the hard ticks *Dermacentor variabilis* and *Dermacentor andersoni* (Acari:Ixodidae). *J. Med. Entomol.* 24, 628-632.

Taylor D., Phillips J.S., Sonenshine D.E. and Hanson F.E. (1991) Ecdysteroids as a component of the genital sex pheromone in two species of hard ticks, Dermacentor variabilis and Dermacentor andersoni (Acari: Ixodidae). *Exp. Appl. Acarol.* 12, 275-296.

Thompson G.D., Davey R.B. and Osburn R.L. (1980) Longevity and fertilization capacity of males and parthenogenesis in females of *Boophilus annulatus* and *B. microplus. J. Econ. Entomol.* 73, 378-380.

Thonney F. (1987) *Etude morphologique et structurale des récepteurs sensoriels du tarse I de la tique Ixodes ricinus L.* Thesis; Univ. Neuchâtel, Neuchâtel, Switzerland. (UnPub)

Thornhill R. and Alcock J. (1983) *The evolution of mating systems.* Harvard University Press, Cambridge, Massachusetts.

Thornhill R. and Gwynne D.T. (1995) The evolution of sexual differences in insects. In *Exploring evolutionary biology, readings form the American Scientist* (ed. Slatkin M.), pp. 234-241. Sinauer Ass.Inc., Sunderland, Massachusetts.

Totze R. (1933) Beiträge zur Sinnesphysiologie der Zecken. *Z. vergl. Physiol.* 19, 110-161.

Treverrow N.L., Stone B.F. and Cowie M. (1977) Aggregation pheromone in 2 Australian hard ticks, Ixodes holocyclus and Aponomma concolor. *Experientia* 33, 680-682.

Uilenberg G. (1992) Veterinary significance of ticks and tick-borne diseases. In *Tick vectar biology. Medical and veterinary aspects* (eds. Fivaz B., Petney T. and Horak I.), pp. 23-33. Springer, Berlin.

Uspensky I.V. and Emeliyanova O.Yu. (1980) Existence of pheromone associations in ticks of the genus Ixodes. *Zool. Zh.* 59, 699-704.

Van der Pers J.N.C., Thomas G. and Den Otter C.J. (1980) Interactions between plant odours and pheromone reception in small ermine moths (Lepidoptera: Yponomeutidae). *Chem. Senses* 5, 367-371.

Waladde S.M. (1978) *A study of the structure and physiology of certain sensary receptors of the adult cattle tick Boophilus microplus (Canestrini) Ixodidae.* Thesis; Univ.Queensland, St.Lucia, Australia. (UnPub)

Waladde S.M. (1982) Tip-recording from ixodid tick olfactory sensilla: responses to tick related odours. *J. Comp. Physiol.* 148, 411-418.

Waladde S.M., Kokwaro E.D. and Chimtawi M. (1981) A cold receptor on the tick Ripicephalus appendiculatus: electrophysiological and ultrastructural observations. *Insect Sci. Appl.* 1, 191-196.

Waladde S.M. and Rice M.J. (1977) The sensory nervous system of the adult cattle tick *Boophilus microplus* (Canestrini). Part III. Ultrastructure and electrophysiology of the chelicceral receptors. *J. Aust. Ent. soc.* **16**, 441-453.

Waladde S.M. and Rice M.J. (1982) The sensory basis of tick feeding behaviour. In *Physiology of ticks* (eds. Obenchain F.D. and Galun R.), pp. 71-118. Pergamon, Oxford.

Webb J.P., George J.E. and Cook B. (1977) Sound as host-detection cue for the soft tick Ornithodoros concanensis. *Noture* **265**, 443-444.

Wharton R.H. (1974) Ticks with special emphasis on *Boophilus microplus*. In *Control of arthropods* (eds. Pal R. and Wharton R.H.), pp. 35-52. Plenum press, London, N.Y..

Wharton R.H. and Norris K.R. (1980) Control of parasitic arthropods. *Vet. Parasitol.* **6**, 135-164.

Wiklund C. and Fagerström T. (1977) Why do males emerge before females? A hypothesis to explain the incidence of protandry in butterflies. *Oecologia (Berl.)* **31**,153-158.

Willadsen P., Bird P., Cobon G.S. and Hungerford J. (1995) Commercialisation of a recombinant vaccine against *Boophilus microplus*. *Parasitology* **110 Suppl.**, S43-S50.

Wood W.F., Leahy M.G., Galun R., Prestwich G.D., Meinwald J., Purnell R.E. and Payne R.C. (1975) Phenols as pheromones of ixodid ticks: a general phenomenon ? *J. Chem. Ecol.* **1**, 501-509.

Yunker C.E., Peter T., Norval R.A.I., Sonenshine D.E., Burridge M.J. and Butler J.F. (1992) Olfactory responses of adult *Amblyomma hebraeum* and *A.voriegatum* (Acari: Ixodidae) to attractant chemicals in laboratory tests. *Exp. Appl. Acarol.* **13**, 295-301.

Yuval B., Deblinger R.D. and Spielman A. (1990) Mating behaviour of male deer ticks *Ixodes dammini* (Acari: Ixodidae). *J. Insect Behav.* **3**, 765-773.

Yuval B. and Spielman A. (1990) Sperm precedence in the deer tick Ixodes dammini. *Physiol. Entomol.* **15**, 123-128.

Zacharuk R.Y. (1985) Antennae and Sensilla. In *Comprehensive insect physiology biochemistry and pharmacology. Vol.6, Nervous system: sensory* (eds. Kerkut G.A. and Gilbert L.I.), pp. 1-69. Pergamon press, Oxford.

ACKNOWLEDGEMENTS

My first thoughts go to Jean Falk-Vairant who had done a considerable amount of initial work on this project and who unfortunately died in 1994 in a tragic road accident. My PhD guide Dr Patrick M. Guerin has been a constant source of inspiration and stimulation and has driven me to give my best in all aspects. I thank Dr Peter A. Diehl for many useful discussions, especially in the later phases of my work and I appreciate the comments of Dr. R. Stocker and Dr. J.-L. Connat, members of the jury who examined my thesis. I am thankful to all those people who largely create the friendly atmosphere at the institute of Zoology and who have helped out on numerous occasions on the more day to day aspects of my work: Josiane Pont, Natacha Hügli, Brigitte Cattin, Serge Durand and Albin Collaud. I am grateful for the technical assistance of Mrs M. Knutti, the bio-statistical expertise of Mrs J. Moret and I acknowledge the input of Michèle Vlimant who has given me some useful insights into the structure of tick chemosensilla.

Working on *Boophilus microplus* would not have been possible without the help of the people at the Ciba-Geigy Agricultural Research Station in St. Aubin especially Messrs Bouvard, Jonczi and Cesari and I am indebted to the University of Neuchâtel and the Swiss National Science foundation for financing my research. I thank all my colleagues who have contributed to the functioning of this lab as well as supplying distraction when required: Barbara, Conor, Frank, Martin, Pablo, Pascal, Peter, Ram, Ruurd, Stoyan and Thomas as well as Gérard and Matthias.

