

**Behavioural responses of the tropical bont tick,
Amblyomma variegatum (Fabricius) to odours of hosts
and of conspecifics**



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(Réponses comportementales de la tique *Amblyomma variegatum*
(Fabricius) aux odeurs de vertébrés et de congénères).

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

IMPRIMATUR POUR LA THÈSE

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Amblyomma variegatum (Fabricius) to odours of
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1. General introduction

Tick history

Ticks are obligate blood-feeders on vertebrates and belong to the Acari, a subclass of the Arachnida. Acari, composed of both ticks and mites, all have a four stage life cycle, the egg, followed by a hexapod larva and succeeded by nymphal and adult stages, which are octopods. Ticks (Ixodida), a suborder of the Acari, constitute just ca. 850 species of the half-million Acari that are believed to exist. They are distinguished from mites by their greater size, the presence of a Haller's organ (the major olfactory organ) on the tarsus I of the forelegs, a highly expandable idiosoma to accommodate blood-meals and the presence of mouthparts adapted to haematophagy. An overview of the systematics of ticks has been reviewed by Keirans (1992). Two phylogenies of ticks based on morphological characteristics (Hoogstraal and Aeschlimann, 1982) and mitochondrial DNA analysis (Klompen et al., 1996) have been proposed, and both are largely in agreement. Ticks are believed to have appeared in the palaeozoic and mesozoic eras, feeding on reptiles. Two major families among tick species are the hard (Ixodidae) and soft ticks (Argasidae), so named because of the toughness of their respective cuticles, and this divergence is believed to have occurred very early in the evolution of this acarine suborder. Ixodidae further differ from Argasidae by the presence of a hard sclerotised scutum covering the entire dorsal region of males and the anterior portion of the dorsum of females. The Ixodidae themselves comprise two major phyletic lineages, the Metastriata and Prostriata, and it is to the Metastriata that *Amblyomma variegatum* belongs. Among other differences between these two lineages, metastriate ticks mate on their hosts: reproduction and feeding are firmly associated.

The evolutionary progress of metastriate tick species is believed to have led to a reduction in body size and length of mouthparts as mammals appeared as alternative sources of blood. More evolved metastriate species also possess fewer sensilla to house olfactory receptor cells, the number of the latter being remarkably similar throughout the Metastriata (Hess and Vlimant, 1986). The sub-family Rhipicephalinae is held to be among the most evolved of the metastriate ticks and these animals exclusively feed on mammals. This group includes *Boophilus microplus*, a one-host cattle tick of huge economic importance. Adult females of this species are typically 3 mm in length and adults possess just 14 olfactory sensilla in the Haller's organ (Hess and Vlimant, 1986). In contrast, members of the sub-family Amblyomminae are quite primitive. The Haller's organ of *A. variegatum* contains 19 olfactory sensilla (Hess and Vlimant, 1986). The Amblyomminae include some of the largest of all ticks, with unfed female adults often measuring 8 mm in length. Most are parasitic on reptiles, 10 % specifically so, a host-ectoparasite association attesting to the early origin of this group.

Life cycle and ecology of Amblyomma variegatum

Amblyomma variegatum, the tropical bont tick, is a typical three-host tick with each life stage (larva, nymph, adult) feeding on a different vertebrate host. The tick inhabits a wide variety of humid and semi-arid ecological zones and is widespread in Africa (Walker and Olwage, 1987; Fig. 1) where it is native and in some Eastern Caribbean islands to which it was introduced. The life cycle (Fig. 2; Varma, 1993) is univoltine in those areas with only one rainy season, and trivoltine in regions with constant rainfall.

The life cycle, host range and the sites of attachment on the host have been summarised by Petney et al. (1987). Briefly, nymphs and larvae are

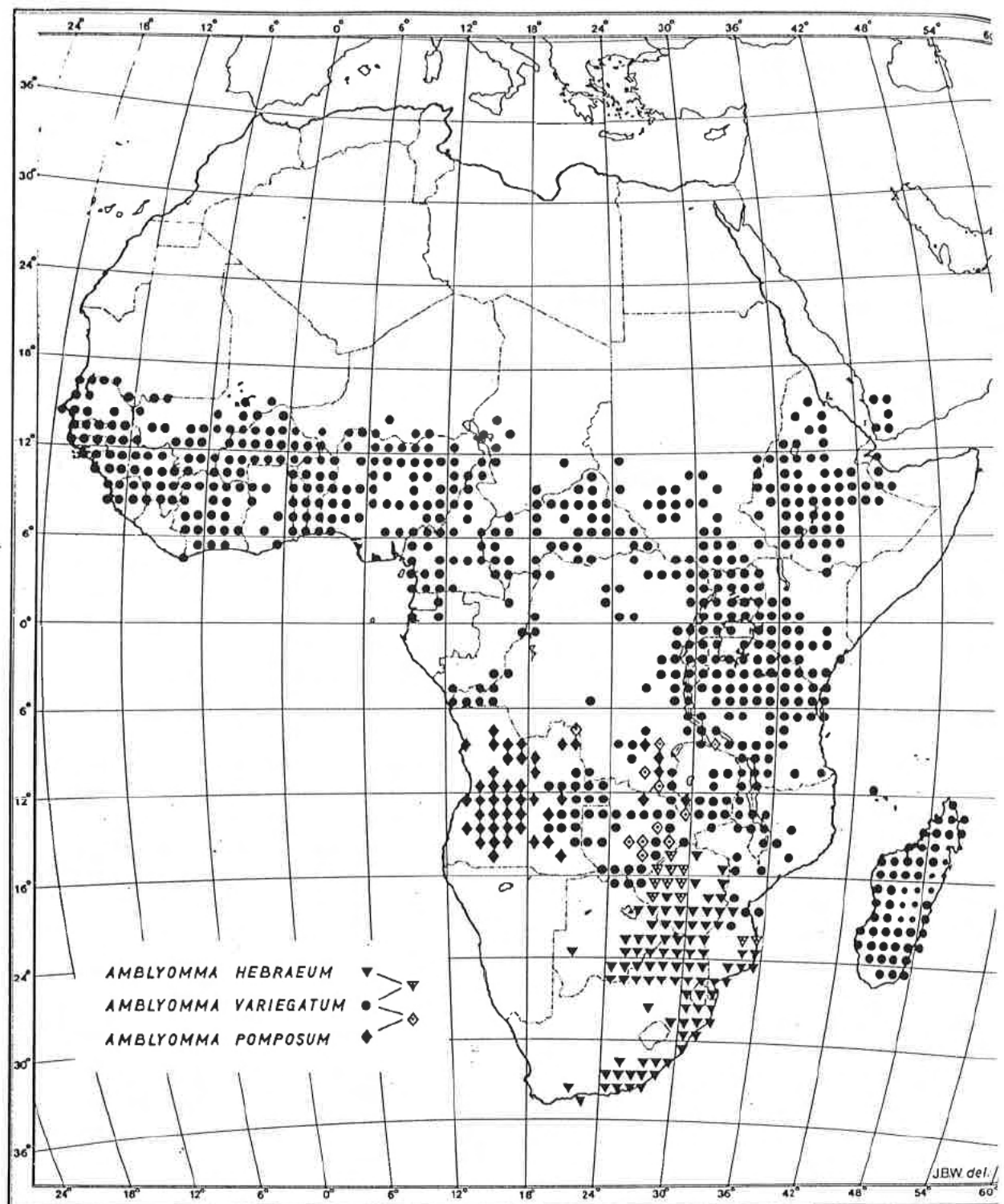


Figure 1. Distribution of *A. variegatum*, *A. hebraeum* and *A. pomposum* in the Afrotropical region, the Yemen Arab Republic and Madagascar.

Symbols combining species indicate geographical proximity. The small symbols for *A. variegatum* in Madagascar indicate highland and coastal rain forest areas where the presence of this tick is questioned (after Walker and Olwage, 1987).

quite indiscriminate, thriving on several vertebrate species, from birds to small mammals. Adults have a preference for large mammals, especially bovids (Barré, 1989). Larvae and nymphs have been found attached to several areas of the host, whereas adults are normally restricted to the ventral surfaces and genitalia, areas that are difficult to groom.

In the laboratory at 25 to 27⁰C, 4 months are needed to complete the three-host life cycle (Fig. 2). In the field this may take far longer due to the influence of climatic variables (reviewed by Yonow, 1995). Male adults emerging from the nymphal stage are the first to appear on the host first just after the rainy season, followed by females some days later (Ekpenyong, 1996). The mechanism whereby males appear before females is not known: male nymphs may feed faster and so emerge earlier as has been shown for *Rhipicephalus evertsi evertsi* (Rechav et al., 1977a). The secretion of immunoglobulin-binding proteins present in the saliva of male ixodid ticks may condition the host immune system to allow females to feed more successfully (Wang et al., 1998). Also, females of most metastriate tick species will not engorge fully without mating. However, in the case of *A. variegatum* and its sister species, *A. hebraeum*, females will not even attach in the absence of an aggregation-attachment pheromone released by feeding males (discussed below). Adult males can stay on the host for several months, whereas females drop off after engorgement following some 12 days on the host. During this period, their weight increases from <0.2g to >1g. Females begin to oviposit for one to two weeks after drop-off, after which they die. Eggs hatch some 50 days after oviposition.

Economic importance

As vectors of several pathogens, ticks represent a serious threat to veterinary health. *A. variegatum* is a vector of diseases such as heartwater (*Cowdria ruminantium*), dermatosis (*Dermatophilus congolensis*) and Q-

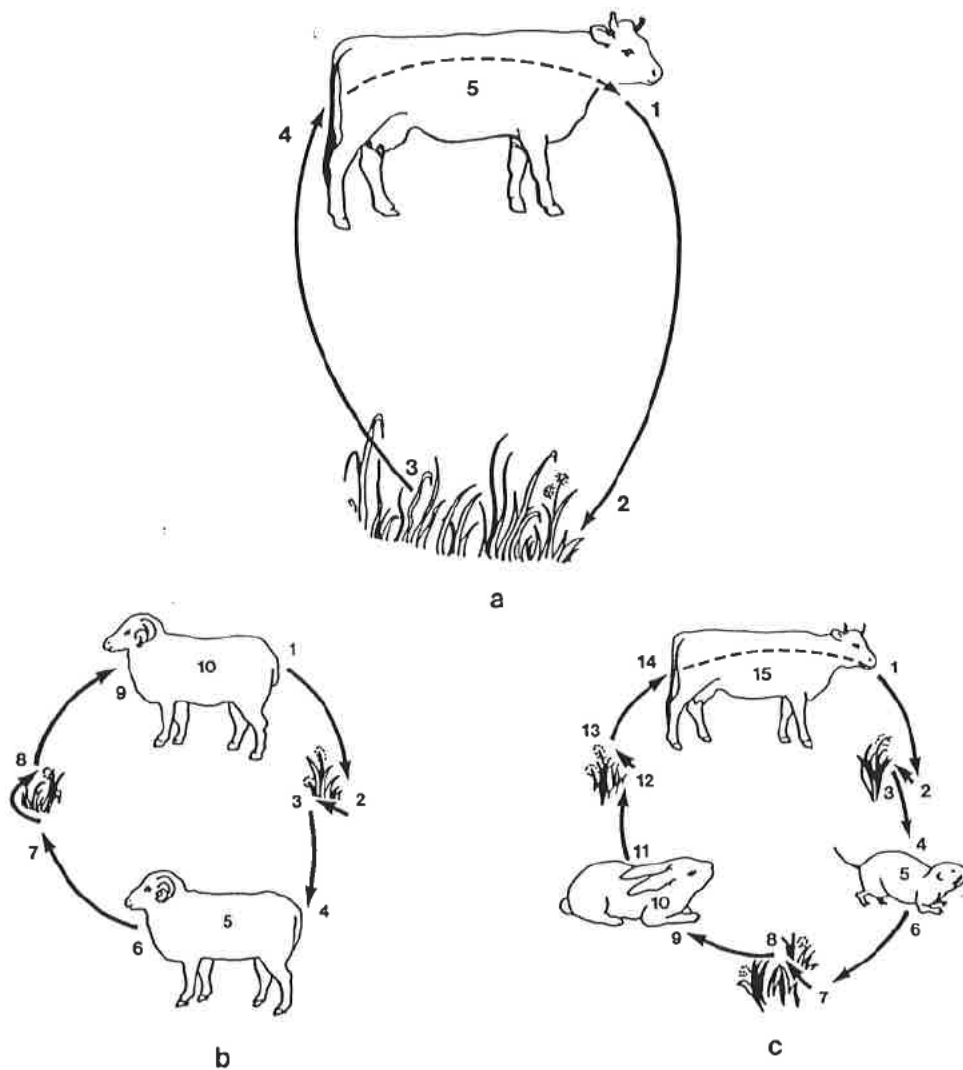


Figure 2. Life cycles of ixodid ticks: (a) one-host life cycle, in which fed female detaches (1), oviposits and dies (2), active larva moves to vegetation tip (3) and climbs on host (4), tick then develops from larva to nymph with all three stages feeding on the same host (5); (b) two-host life cycle, sequence as in one-host life cycle up to the nymphal stage (5), then fed nymph detaches from host (6), develops to adult in vegetation (7), adult moves to vegetation tip (8) and climbs on the host (or ambushes the host), attaches to second host (9) and feeds (10); (c) three-host cycle, sequence as in one-host cycle up to larva feeding on host (5), then larva detaches (6) and moults to nymph in vegetation (7), active nymph moves to vegetation tip (8) and climbs on the host (or ambushes the host) (9), feeds on second host (10), detaches (11), moults into adult in vegetation (12), adult moves to vegetation tip (13) climbs onto host (14) (or ambushes the host) and feeds (15). (After Varma, 1993. In *Medical Insects and Achnids*, p 617, Chapman and Hall, London).

fever (*Coxiella burnetti*). Ticks may also cause harm by exsanguination and secondary infections of feeding wounds. A loss of 40-61g in live weight gain of young cattle was estimated by Pegram et al. (1989) as being equivalent to the successful engorgement of one *A. variegatum* female.

Host seeking by A. variegatum

More than 90% of all known ixodid ticks, including *A. variegatum*, exhibit the three-host life cycle where each mobile life stage (larva, nymph and adult) feed on different animals and drop to the ground between bloodmeals to moult (Hoogstraal and Aeschlimann, 1982). Parasitising vertebrates is no easy task since the potential host is always larger and mobile, so specific physiological adaptations and the involvement of various sensory modalities are necessary for a tick to maintain contact with hosts. Tremendous resistance to desiccation and starvation enable ticks to survive for months off the host. While nidicolous ticks share a microenvironment with their hosts, others such as *A. variegatum* must await their arrival. The sensory modalities involved and the events that lead to host seeking have been reviewed by Waladde and Rice (1982). Ticks may either quest on vegetation waiting for a host to pass, as with *Ixodes ricinus*, or run to engage their host, as with *Hyalomma dromedarii* (Kaltenrieder, 1990). These two strategies have been described for 'ambush' or 'hunter' ticks but this division is somewhat arbitrary as it is based on the relative mobility of the free-living stages. Accordingly, larvae of *A. variegatum* are considered as ambushing ticks, adults as hunting ticks while nymphs are intermediate. The modalities that ticks employ to perceive their host include thermal (Lees, 1948), acoustic (Webb et al., 1977), visual and mechanical cues as well as odour. Different sensory inputs must be weighted by the tick in preparing to orientate to a host. Mechanical disturbance is particularly important for the activation of immature *A. variegatum*, which they use to enable them to

grasp their host (Barré, 1989) whereas olfaction is considered the primordial modality for activating adults of this species (Barré et al., 1997; Norval et al., 1989a). Although olfaction is the better studied modality in *A. variegatum*, this species is sensitive to infrared radiation emitted by vertebrates (Poffet, 1988) and acutely sensitive to visual cues (Kaltenrieder, et al., 1989; Kaltenrieder, 1990).

Host odour attractants for haematophagous arthropods

In this century, the study of host seeking by haematophagous arthropods has been confined to those relatively few ectoparasites (< 300) that transmit diseases of economic importance, particularly tsetse flies. Several attractants have been identified on the basis of electrophysiological studies where olfactory organs of arthropods are stimulated with host odours. Particular attention has been given to components of vertebrate breath, especially CO₂, 1-octen-3-ol and acetone. CO₂ has been shown to attract a wide range of bloodsucking arthropods, pertinent examples include tsetse flies (Paynter and Brady, 1993), triatomines (Taneja and Guerin, 1995), mosquitoes (Braverman et al., 1991) and ticks (Gray, 1985). 1-Octen-3-ol, a breath component present in very low amounts, is known to increase field catches of several haematophagous diptera such as tsetse flies (Hall, 1984), horseflies (Leprince et al., 1994), stable flies (Schofield and Brady, 1997), biting midges (Blackwell et al., 1996) and mosquitoes (Becker et al., 1995). Acetone has been found to induce upwind responses of tsetse flies (Paynter and Brady, 1993) and stable flies (Schofield and Brady, 1997). Phenolic compounds present in vertebrate urine, particularly *p*-cresol and 3-*n*-propylphenol, attract tsetse flies (Vale et al., 1988).