My parents, largely absent during the years I spent in Switzerland, have nevertheless continued to be a guiding presence, always gently showing me the right way in life. Finally, I am deeply grateful to my wife Jyotika who in many ways has been my *raison d'être* when not thinking about *Boophilus microplus*.

SUMMARY

Boophilus microplus (Canestrini) is a one-host tick species and an important ectoparasite of cattle. Larvae of this species colonise the host and subsequent stages, nymphs and adults, develop on the same host. Mating takes place on the host and fertilisation is a prerequisite for female engorgement. Males moult one day earlier than females and need 2-3 days of development before they will copulate with females. However they form pairs earlier on, even with females still enveloped in the nymphal cuticle before moulting.

Adult *B. microplus* can be reared on laboratory rabbits and mating behaviour has been studied by placing males near females feeding on rabbit ears. The mating behaviour of *B. microplus* is described here as a process that can be divided in four phases, separated by distinct events. 1) Male detachment is followed by searching behaviour. Males are capable of locating attached females from a distance of *ca.* 1 cm but under these circumstances also move towards attached males. 2) When a male contacts a female he mounts her and intensively investigates her dorsal side by probing the surface with his tarsi and mouthparts, both bearing sensilla believed to be involved in chemoperception. 3) While staying in close contact with the female, he then proceeds to crawl to the ventral side. This behavioural event is referred to as tip-over. It is followed by a phase of ventral positioning till 4) the gonopore is located and he inserts his mouthparts, after which gonopore exploring leads to spermatophore production and transfer.

This characteristic behaviour is clearly mediated by chemical as well as mechanical factors since elements of it can also be observed using a small glass bead as dummy in a host-simulating arena. Positioned on a biological membrane, stretched over a heated physiological salt solution which assures high temperature and humidity, it can be treated with extract (test) or with solvent only (control). Significant arrestment, evaluated as the ratio between staying time on test and control of individual males, as well as tip-over behaviour could be observed in unfed and fed males responding to the extract of females but not in females. These responses can be used as a bioassay for chemical stimuli relevant to male mating behaviour.

We can thus conclude that mating of the cattle tick *Boophilus microplus* is mediated by chemical stimuli on the cuticle of female ticks

Since males were able to locate females from a short distance the role of volatiles in mating behaviour was investigated by making electrophysiological recordings from two olfactory hairs on the tarsus of the first pair of legs. These wall-pore single walled sensilla together house *ca.* 25% of the total population of olfactory receptor neurones in this tick. Receptor cells in the two sensilla responded to 2,6-dichlorophenol, known as a volatile sex pheromone for several metastriate tick species, in a dose-dependant manner. Using these receptors as specific detectors for compounds in the effluent of a gas chromatograph (GC), this compound was detected in extracts of females. In addition it was isolated from males, engorged nymphs and larvae, and is therefore present in all life-stages of this tick, but was not found in an extract of eggs. No other product from tick extracts elicited responses from these olfactory sensilla.

However, male *B. microplus* were not arrested on a glass bead treated with 2,6-dichlorophenol in the host-simulating arena, nor did they stay longer on the arena around this bead than in the control. In addition, no odour-conditioned anemotaxis,

change in angular velocity or speed of males walking on a locomotion compensator was observed in response to this compound when delivered in a conditioned air-stream. We could therefore not establish a role for 2,6-dichlorophenol on its own as a semiochemical in males of this species.

The nature of contact stimuli present on the cuticle of female ticks was investigated using the glass bead bioassay. Activity was shown to be present in extracts of females, males and larvae, as well as nymphal exuviae but not in exuviae of *Ixodes ricinus*. Using thin layer chromatography, one apolar fraction could be isolated showing light arrestment activity. This fraction consisted of a mixture of cholesteryl esters. After transmethylation, the fatty acid methyl esters from this fraction were separated on GC and quantified using an FID detector. The fatty acid moieties of the cholesteryl esters range from C12 to C24 and mainly contain unsatured compounds. One cholesteryl ester - cholesteryl oleate - has been known as a mounting pheromone in several species of ticks. Testing the major components of this mixture, including cholesteryl oleate, showed that none of the components on their own could induce activity at doses comparable to those found in the extract. The activity of the fraction was also not due to saturated or unsaturated esters. Only the total mixture was active at natural concentrations. However, even though the activity of this fraction represented the activity of all the fractions combined, it was far less active than the unseparated extract.

When female extract was separated by means of a small silica column all fractions recombined showed activity comparable to the unseparated extract. In addition to an early eluting apolar fraction containing cholesteryl esters, a second set of active more polar fractions was isolated. Together they reproduce the original activity of the extract and tip-over behaviour appears to be associated with the more polar material. Since male ticks frequently probe the surface of the treated bead with their mouthparts, palpal contact chemoreceptors may be involved in male courtship behaviour. On the last moveable segment of the palps these ticks carry a group of nine terminal pore sensilla. Tip recordings can be made from six of these gustatory sensilla belonging to one type and sensory cells in at least two of them respond to dilutions of the extract as well as to the polar fractions that were active in the bioassay. Chemical stimuli play a crucial role during male exploration of the dorsal surface of female ticks and initiate tip-over behaviour that brings the male into a position to probe the female gonopore

RÉSUMÉ

Boophilus microplus (Canestrini) est une tique à simple hôte et un ectoparasite important du bétail. Les larves de cette espèce colonisent l'hôte et les stades suivants, nymphes et adultes, se développent sur le même hôte. L'accouplement se fait sur l'hôte et la fertilisation est une condition pour l'engorgement de la femelle. Les mâles muent un jour avant les femelles et ils ont besoin de 2-3 jours de développement avant de copuler avec les femelles. Cependant, ils forment des paires déjà plus tôt, même avec des femelles encore enveloppées dans une cuticule nymphale avant la mue.

Les adultes de *B. microplus* peuvent être élevés sur des lapins de laboratoire et le comportement de l'accouplement a été étudié en plaçant des mâles près des femelles qui se nourrissent sur une oreille d'un lapin. Le comportement de l'accouplement est décrit ici comme un processus qui peut être divisé en quatre phases, séparées par des événements distincts. 1) Le détachement du mâle est suivi par le comportement de recherche. Les mâles sont capables de localiser des femelles attachées à une distance d'environ 1 cm mais sous ces conditions s'approchent aussi vers des mâles attachés. 2) Quand un mâle contacte une femelle il la monte et examine intensivement sa face dorsale en tâtant la surface avec ses tarses et pièces buccales, portant des sensilles impliquées dans la perception chimique. 3) En restant en contact intime avec la femelle il rampe vers le coté ventral. Ce comportement est déterminé "bascule". Il est suivi par une phase de positionnement ventral jusqu'à 4) le gonopore est localisé et il introduit ses pièces buccales, pour l'exploration du gonopore qui mène au transfert du spermatophore.

Ce comportement caractéristique est clairement régulé par des facteurs chimiques de même que mécaniques puisque des éléments de ce comportement peuvent être aussi observée en employant une petite bille de verre comme modèle dans une arène simulant l'hôte. Celle ci, positionnée sur une membrane biologique étiré sur une solution de sel physiologique assurant une température et humidité élevées, peut être traité avec un extrait (test) ou avec solvant (contrôle). Une arrestation significative, évalué par le rapport entre temps d'arrêt sur test et sur contrôle, aussi bien que le comportement de bascule pouvaient être observés chez les mâles nourris et non-nourris en réponse à l'extrait de femelles mais pas chez les femelles. Ces réponses peuvent être utilisées comme test biologique pour les stimuli chimiques relevants pour le comportement sexuel du mâle.

Ainsi on peut conclure que l'accouplement de la tique du bétail *Boophilus microplus* est régulé par des stimuli chimiques sur la cuticule de la femelle.

Puisque les mâles étaient capables de localiser des femelles à une courte distance le rôle des produits volatiles a été examiné en faisant des enregistrements électrophysiologiques de deux poils olfactifs sur le tarse de la première paire des pattes. Ces sensilles à paroi simple avec pores abritent environ 25% de la population totale des récepteurs olfactifs chez cette tique. Des cellules réceptrices dans les deux sensilles répondent au 2,6-dichlorophénol, connu comme phéromone sexuelle volatile de plusieurs espèces de tiques métastriates, suivant la dose. En utilisant ces récepteurs comme détecteurs spécifiques pour des composant dans l'effluent d'un chromatographe à phase gazeuse (GC), ce composant était détecté dans des extraits de femelles. En outre, il était isolé des mâles, des nymphes gorgées et des larves, et est

donc présent dans toutes les stades de cette tique, mais n'a pas été trouvé dans un extrait d'oeufs. Aucun autre produit n'a donné de réponse sur ces sensilles olfactives.

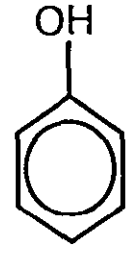
Cependant, les mâles de *B. microplus* n'étaient pas en arrêt sur une bille de verre traitée avec du 2,6-dichlorophénol dans l'arène simulant l'hôte et ils ne restaient pas plus longtemps dans l'arène autour de cette bille que dans le contrôle. En outre aucun anémotaxis conditionnée par odeurs, aucun changement de vélocité angulaire ni de vitesse des mâles marchant sur un compensateur de locomotion n'étaient observés en réponse à ce composant dans un flux d'air conditionné. Nous ne pouvons donc pas établir un rôle comme produit sémiochimique chez les mâles de cette espèce.

La nature des stimuli de contact qui se présentent sur la cuticule des tiques femelles a été examiné en utilisant le biotest avec bille de verre. Une activité a été démontrée présente dans des extraits des femelles, mâles et larves, de même que des exuvies nymphales mais pas dans des exuvies d'*Ixodes ricinus*. En utilisant la chromatographie sur couche mince, une fraction apolaire pouvait être isolée montrant une faible activité d'arrestation. Cette fraction consiste en une mélange d'esters de cholestérol. Après transméthylisation, les esters de méthyl de cette fraction étaient séparés sur GC et quantifiés en utilisant un détecteur FID. Les parties acides gras des esters de cholestérol varient entre C12 et C24 et constituent surtout des composants non-saturé. Un ester de cholestérol - l'oléate de cholestérol - était déjà connu comme "phéromone de monte" dans différentes espèces de tiques. Les tests des composants majeurs de ce mélange, incluant l'oléate de cholestérol, montrent que aucun de ces composants était capable de déclencher une activité à des doses comparables a celles trouvées dans l'extrait. L'activité n'était aussi pas due aux esters saturés ou non-saturés. Seul le mélange total était actif à des concentrations naturelles. Cependant, malgré le fait que l'activité de cette fraction représente l'activité de toutes les fractions combinées, il était beaucoup moins actif que l'extrait non-séparé.

Quand l'extrait de femelles était séparé au moyen d'une petite colonne de silice toutes les fractions combinées montraient une activité comparable à l'extrait non-séparé. En plus d'une fraction apolaire éludant tôt contenant des esters de cholestérol, un second groupe de fractions actives et plus polaires était isolé. Ensemble ils reproduisent l'activité originale de l'extrait et le comportement de bascule est apparemment associé avec le matériel plus polaire. Puisque les tiques mâles tâtent fréquemment la surface de la bille traitée avec leurs pièces buccales, des chémorécepteurs de contact peuvent être impliqués dans le comportement sexuel du mâle. Sur le dernier segment mobile des palpes, ces tiques portent un ensemble de neuf sensilles à pore terminal. Des enregistrements peuvent être faits de six de ces sensilles gustatives qui appartiennent à un seul type et des cellules sensorielles dans aux moins deux d'entre elles répondent aux dilutions d'extrait de même qu'aux fractions polaires qui étaient actives dans le biotest. Les stimuli chimiques jouent alors un rôle crucial pendant l'examination de la surface dorsale de la femelle par les mâles et lancent le comportement de bascule qui met un mâle dans la position de explorer le gonopore de la femelle.

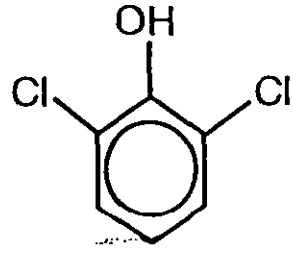
Chemical compounds in tick sensory physiology and chemical ecology

volatile phenolics and other aromatics

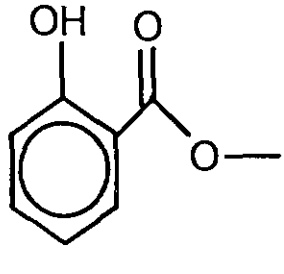


P

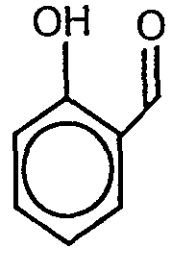
4MP



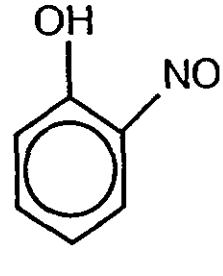
26DCP



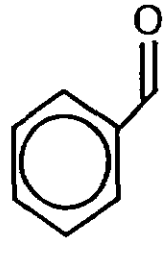
MS



Sal



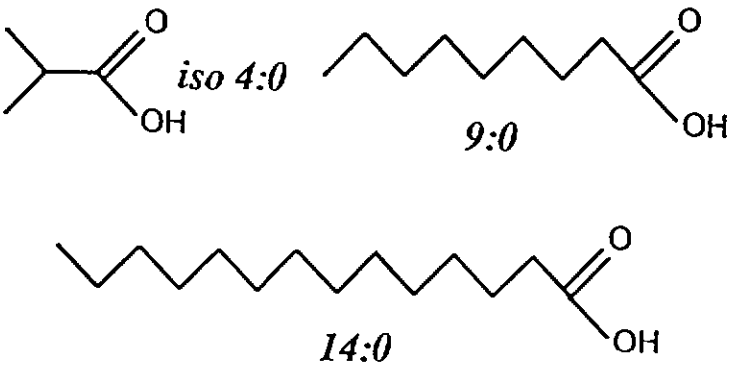
2NP



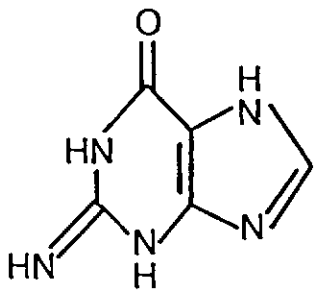
Bal

label	*name*	*formula*	*MW*	*synonyms*
P	phenol	C6H6O	94.1	
4MP	4-methylphenol	C7H8O	108	p-cresol
26DCP	2,6-dichlorophenol	C6H4Cl2O	163	
2NP	2-nitrophenol	C6H5NO3	139	o-nitrophenol
MS	2-hydroxybenzoic acid methyl ester	C8H8O3	152	methyl salicylate
Sal	2-hydroxybenzaldehyde	C7H6O2	122	salicyl aldehyde
Bal	benzaldehyde	C7H6O	106	