Interaction between host odour compounds: Examples of synergism between products in attracting blood feeding arthropods include the interaction of

phenols present in urine for the attraction of tsetse flies (Vale et al., 1988), CO₂ plus 1-octen-3-ol for mosquitoes (Kline et al., 1991) and CO₂ plus o-nitrophenol for the tick *A. variegatum* (Barré et al., 1997). The release of blends of compounds (CO₂, 1-octen-3-ol, acetone and phenols) simulating an individual host have thus far been found to be only 50% as attractive as host odour for tsetse flies although attraction could be increased to that of a natural source by increasing the concentrations of ketones by fifty times (Torr et al., 1995).

Host specificity: It is known that individuals of a species vary considerably in attractivity to haematophagous arthropods (Vale, 1974; Knols et al., 1995) yet the contribution of host odour to this specificity is unknown. Host-associated microflora may be implicated. Several short-chain fatty acids are produced on the skin by bacterial degradation (Kanda et al., 1990), and mixtures of such fatty acids attract mosquitoes (Knols et al., 1977). Further, Nicolaides (1974) has pointed out that humans secrete more than 200 types of longer chain fatty acids, many of which have an unusual degree of branching, that may contribute both to an individual odour and also to the profile of the bacteria residing on the skin. Other bacterial by-products such as methyl sulphides and H₂S have, respectively, proved behaviourally active in the laboratory for blow flies (Nilssen et al., 1996) and ticks (Steullet and Guerin, 1992). So far, however, none of the compounds which attract haematophagous arthropods could be said to be specific to vertebrates. Microbial cultures attract arthropods such as triatomines (Guerenstein et al., 1995), mosquitoes (Knols and de Jong, 1996) and blowflies (Morris et al., 1997), providing evidence that microflora resident on or in the host may furnish attractants, but which in the end contribute to a very unique individual odour profile for each vertebrate (as any visitor to a zoo knows).

The quantity of specific volatiles released from the host may also contribute to host specificity. Mullens and Gerry (1998) captured equal numbers of the mosquito *Culex quinquefasciatus* from calves and a trap containing their equivalent CO₂ output. Lactic acid, a principal constituent of human sweat, but a minor constituent of the sweat of several other mammals, is a known component of the host attractant for the anthropophilic mosquito, *Aedes aegypti* (Geier et al., 1996). Another example is H₂S, liberated from the anaerobic rumen which can contain up to 100 ppm in bovids (Hungate, 1966) compared to less than 1ppm in human breath (Steullet and Guerin, 1992). However, the relative proportions of a given volatile in a host odour is constantly changing. Contents of a bovid rumen vary with diet and time of day (Funk et al., 1987) and the concentration of acetone and other ketone bodies in mammalian breath increases with starvation (Kundu et al., 1993; Dobbelaar et al., 1996), rising to levels 15 times higher in human breath after fasting for 24 hours (Skrupskii, 1995).

Although highly specific host-ectoparasite associations exist, the specificity of an ectoparasite for a given host species should not be assumed. This has been proven several times in human history, the classic example being the spread of the Black Death in the fourteenth century where black rats were struck with plague carried by fleas, who then infested humans.

Host odours in other behaviours of haematatophagous arthropods: Host odours can mediate other behaviours of a blood-feeding arthropod apart from host seeking. Evidence exists that the site of swarming of males (which do not feed) of the biting midge, *Culicoides variipennis sonorensis* is determined by host odour and not by the odour of nulliparous females (Gerry and Mullens, 1998). Dougherty et al., (1995) identified specific compounds from host faeces that elicit an oviposition response from the sandfly *Lutzomyia longipalpis*. Further, host faecal odour extracts synergise

the effect of the oviposition pheromone in this species (Dougherty et al., 1993). Triatomines are attracted to NH_3 (Taneja and Guerin, 1997), a host odour, but also liberated from triatomine faeces which may serve as a refuge marker (Lorenzo and Lazzari, 1996). Arthropods may synthesise compounds associated with vertebrates themselves. Isobutyric acid, a known host odour which is behaviourally active in triatomines (Schofield, 1975), is the principal component of a gland that releases volatiles in these animals (Cruz-López et al., 1995). o-Nitrophenol, a component of bovid odour, is synthesised and released in amounts up to 0.03% of body weight per day (Diehl et al., 1991) by adult males of *A. variegatum* feeding on a host. This compound represents a particularly potent demonstration of parsimony, as it attracts con-specifics (Hess and De Castro, 1986), promotes their aggregation (Schöni et al., 1984) and attachment at the feeding site (Norval et al., 1992).

Conclusions regarding host odour attractants for haematophagous

arthropods: The study of host odours is a daunting task: Krotoszynski et al. (1977) identified over 100 compounds as being present in human breath and Ellin et al. (1974) found over 40 compounds in human effluvia. There also remains considerable technical difficulty in identifying low molecular weight gases, and some analytical problems associated with the isolation of basic polar compounds such as amines. Even simple molecules such as ammonia have not received due attention: this gas is an attractant for both horseflies (Hribar et al., 1992) and triatomines (Taneja and Guerin, 1997), and amines have not yet been studied as possible attractants. This may be an oversight, but Heath et al. (1997) found potent synergism between trimethylamine and a mixture containing putrescine and ammonium acetate for trapping Mediterranean fruit flies. It may also be said that responses by haematophagous arthropods to the few known attractants (e.g., CO_2 , acetone,

1-octen-3-ol) have not received due attention. Behavioural responses to attractive chemicals at different doses in the laboratory (e.g., Schofield and Brady, 1997) or in the field (e.g., Barré et al., 1997) have been investigated in only a handful of studies.

The range of compounds tested behaviourally on haematophagous arthropods remains extremely limited in spite of abundant electrophysiological evidence indicating biological activity of a range of chemostimulants. Cork (1994) has demonstrated several electrophysiologically active compounds in wound fluid for screwworms, and in human sweat for mosquitoes (Cork and Park, 1996). Steullet and Guerin (1994a,b) also found a wide range of olfactory chemoreceptors for host volatiles in the tropical bont tick, *A. variegatum*, yet it still remains the case that there are probably several compounds, as yet unidentified, which obscure our view of the volatile-mediated relationship between a haematophagous arthropod and its host.

Olfaction in A. variegatum

The main, probably the only, olfactory receptors of ticks are located on the first tarsus of each of the forelegs. This was established early in the century (Hindle and Merriman, 1912), as ticks were seen to wave their forelegs to sample their environment much as insects do with their antennae. As in other arthropods, olfactory sense organs in ticks are normally innervated setae with porous walls. Porous walls favour the diffusion of molecules from the air to the dendritic membrane of the olfactory receptor cells(s) within the sensillum, across the sensillar lymph. The binding of the odour to the dendritic membrane initiates a complex transduction process within the neuron which in turn leads to production of action potentials that travel to the synganglion for processing. The majority of olfactory sensilla are located in the Haller's organ, a compound sensory organ consisting of a

capsule and an anterior pit on the dorsal aspect of the tarsus of leg 1. Olfactory sensilla are also present on the surface of the anterior tarsus outside Haller's organ, but none have been found elsewhere in ticks. The ultrastructure of these sensilla have been extensively studied and reviewed (Hess and Vlimant, 1982; 1986). Ixodid ticks possess relatively few (<100) olfactory receptors, about half of which are located in the Haller's organ (Hess and Vlimant, 1986). This contrasts sharply with the 12000 receptors estimated to exist for the stable fly, *Stomoxys calcitrans* (McIver, 1987), or the ca. 25000 receptors specialised in the perception of one or two products in the silk moth, *Bombyx mori* (Kaissling and Priesner, 1970). Yet even with this small number of receptors, ticks can still locate a host. In contrast to the knowledge of the ultrastructure of olfactory sensilla, studies of the chemical specificity of olfactory receptors are scarce, largely limited to the aggregation-attachment pheromone components (see below) and 2,6 dichlorophenol (Waladde, 1982; De Bruyne and Guerin, 1994) and the characterisation of ammonia receptors (Sinitsina, 1974; Haggart and Davis, 1979; 1980). Nonetheless, it appears that receptors are highly conserved among tick species (see discussion, chapter 2).

The aggregation-attachment pheromone

The aggregation-attachment pheromone (AAP) is secreted by feeding male ticks to attract conspecifics and was first described for *Amblyomma maculatum*, its presence being necessary for females to attach to a host (Gladney, 1971, Gladney et al., 1974). Such a signal is also present in *A. hebraeum* and attracts nymphs (Rechav et al., 1976) as well as adults (Rechav et al., 1977b). Schöni et al. (1984) isolated *o*-nitrophenol (ONP), methyl salicylate (MS) and nonanoic acid from extracts of fed *A. variegatum* males and showed that this mixture of compounds induced aggregation as well as pairing of males and females in a bioassay off the host. Diehl et al.