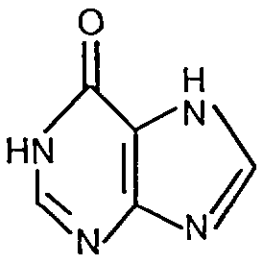
fatty acids



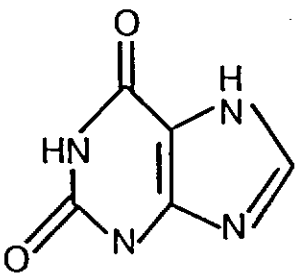
purines



GUA



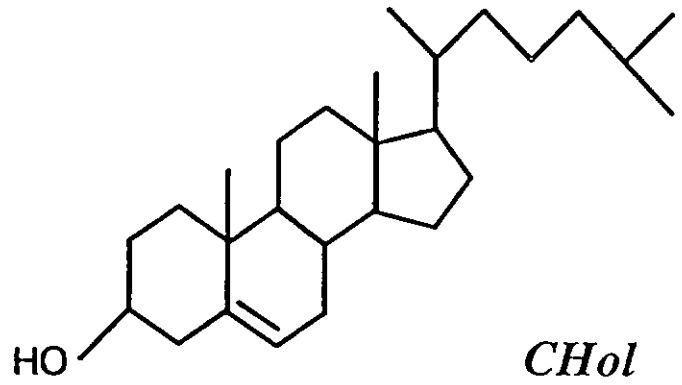
HXA



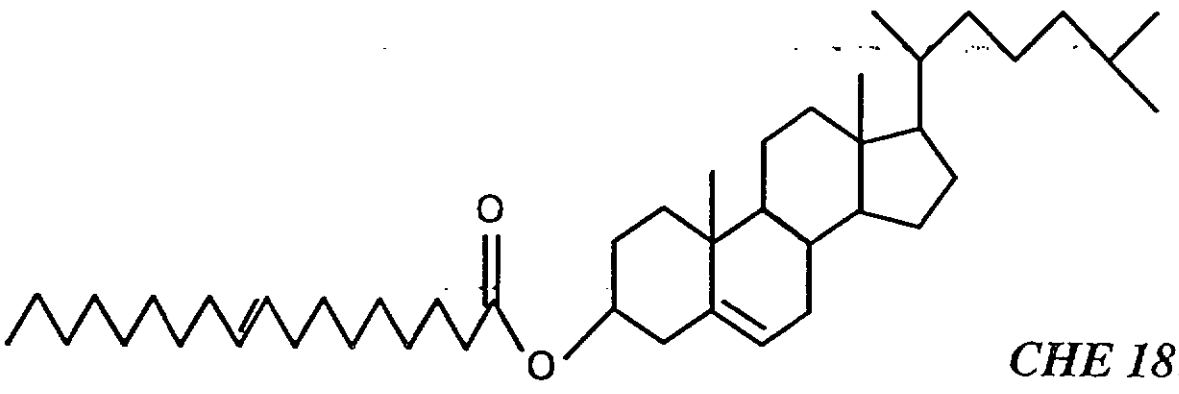
XAN

label	*name*	*formula*	*MW*	*synonyms*
		fatty acids		
iso 4:0	2-methylpropanoic acid	C4H8O2	88.1	*isa*-buteric acid
9:0	nonanoic acid	C9H18O2	158	pelargonic acid
14:0	butadecanoic acid	C14H28O2	228	myristic acid
		purines		
GUA	2-amino-6-oxopurine	C5H5N5O	151	guanine, 2-aminohypoxanthine
XAN	2,6-dioxopurine	C5H4N4O2	152	xanthine, 2,6(1H,3H)-purinedione
HXA	6-oxopurine	C5H4N4O	136	hypoxanthine, purine-6(1H)-one

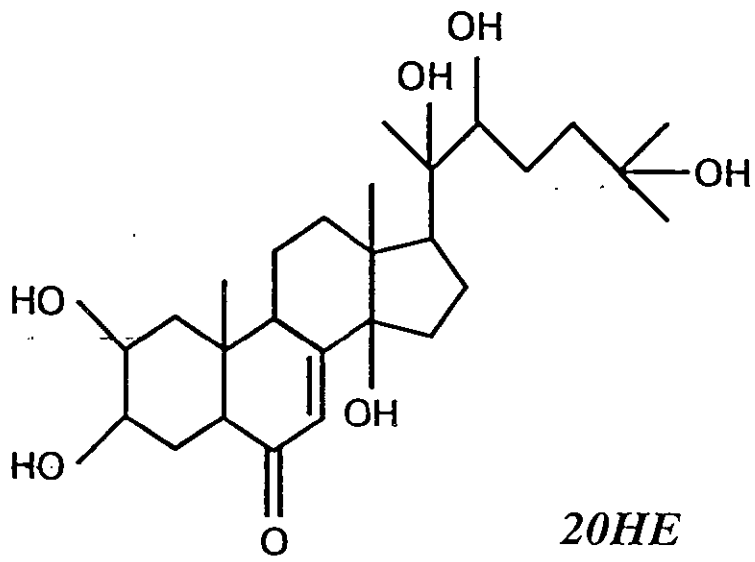
steroids



CHol



CHE 18:1



20HE

label	*name*	*formula*	*MW*	*synonyms*
CHol	cholest-5-en-3ß-ol	C27H46O	386.6	cholesterol
CHE 18:1	cholest-5-en-3ß-yl octadec-(Z)9-enoate	C45H78O2	650	cholesteryl oleate
20HE	2,3,14,20,22,25-hexahydroxy cholest-7-en-6-one	C27H44O7	470.6	20-hydroxyecdysone

Distribution of chemoreceptive sensilla in *B. microplus*

cluster	No	Walladde	type	cells
d I	1	md 3	wp-sw	5
d II	1	ap 1	wp-sw	15
d II	5	ap 3	wp-dw	3
d II	6	ap 2	wp-dw	3
c	?	cap 1	wp-sw	4
c	?	cap 2	wp-sw	17
c	?	cap 3	wp-sw	4
c	?	cap 4	wp-sw	4
la II	1	md 4	wp-dw	7
d III	2	md 6	wp-dw	7
d IV	1	md 10	wp-dw	2
d IV	2	md 9	wp-dw	2
v III	1	mv 6	wp-dw	4
v III	4	mv 3	wp-dw	4
all wp-sw combined				49
all wp-dw combined				32
total				81