(1991) identified ONP and MS, but not nonanoic acid, in the headspace over feeding *A. variegatum* males only after 3-4 days of feeding and reported that the AAP is probably produced in dermal glands on the ventro-lateral cuticle of male ticks. Apps et al. (1988) isolated ONP, benzaldehyde and isobutyric acid in the headspace of *A. hebraeum*. Olfactory receptors for ONP, MS, benzaldehyde and isobutyric acid are present in the Haller's organ of *A. variegatum* (Schöni, 1987; Steullet and Guerin, 1994a,b) and field experiments confirm earlier findings that ONP attracts females of this species over a considerable distance (Hess and De Castro, 1986) whereas methyl salicylate and nonanoic acid may play a role in pairing and attachment (Schöni et al, 1984). Dongus and Gothe (1995) demonstrated the existence of a volatile signal from fed *Hyalomma truncatum* males that attracts conspecific ticks. Consequently, a signal combining attraction to the host with aggregation and sexual pairing on the host may not be exclusive to *Amblyomma* species.

Behavioural assays for testing the responses of arthropods to olfactory stimuli

Various experimental designs exist for measuring the behavioural responses of arthropods to olfactory stimuli, ranging from test chemicals applied to sectors of a petri dish, to one arm of a Y-tube olfactometer or in an air stream such as in a wind tunnel or over the apex of a locomotion compensator. While each kind of olfactometer has its own advantages, each also varies considerably in the behaviours that animals are allowed to employ in orientating towards a source. The most 'indiscriminate assays' (Kennedy, 1977) allow the animal to use several taxes (e.g., chemotaxis, anemotaxis, phonotaxis, phototaxis) to orientate but such results are difficult to interpret. If there is confusion about the mechanism the animal uses to approach the source, it renders experimental reproducibility more

problematic. Field trapping is an example of a particularly indiscriminate assay as arthropods can deploy their full range of behaviours to the task of approaching a stimulus source. As the level of discrimination of a given experimental design increases, some behaviours are excluded.

Anemotaxis and chemotaxis are two main mechanisms that are believed to be involved in the orientation to an olfactory stimulus in an air stream. However, it is difficult to exclude one from the other, as seen in several wind tunnel studies where animals try to locate an attractant delivered from a point source. A truly discriminating assay (Kennedy, 1977) would (1) allow the exclusion of either the anemotactic or chemotactic component of the orientation (2) permit the animal to move freely in all directions yet ensure that the concentration of the olfactory stimulus remains independent of the locomotion undertaken (open-loop design) (3) permit the immediate onset or cessation of the olfactory stimulus.

Most experimental designs to date have emphasised the anemotactic component of olfactory orientation as experiments involving chemotaxis alone are very difficult to realise. It is technically far simpler to deliver odour in an air stream to give a more homogenous and stable plume than would be possible by diffusion.

The locomotion compensator or servosphere: All behavioural measurements in this study have been made on a locomotion compensator, or servosphere (Fig. 3), invented by Kramer (1976). A more complete description of this apparatus (Kramersphere) is described by Taneja and Guerin, (1995). Briefly, a walking arthropod is maintained at the apex of a sphere by compensating for any displacement made by the arthropod in the x-y plane. The compensator monitors movements of the animal at regular intervals allowing reconstruction of the tracks described and an evaluation of various parameters of locomotion such as speed, direction and angular

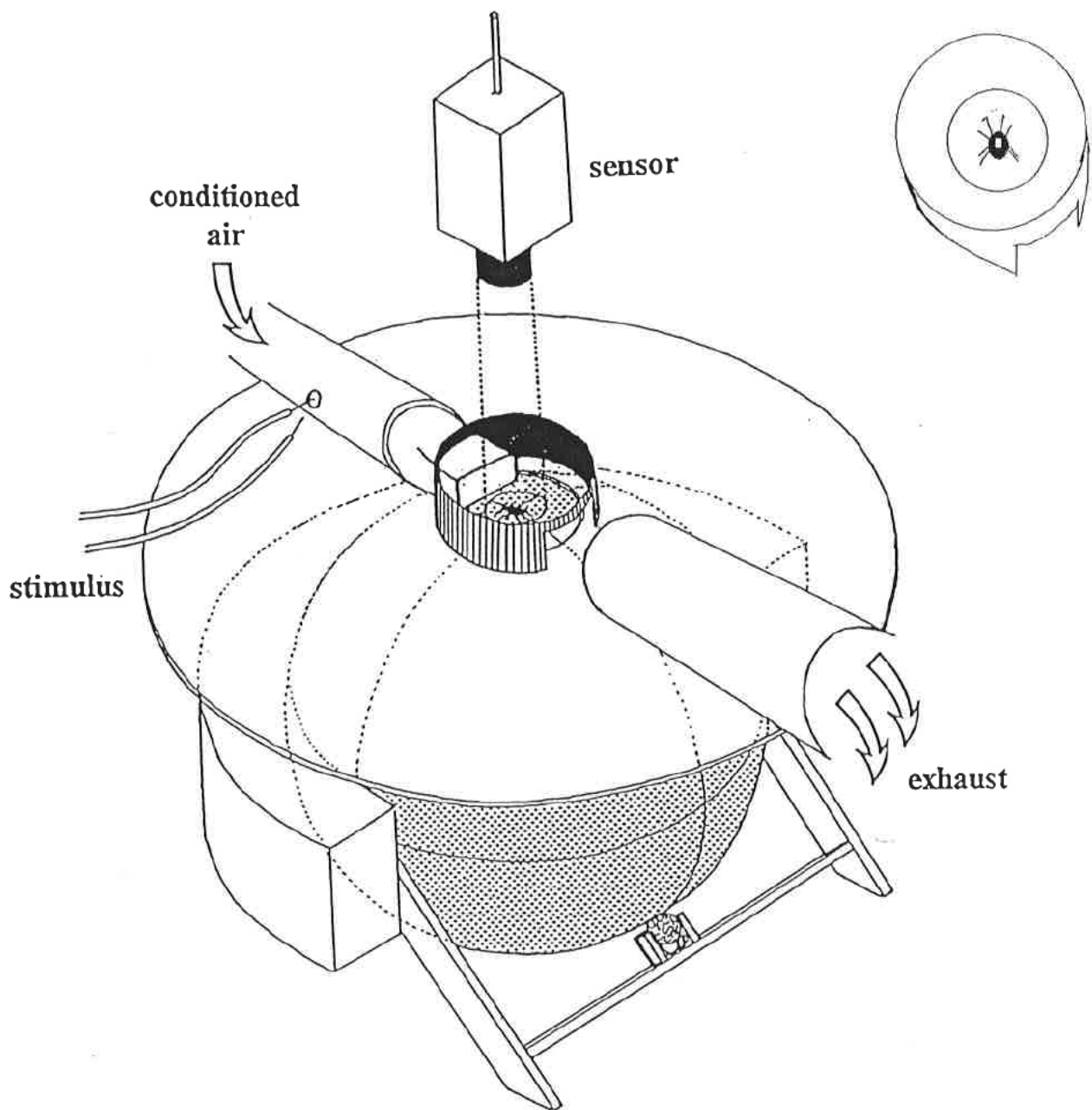


Figure 3. The set-up for behavioural studies consisting of a locomotion compensator (a perspex servosphere linked to a position sensor) and a horizontal air-stream delivery with stimulus injection and exhaust. The arthropod walks on the apex of the sphere. A metal dome (28 cm diameter and 4 cm high) coated with a non-reflecting black paint with a hole (5.5 cm diameter; roof of dome not drawn on sphere diagram) at its centre to permit passage of light from the sensor, covered the apex of the sphere to minimise interference from air-currents and residual light in the room (inset).

velocity. The apex of the sphere is bathed in a horizontal air stream into which chemical stimuli can be introduced or withdrawn. This experimental design can be described as a discriminating assay for odour-mediated anemotaxis and offers several advantages over other olfactometers Kennedy (1977). One advantage is that the arthropods walk on the sphere in an open-loop design with respect to odour, i.e., the animal cannot adjust its position with respect to the odour concentration as it might in a Y-tube olfactometer or flying or walking on the edge of an odour plume. Another advantage is that the onset and cessation of the odour is completely under the control of the operator allowing a temporal presentation of the odour. This allows the observation and interpretation of 'on- or 'off-responses' when the odour is supplied or withdrawn. The Kramersphere has been used to study the behavioural responses of several arthropods to olfactory (e.g., Kramer, 1992), acoustic (e.g., Hennig and Weber, 1997) and anemotactic stimuli (e.g., Bell and Kramer, 1979). The complete liberty of an animal to move unimpeded in all directions in the Kramersphere design is a particular advantage over other walking arenas where the animal is tethered at the apex of a sphere (below).