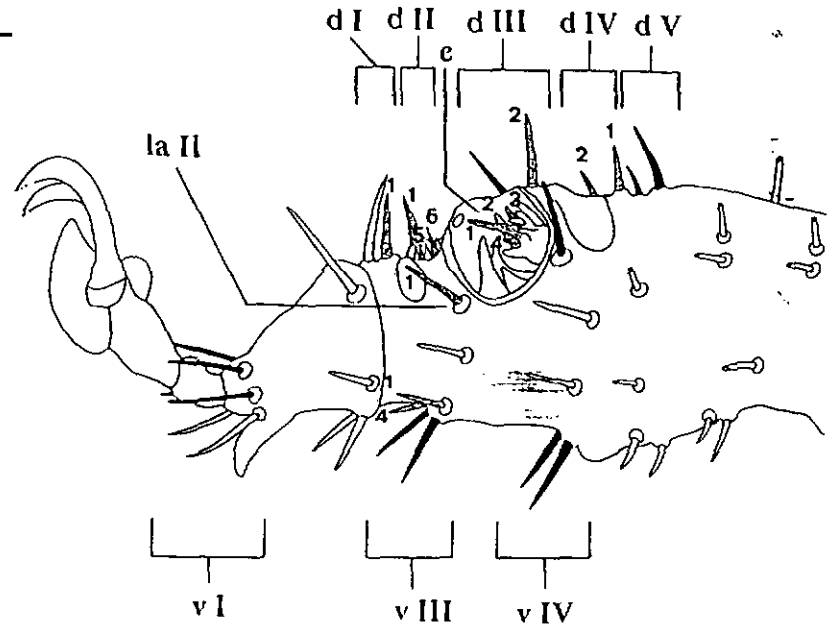


FIGURE 1. Schematic drawing of the distribution of olfactory (grey) and gustatory (black) sensilla on the inner surface of the right tarsus of the first pair of legs. Modified after Waladde (1978). Nomenclature of sensillum clusters after Hess and Vlimant (1986). The table compares this nomenclature with that of Waladde (1978) and gives an overview of the olfactory hairs on the tarsus with their innervation. The numbers indicate shaft innervating sensory neurones (cells), tp = terminal pore, wp = wall-pore, sw = single wall, dw = double wall.

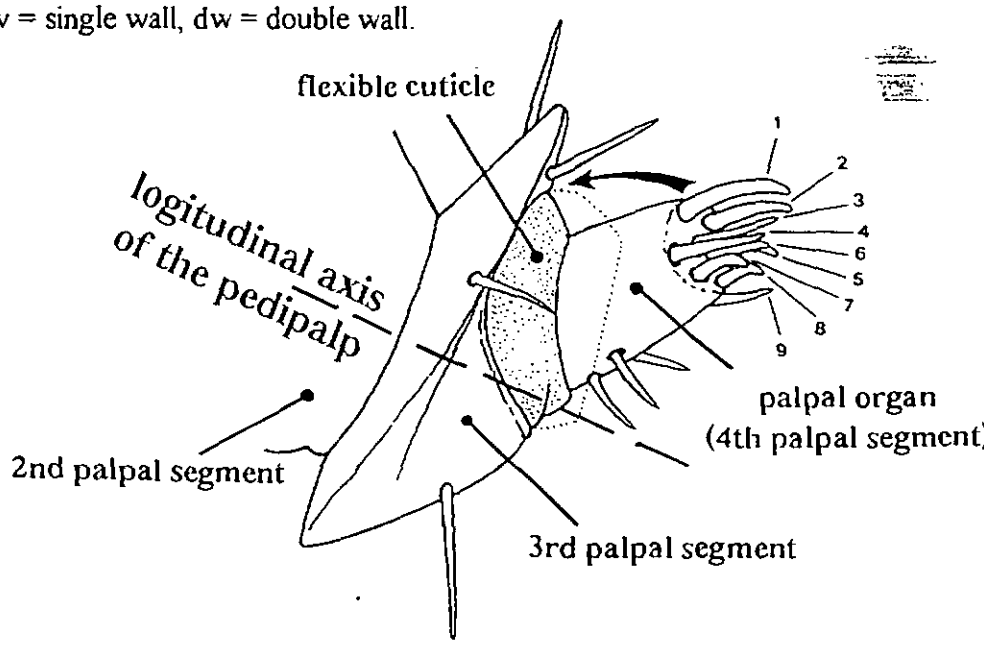


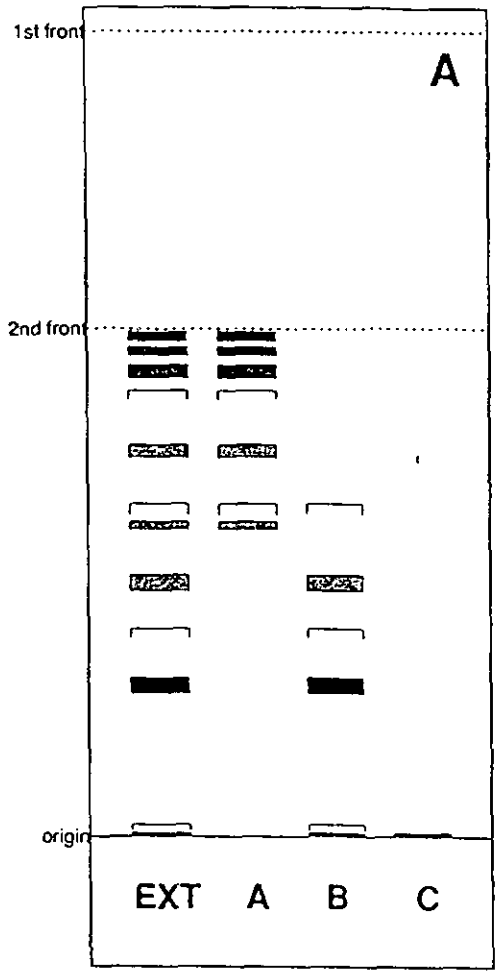
FIGURE 2. The position of gustatory sensilla, labelled 1 to 9, on the palpal organ as they were observed under the microscope during electrophysiological recordings in a lateral view of the right pedipalp of a male *B. microplus* placed upside down. The dotted line indicates the position of the palpal organ in folded position. The arrow is the direction of movement of the palpal organ when folding back.