Previous designs of spheres used for recording locomotion include the Y-maze globe (Rust et al., 1976; Fig. 4a). In this design, the animal is suspended from a height and clings to the frame of a globe which is also above the ground. The animal then manipulates the globe to travel in three directions. This has been used to study the walking speed of a cockroach and its response to pheromone (Rust et al., loc.cit.) and was also employed in investigating the responses of the blowfly, *Lucillia cuprina*, to bacterial odours associated with its host (Morris et al., 1997). Kerfoot (1968) designed a sphere where animals are tethered and walk on a table-tennis ball held in place by ball point pens (Fig. 4b). The ball is connected to a wire,

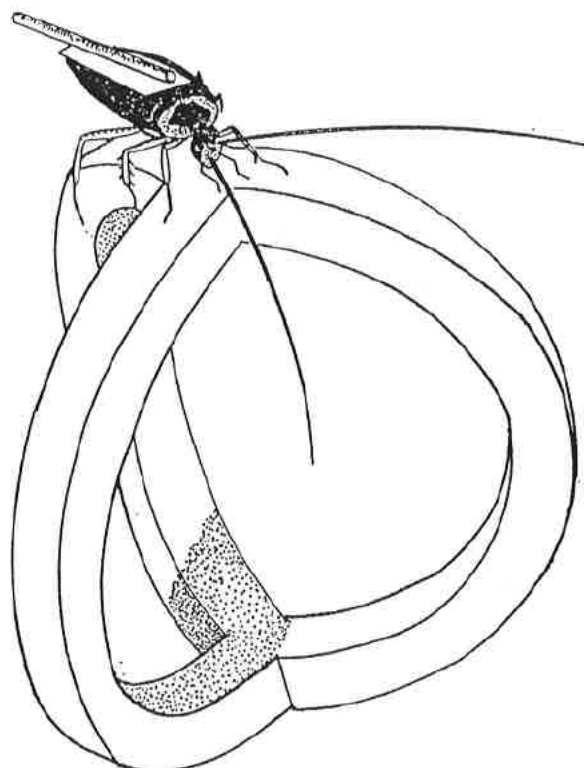


Figure 4. Various non-compensatory systems employed to study the walking behaviour of arthropods.

Figure 4a. The Y Maze globe (after Rust et al., 1976). The arthropod clings to the globe and turns the sphere while orientating along one of the three hoops. The animal is suspended and the globe is above the ground (not shown). The number of rotations of the globe provides an estimate of walking velocity. Turns left and right can also be recorded, but this system does not measure angular velocity.

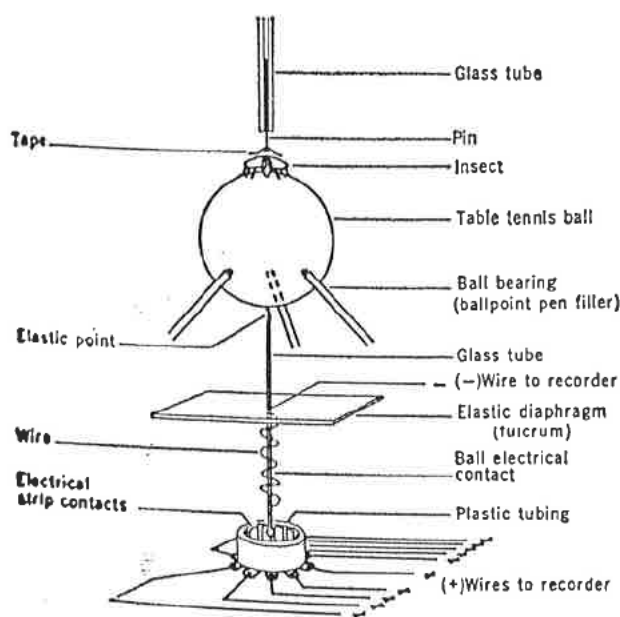


Figure 4b. The Kerfoot sphere (after Kerfoot, 1968). In contrast to the Y maze globe, this apparatus measures angular velocity, but cannot measure linear velocity. A change in the walking direction of the arthropod causes torsion in a sensor wire which in turn activates electrical strip contacts at the base that corresponding to chosen intervals between 0-360°. Therefore, if an arthropod walks in a constant direction, or stops, these events will not be registered.

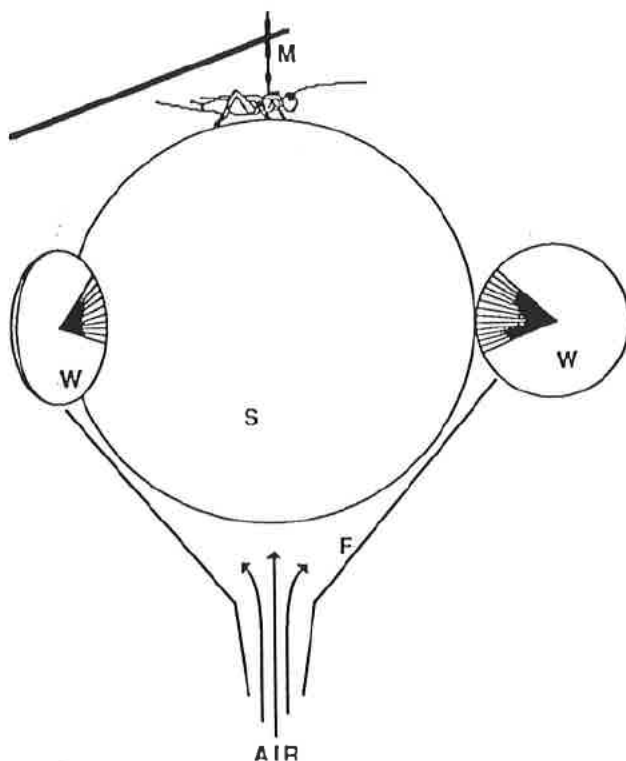


Figure 4c. The Dahmen sphere. The apparatus consists of a styrofoam sphere (S), a supporting funnel (F), rotation detection wheels (W) and mounting apparatus (M) (after Walikonis et al., 1991). The wheels lightly contact the equator of the sphere and are positioned at right angles to each other so as to record displacements in the x,y plane. In this design, the rotation of the wheels can be detected by the passage of light through the spokes of the wheels onto an electronic photoreceptor.

the movement of which activates electrical contacts at the base of the system that correspond to changes in direction. The most elegant of the alternative sphere designs is the Dahmen sphere (Fig. 4c; Dahmen, 1980). A light, hollow styrofoam ball is let float in air so that it can be handled, turned and moved easily by the animal itself. The ball swims in an air cushion of diameter 2 mm greater than the ball. An air stream enters and supports the ball with nearly no friction, through a small hole in the bottom of the cup. Animals are tethered and movements in the x-y plane are collected by disks with a spoke pattern in two dimensions. Dahmen (1980) used the apparatus to record tracks of the beetle *Cicindella silvatica* to visual stimuli. The advantage of these systems (Fig. 4) over the Kramersphere (Fig. 3) is that they are non-compensatory, i.e., they do not employ a servosphere mechanism as the animal displaces the sphere or globe directly.

Aims of the present study

This study aimed to investigate the effect of host-animal volatiles on the behaviour of the bont tick, *Amblyomma variegatum* (Fabricius), an ectoparasite of bovids. Host location by blood-feeding arthropods, many of which are vectors of disease, is based on several modalities including heat (Lees, 1948), sound (Webb et al., 1977), light (Kaltenrieder et al., 1989) and olfaction (Lehane, 1991). Although olfactory stimuli derived from host odour components have been studied for haematophagous arthropods as diverse as triatominae (Taneja and Guerin, 1995, 1997), tsetse flies (Paynter and Brady, 1993) and mosquitoes (Cork and Park, 1996), ticks have been largely neglected. Volatiles released from the host are known to be involved in the attraction of ticks to vertebrates (Hafez and Bishara, 1982; Downes, 1984; Norval et al., 1989b), but no attractant based on host odour components has been developed for this group of arthropods. However, ample evidence exists for the attractivity of conspecifics to known components of the aggregation-attachment pheromone released by feeding *A. variegatum* males. Having established the behavioural responses of *A. variegatum* adults to these pheromone components presented in an air stream on a servosphere, we set out to examine the responses of *A. variegatum* to several host compounds and their mixtures.

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2. Results

Chapter 1. Responses of the tropical bont tick, *Amblyomma variegatum* (Fabricius), to its aggregation-attachment pheromone presented in an air stream on a servosphere.

submitted

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Abstract

Male *A. variegatum* ticks release a mixture of *o*-nitrophenol (ONP) and methyl salicylate (MS) from dermal glands which develop soon after a host has been infested and which serve to attract conspecifics to the attachment site. The behavioural responses of *A. variegatum* to these volatiles presented in an air stream on a servosphere are detailed here. In the absence of wind, *A. variegatum* walked on all eight legs, but with long halts. Wind caused continuous walking and induced a reaching response where the first leg pair was used to actively sample the air. This increased the angular velocity and reduced walking speed. These wind induced effects were amplified in the presence of vapours from mixtures of ONP and MS in the air flowing over the ticks. Both genders responded in the same manner to vapours from source doses of ONP (down to 1 pg) and MS (down to 100 pg) presented individually. Vapour from a binary 1:1 mixture of ONP and MS was attractive over a 10^4 fold concentration range (10 ng to 10 μ g) providing an increase in upwind displacement of 20-40% over controls, significantly higher than the natural ratio where ONP vapour predominates. Source doses of 1 mg of the binary mixture were not attractive. Although the response to ONP vapour was variable on its own, it was consistently attractive when delivered with steer hair odour which was unattractive on its own. Moreover, the upwind walk to this combination did not cause a change in speed or angular velocity. This supports the hypothesis that the response to the pheromone is enhanced by host odour.

Keywords: *Amblyomma*, tick, pheromone, behaviour, servosphere

Introduction

Amblyomma variegatum Fabricius (Acari, Ixodidae), the tropical bont tick, is an obligate haematophagous ectoparasite that causes extensive economic damage to bovids both in the Caribbean and in sub-saharan Africa (where it is native). It is a typical three-host tick species with each stage (larva, nymph and adult) parasitising different mammals for several days. As a metastriate tick species, *A. variegatum* mates on its host and feeding adult males secrete an aggregation-attachment-pheromone (AAP), which is perceived by the Haller's organ on the forelegs, the principal olfactory organ of ticks. The secretion of an AAP is fundamental to the life cycle of this tick, for females on the host will only attach and feed near a source of pheromone (Norval and Rechav, 1979; Barré et al., 1998). The AAP also promotes the aggregation and attachment of other males (Norval and Rechav, 1979). Field tests with *Amblyomma hebraeum*, a sister species of *A. variegatum* with a similar AAP composition (Diehl et al., 1991), indicate that the semiochemical helps to mediate long-range attraction of conspecifics of both sexes to the host: an extract of feeding male *A. hebraeum* applied to cattle preferentially attracts wild conspecifics to the host and even to the area of application (Rechav and Whitehead, 1978). Further, Norval et al. (1989) demonstrated that cattle infested with *A. hebraeum* were more attractive to conspecifics than uninfested cattle, and postulated that the AAP may aid in marking a suitable host for further infestation. *A. hebraeum* and *A. variegatum* adults were also preferentially attracted to rabbits on which ticks were feeding (Yunker et al., 1990). Although such 'host baiting' as suggested by Norval et al. (1989) by males feeding on bovid hosts still remains to be proven for *A. variegatum* (Barré et al., 1998), extracts of feeding males of this species proved very attractive in the field at a distance of over 10 m when delivered with CO₂, whereas the latter was a poor attractant on its own (Norval et al., 1992).

The principal volatile components of the AAP of *A. variegatum* have been identified as *o*-nitrophenol (ONP) and methyl salicylate (MS), both released at an average rate of $15 \mu\text{g}\cdot\text{day}^{-1}$ and $4 \mu\text{g}\cdot\text{day}^{-1}$ or, respectively ca. 0.2% and 0.05% of body weight $\cdot\text{day}^{-1}$ by males from their dermal glands type 2 after feeding for 3-4 days (Diehl et al., 1991). Olfactory receptors have been located in Haller's organ for both ONP and MS (Schöni, 1987), but behavioural studies on the responses of *A. variegatum* to these compounds have yielded mixed results. ONP has been shown to induce aggregation in still air (Schöni et al., 1984), and to attract the tick in the field (Hess and De Castro, 1986), probably via odour-mediated anemotaxis. Hess and De Castro (1986) did not find MS to be attractive in the field, although Norval et al. (1991) reported attraction to this compound in the presence of CO_2 . Yunker et al. (1992) reported strong attraction by *A. variegatum* to MS vapour in a two-choice olfactometer, but not to the vapour of ONP.

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

Materials and Methods

Ticks

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

Before an experiment, a small piece (ca. 4 mm²) of an adhesive reflector (3M, No. 7610, Switzerland) was placed over 2 mm posterior to the capitulum on the back of each tick before the start of an experiment. The tick was then placed on the servosphere, and where necessary, was activated to walk by gently nudging its body with forceps. Unless otherwise specified, ticks were allowed at least 3 min to acclimatise in the air stream on the sphere so as to record walks at a stable speed. All tests were carried out in the dark under filtered incandescent light (filter cut-off, 780nm) as these animals are acutely sensitive to visual stimuli. Each tick was only tested once.

Stimulus and stimulus delivery for behavioural recordings.

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

Vapours from the gas-wash bottles were introduced upwind via teflon tubing (2 mm i.d. x 4 mm o.d.) attached to a small metal tube blocked at one end (3.8 cm long, 1.7 mm i.d. x 2 mm o.d.) with four 0.5 mm diameter holes along one side of its length. The needle was inserted horizontally, with its holes positioned against the air-flow, 26 cm upwind of the apex of the sphere through a rubber septum in the wall of the stainless-steel tube conducting the main air stream to the sphere. Computer-controlled solenoid valves switched the charcoal-filtered air flow (150 ml $\cdot$ min⁻¹) between control and test gas-wash bottles for consecutive 1 min control, test and end-control periods, thus voiding 30% of the volume of the gas-wash bottle in one minute. To determine if the consecutive presentation of the control followed by test treatment might bias results, 15 ticks were presented with

charcoal filtered air ($150 \text{ ml}\cdot\text{min}^{-1}$) for two consecutive 1 min recording periods. In another experiment, 24 ticks were presented with air ($150 \text{ ml}\cdot\text{min}^{-1}$) passing through a control gas-wash bottle for 1 min (i.e., one containing filter paper from which solvent had been evaporated) followed immediately for another minute by air flowing through a second control gas-wash bottle, as in the normal test procedure. No difference between the ticks' walk in the first and second minute was apparent in either experiment.

Estimation of release rates of chemicals in behavioural tests

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

Response to o-nitrophenol in the presence of host odour

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

Servosphere and recording of behaviour

A servosphere (Kramer, 1976) was used to record the responses of *A. variegatum* adults to volatile chemical stimuli. This apparatus functions in such a way as to keep the arthropod at the same position in space, i.e., at the apex of a perspex sphere (50 cm diameter) to which a stimulus delivery tube is directed. The detail of the equipment and procedure followed was as for Taneja and Guerin (1995) with certain modifications. The feedback and the reaction time of the servosphere motors were adjusted to allow the ticks to walk unimpeded in all directions within a circle (2 cm diameter) with the photosensor placed 25 cm directly above the animal. A metal dome (28 cm diameter and 4 cm high) coated with a non-reflecting black paint and with a hole (5.5 cm diameter) at its centre to permit passage of light from the sensor, covered the sphere apex to minimise interference from air-currents

and residual light in the room. The air stream ($3.5 \text{ l}\cdot\text{min}^{-1}$) flowing over the ticks exited from a rectangular orifice (4 cm long, 2.5 cm high) positioned 6 cm from the apex of the sphere. This flow rate was necessary to provide a stable wind speed of $0.18 \text{ m}\cdot\text{s}^{-1}$ ($\pm 0.01 \text{ m}\cdot\text{s}^{-1}$; 70% RH) at the apex as measured by a hot-wire anemometer (Thermo-air, Schiltknecht Messtechnik, Switzerland, response time $< 1 \text{ s}$). This wind velocity is more than five times greater than the maximum walking velocity of *A. variegatum* adults in these conditions. The air stream was always delivered at room temperature ($23\text{-}25^{\circ}\text{C}$). Temperature and humidity were measured with a thermo-electric hygrometer (Hygro-Air II, Schiltknecht Messtechnik, Switzerland, response time ca. 1 s). Two black and white infrared-sensitive video cameras (Canon Ci 20PR, Japan and PCO Computer Optics 77 CE), one positioned at a 70° angle over the orifice, another looking tangentially to the apex of the sphere from a downwind position, were employed to view the behaviour of the ticks. Both views were observed simultaneously by entering the CCD camera signals to a screen splitter (Panasonic production mixer, WJ-MX12, Japan), to a video recorder (Panasonic VCR NV-180, Japan) and a video monitor (Panasonic, WV-5360, Japan).