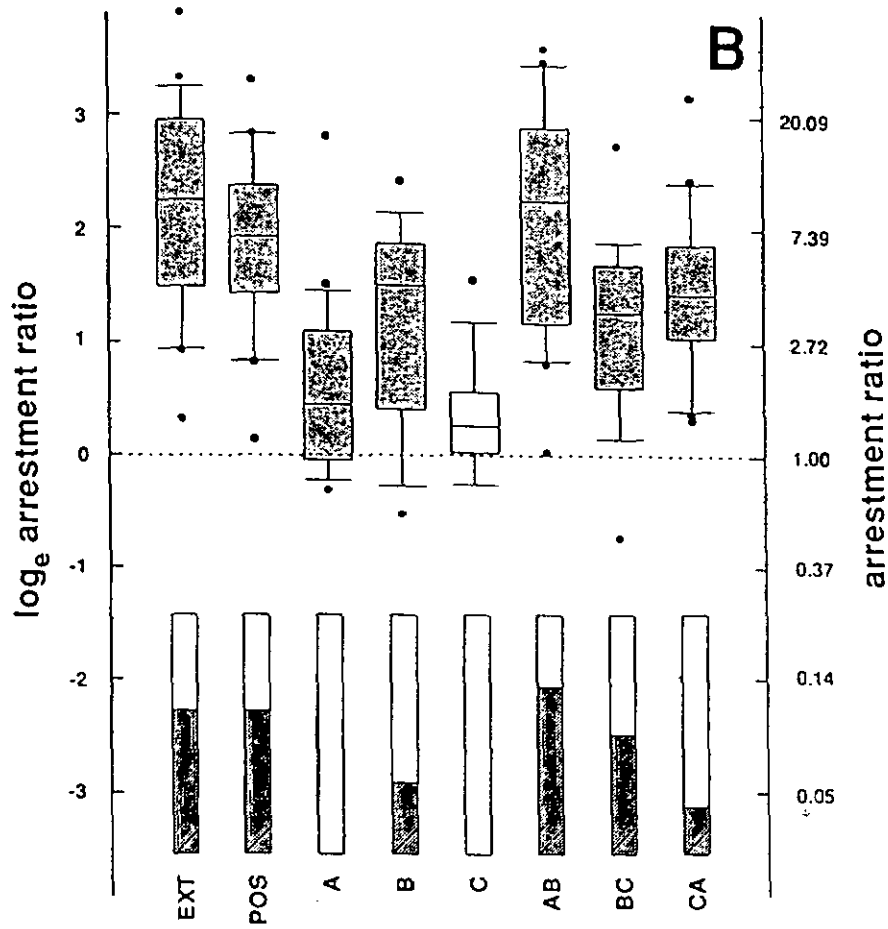
Bioassay results with other SPE column fractionations

Bioassay results of three SPE column separations, here referred to as SPE I, SPE II and SPE III, using the same method as mentioned in RESULTS 3 but with different solvent systems. SPE III is the fractionation given in RESULTS 3 the other two rendered results that are different. The major difference being that the highest activity is found back in a different combination of sets of fractions (AB, BC, or AC). However some important elements remain the same.

1) In each fractionation some arrestment activity is associated with the apolar fractions, confirming the activity of cholesteryl esters.
2) The maximum activity after fractionation which corresponds to the activity of the extract before separation is each time found in a combination of apolar with more polar material.
3) Finally tip-over behaviour is never associated with cholesteryl esters, always found in one of the two more polar sets (B or C) when tested on their own and both arrestment and tip-over need to be enhanced by the addition of one other set of fractions.

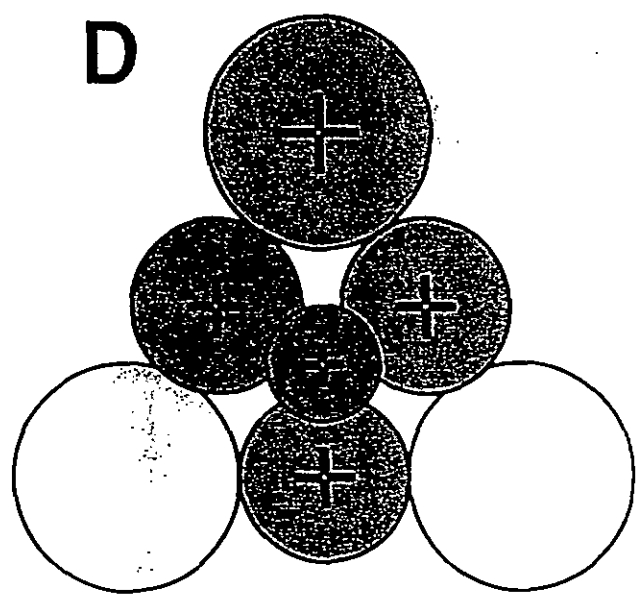
FIGURES. Preparative solid phase extraction (SPE) on a small silica gel column of a chloroform:methanol (1:1) extract of female *B. microplus* and bioassay of fractions. **A:** Thin layer chromatography (TLC) of whole extract (EXT) and SPE fraction sets A B and C. **B:** Behavioural responses of individual male *B. microplus* ticks to a glass bead treated with the female extract and its SPE fractions at 3 equivalents. EXT, extract before separation, POS, positive control, *i.e.* all fractions A, B and C combined. **C:** The SPE elution procedure and the way the individual fractions were recombined in the three sets A, B and C. Solvents are HEX hexane, DCM, dichloromethane, CH chloroform, M, methanol and WA water **D:** A graphical summary of the bioassay results with the three fractions and the different combinations presented in a triangular diagram. The three corner circles represent the individual sets of fractions with A left B top and C right. The smaller circles are the combinations of those sets with the total mix ABC in the centre. The grey shade of each circle indicates the level of arrestment whereas the cross stands for incidence of tip-over.