Data analysis

The co-ordinates of the sphere's displacement while compensating for the displacement of the tick were collected by computer and the vectors obtained were used to reconstruct the tracks described by the tick (Taneja and Guerin, 1995). It was established from video and track analysis of several ticks' walks at different speeds that the walking behaviour was best described by choosing a sampling rate of 0.2 s per vector and by applying a running mean over five successive vectors (Fig. 1). Movements of less than 0.1 mm (the lowest increment sampled by the

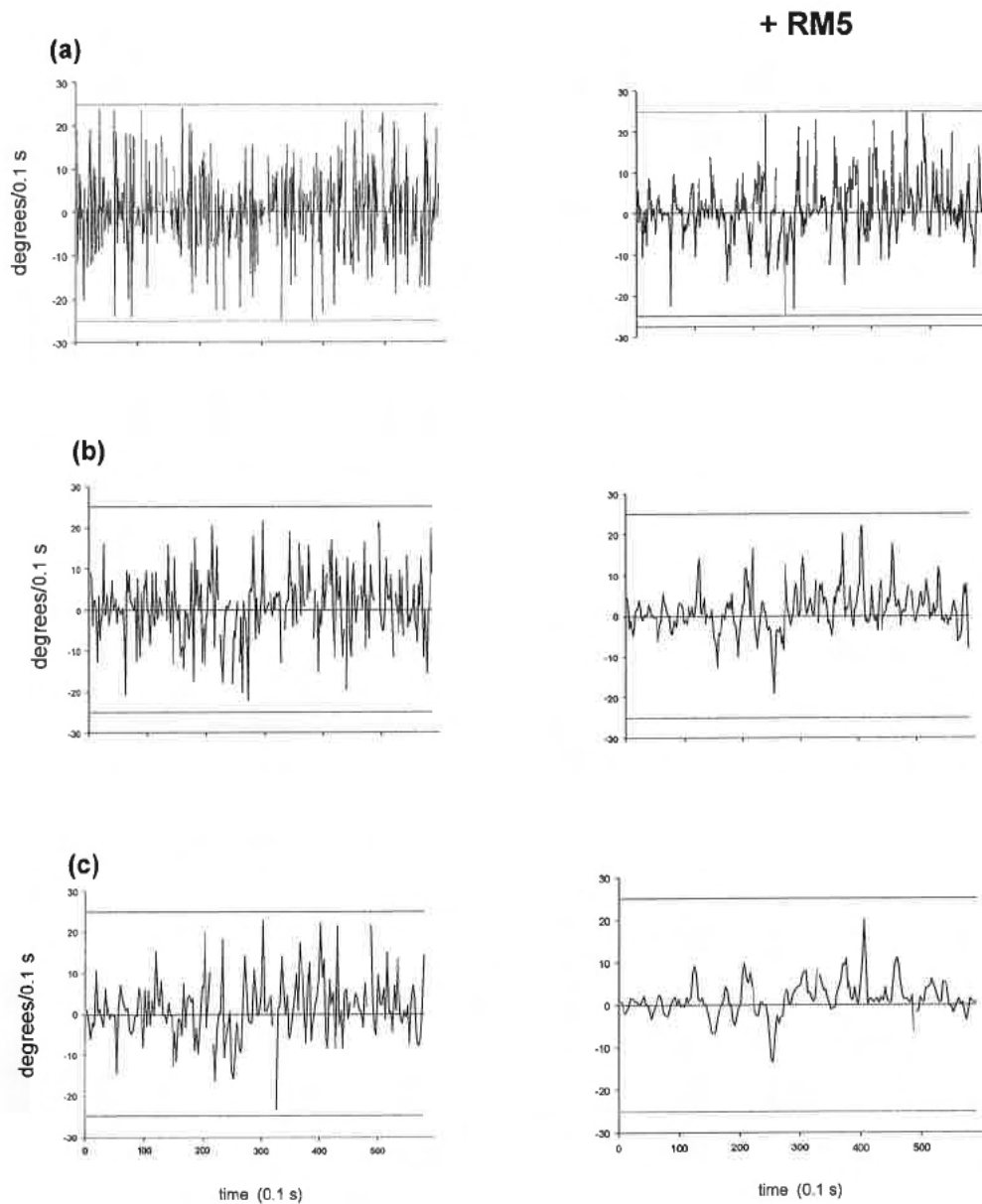


Figure 1. The angular velocity of a single *A. variegatum* adult walking for one minute on the servosphere viewed as a function of sampling rate (s). A running mean over five successive vectors was applied to the right hand graphs; (a), (b) and (c) represent the instantaneous angular velocities at sampling rates of 0.1, 0.2 and 0.3 s respectively. The horizontal lines presented above and below the traces of the angular velocities are the maximum values for any tick i.e., $250^\circ/\text{s}$. Applying a sampling rate of 0.2 s per vector and then applying a running mean of five such successive vectors was found to give the most realistic description of the walk of *A. variegatum* adults.

servosphere) were excluded. In this manner, some noise was removed from the track records to give a realistic picture of the turning angles described. Any remaining high angles described when the tick pivoted were deleted manually. These modifications typically removed < 6% of all vectors. As the overall speed of individual ticks fell in the range 10 to 30 mm•s⁻¹, the chosen sampling rate provided a resolution of 40 to 120% of the body length of the tick per vector. These vectors were used to calculate speed, angular velocity (⁰•s⁻¹), stop time and direction (ϕ) vis-à-vis wind and path straightness r , (the variance of ϕ , circular statistics, after Batschelet, 1981).

Apart from speed, parameters calculated from the vectors in control and test periods were not normally distributed and were therefore compared using non-parametric statistical tests (two-tailed). Differences between walks in test and control periods were analysed pair-wise using the Wilcoxon signed rank test. The significance of differences between treatments was determined using the Wilcoxon-Mann-Whitney test (MWU) by comparing differences pairwise between the test and control for different parameters. An analysis (Spearman test) undertaken to investigate any inter-relationships between the relevant parameters (above) showed that they were poorly correlated (correlation coefficient < 0.15, n>1600).

The increase in time spent walking in the cone (60⁰ either side of due upwind) over the relative time spent walking there in the preceding control period of equal duration was chosen as a means of measuring attraction and, when significant, termed an upwind response. A “target vector” was also calculated by multiplying the cosine of the mean direction (ϕ) by the path straightness (r), or the mean direction multiplied by the deviation from that direction. This provides an estimate of the efficiency with which a particular direction was followed and is independent of the arbitrarily chosen upwind cone. The target vector was in close agreement with the relative time spent walking in the cone 60⁰ either side of due upwind (Spearman correlation

coefficient 0.93, $p < 0.0001$, $n > 400$, two-tailed). The paired differences in the time spent walking in the upwind cone between test and control were also closely correlated with the paired differences in target vector between test and control (Spearman correlation coefficient 0.91, $p < 0.0001$, $n > 1600$, two-tailed).

Results

Walk of A. variegatum on the servosphere without wind

In the absence of wind, *A. variegatum* adults always walked on eight legs and tended to follow a chosen direction for at least one minute before switching to another direction in a trajectory that was nonetheless circular. The tick maintained its rostrum parallel to the surface of the sphere and executed a walk at constant speed with shifts to the left and right of the path (Fig. 2). These shifts consisted of $30 \text{ half-turns} \cdot \text{min}^{-1}$, with each half-turn between 20° and 40° for males and 20° - 30° for females. Walking bouts were, however, interrupted by longer pauses.

Walk of A. variegatum on the servosphere in wind

In wind, *A. variegatum* normally walked continuously, often for some hours. Circular trajectories were uncommon ($< 10\%$). The gait of *A. variegatum* walking in an air flow alternated between two distinct categories, an eight-legged walk as in still air and a six-legged walk. The six-legged walk consisted of the animal lifting its forelegs above the ground to sample the air vigorously. To achieve this, the alloscutum was fixed as