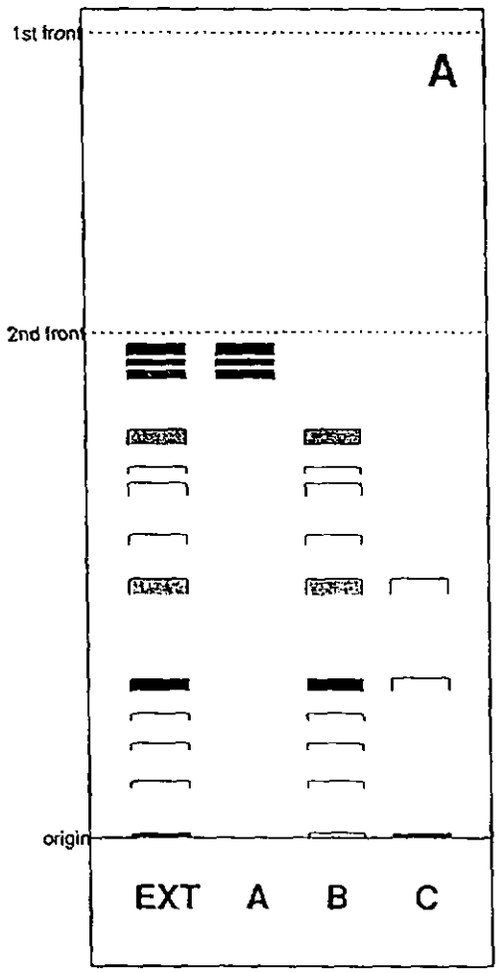


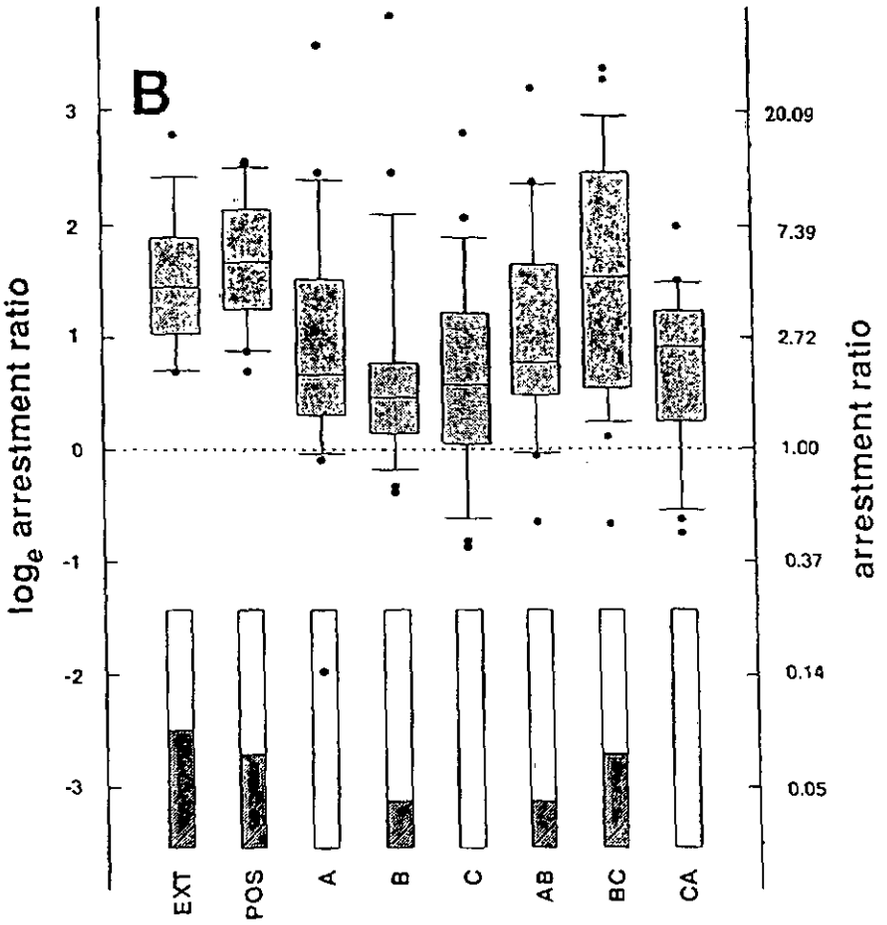


SPE I

solvent	*ml*	*fractions*	*set*
M	2	CONDITIONING	
CH	2	id	
HEX:CH	2	id	
HEX	3	id	
application of 100ul extract (CH)			
HEX	3	1	A
HEX:CH (1:1)	2	2	B
CH	3	3	B
CH:M (1:1)	3	4	B
M	2	5	C
M:WA (1:1)	2	6	C
WA	2	7	C

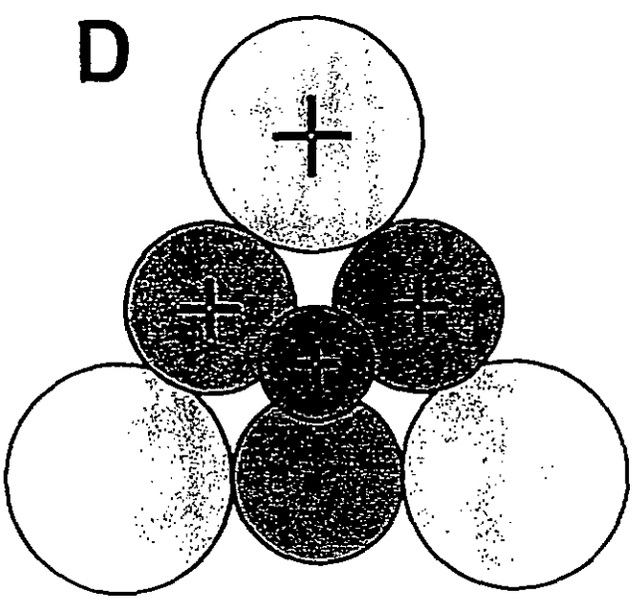


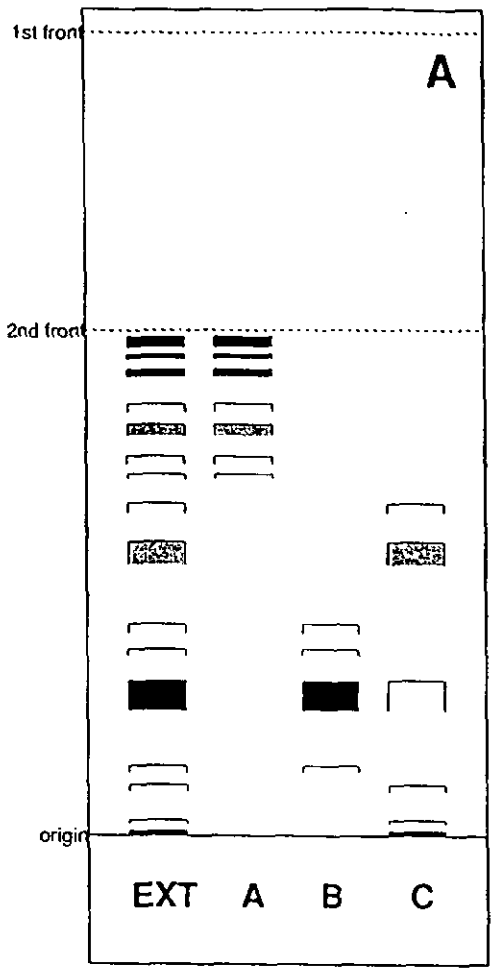


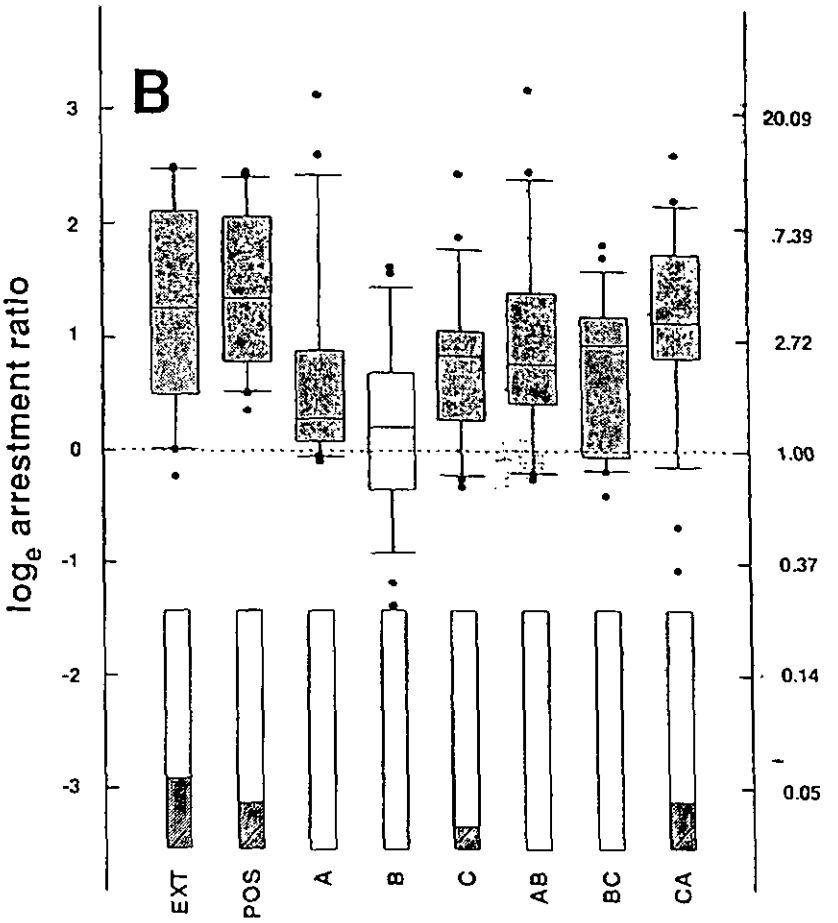


SPE II

solvent	*ml*	*fractions*	*set*
M:WA (1:1)	2	CONDITIONING	
M	2	id	
CH	2	id	
HEX:DCM (1:1)	2	id	
HEX	3	id	
application of 100ul extract (CH)			
HEX:DCM (1:1)	3	1	A
DCM	1	2	B
CH:M (9:1)	2	3	B
CH:M (1:1)	2	4	C
M	2	5	C
M:WA (1:1)	2	6	C

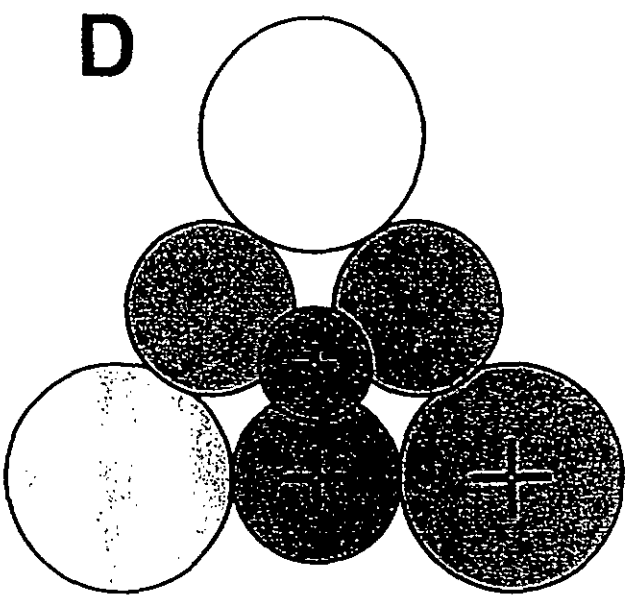






SPE III

solvent	*ml*	*fractions*	*set*
M:WA (1:1)	2	CONDITIONING	
M	2	id	
CH:M (1:1)	2	id	
CH	2	id	
HEX:CH (75:25)	2	id	
application of 100ul extract (CH)			
HEX:CH (75:25)	4	1, 2, 3, 4	A
CH	3	5, 6, 7	B
CH:M (1:1)	3	8, 9, 10	C
M	2	11	C
M:WA (1:1)	2	12	C



Bioassay results with cholesterol, cholesteryl oleate and guanine

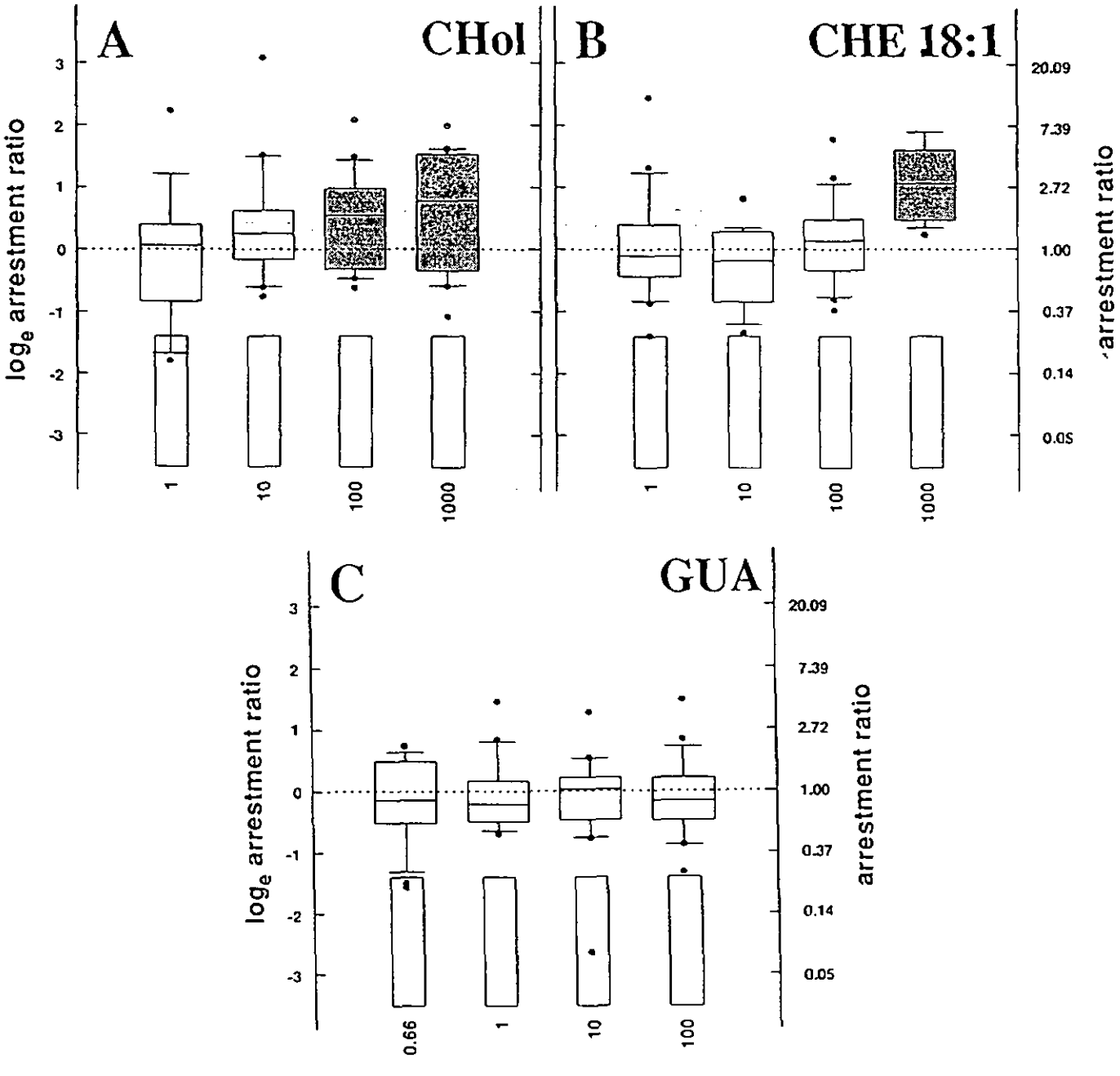


FIGURE 1. Behavioural responses of individual male *B. microplus* ticks ($16 \leq n \leq 29$) to a glass bead treated with synthetic compounds at different doses (test) or solvent only (control). Box plots show the distribution of the natural logarithm of the arrestment ratios, *i.e.* the ratios between the time spent on the test versus the control bead. Horizontal lines of one box mark the 10th, 25th, 50th, 75th and 90th percentiles while 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 indicate the proportion of males exhibiting tip-over behaviour on the test bead. **A:** cholesterol, **B:** cholesteryl oleate and **C:** guanine.