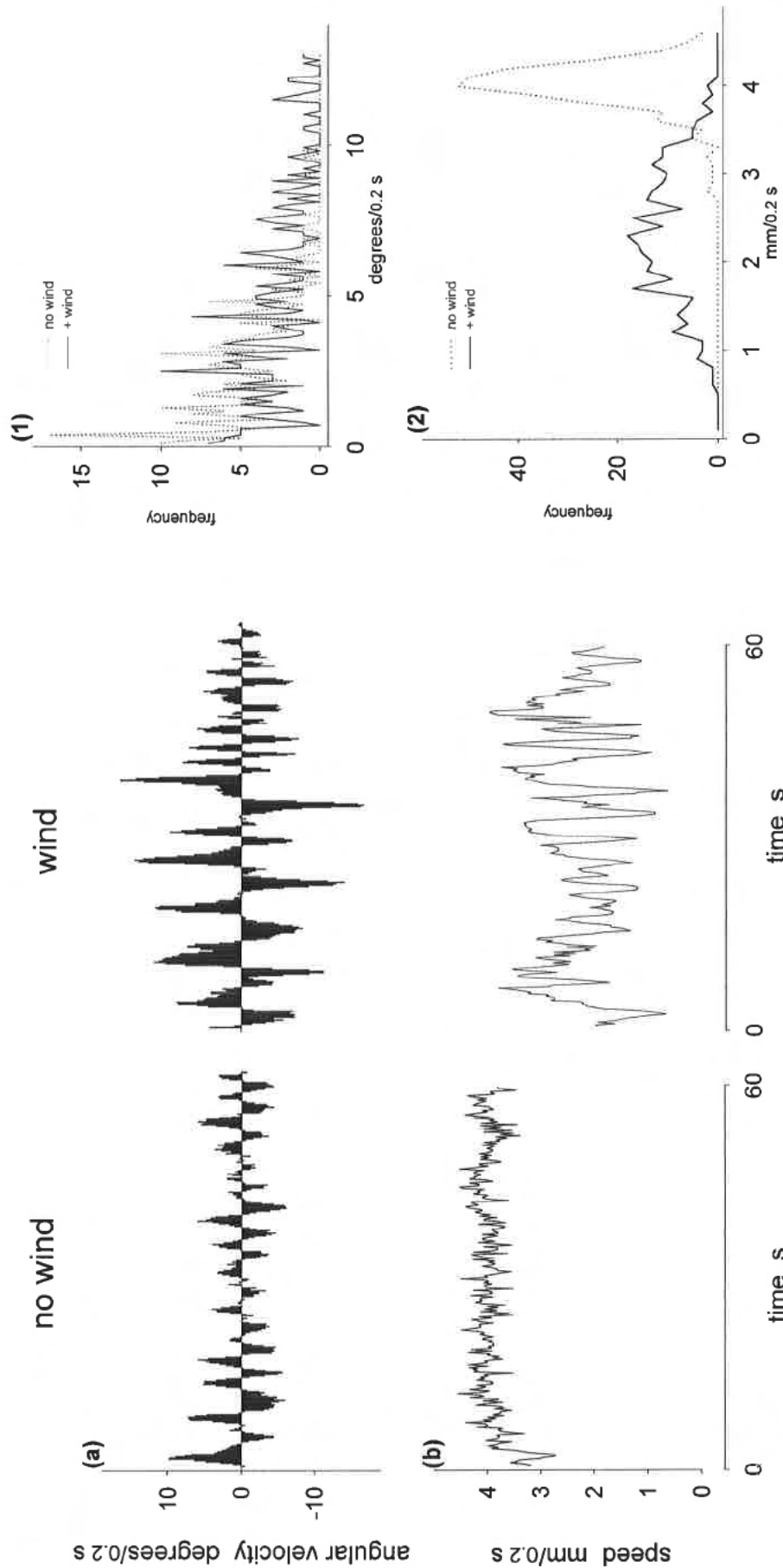


Figure 2a. The influence of wind ($0.18 \text{ m}\cdot\text{s}^{-1}$) on the angular velocity (a) and speed (b) of an *A. variegatum* adult walking on a servosphere as recorded using 0.2 s successive vectors. A running mean over 5 vectors was applied once to speed and twice to angular velocity for graphical representation. Note the reduced less stable speed and increased angular velocity of the walk in the presence of wind.

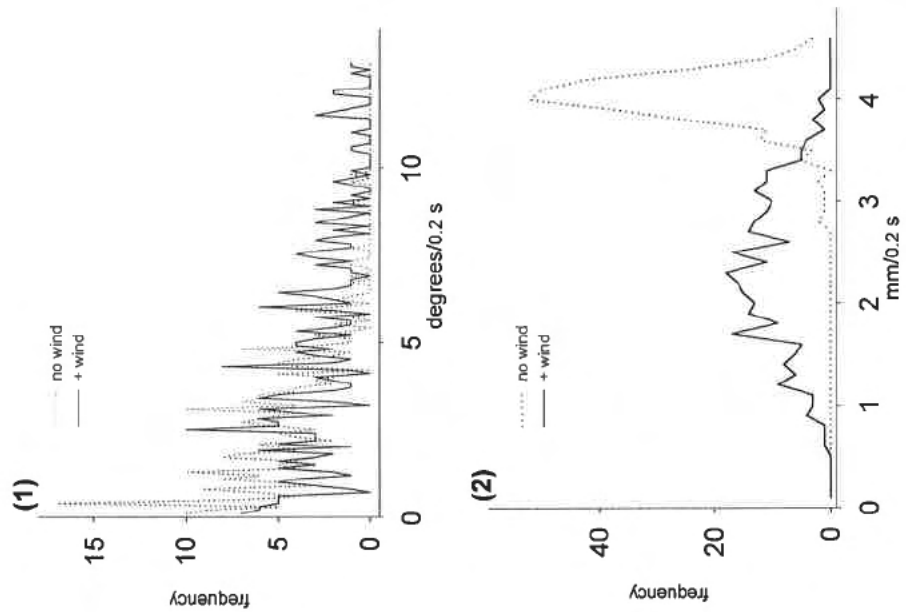


Figure 2b. Frequency distribution of instantaneous (0.2 s) angular velocity (1) and speed (2) of data in Fig. 2a. Note the reduced speed and increased frequency of large angles in the presence of wind. The distributions of angular velocity and speed were significantly different ($p < 0.001$, Kolmogorov-Smirnov two-sample test) between walks in the presence and absence of wind.

vertically as possible using the hind legs as a buttress. This laboured walking was always seen before the tick made sharp angles or pivoted, i.e., turns $>150^{\circ}\cdot\text{s}^{-1}$, and was associated with a lower speed (ca. 20%) and higher angular velocity (ca. 30%) compared to the eight-legged walk. Generally males exhibited this behaviour more than females, and this reaching behaviour was only seen in the presence of wind. This wind-induced pattern of locomotion was also observed for other tick species running on the sphere (*A. hebraeum*, *Rhipicephalus sanguineus*, *Ixodes ricinus*, *Argas persicus* and *Ornithodoros moubata*). Straighter avoidance walks for *A. variegatum* were recorded when the ticks were presented with stimuli such as a light source (ca. 400 lux) and high wind speeds ($0.5\text{ m}\cdot\text{s}^{-1}$) of low humidity.

To investigate the effects of wind, five ticks were left to walk in still air followed by an air stream ($0.18\text{ m}\cdot\text{s}^{-1}$), and this order was reversed for five other ticks. Tracks were recorded after 2 min acclimatisation to either condition. The order of the experiments had no discernible effect on the subsequent walking, so the data for the two lots of ticks were pooled for analysis. Wind affected tick walking behaviour: the average speed was significantly reduced from $16\text{ mm}\cdot\text{s}^{-1}$ to $11\text{ mm}\cdot\text{s}^{-1}$ ($p < 0.0001$, Wilcoxon signed rank test) and there was a tendency towards an increased angular velocity (Fig. 2), due in large part, to the induction of the 6-legged walk in wind. No difference in walking behaviour was evident at wind speeds in the range $0.1\text{--}0.5\text{ m}\cdot\text{s}^{-1}$. Wind did not evoke anemotaxis, but when offered a choice, ticks were acutely sensitive to small variations ($> 0.02\text{ m}\cdot\text{s}^{-1}$) in the wind speed, orientating to contact zones of higher wind velocities in the air stream passing over the sphere. This taxis was seen both in the presence and absence of attractants and may allow ticks seek to increase the flux of odours over the olfactory organs on the forelegs while reaching. Although all tests

were carried out in an air stream at 70% RH, a comparison with the walk of twenty ticks ($n = 20$) at 100% RH yielded no significant differences.

One minute walks of over 1600 ticks walking in an air stream at $0.18 \text{ m}\cdot\text{s}^{-1}$ preceding test periods for chemicals (below) were analysed. There was no preference for upwind walks (median time in the upwind cone 22.7%) and the circular mean (ϕ) was 177° relative to upwind (0°) with a straightness index (r) of 0.26, and a median target vector of -0.21 . Ticks usually walked continuously with a median speed of $19.3 \text{ mm}\cdot\text{s}^{-1}$ at an angular velocity of $24.7^\circ\cdot\text{s}^{-1}$, but walked slower in the upwind cone (median $16.9 \text{ mm}\cdot\text{s}^{-1}$, $p < 0.0001$, Wilcoxon sign rank test) and at a lower angular velocity ($22.4^\circ\cdot\text{s}^{-1}$, $p < 0.0001$).

A comparison between male and female ticks showed that females ($n = 478$) walked slightly faster (median $21.3 \text{ mm}\cdot\text{s}^{-1}$) than males (median $18.9 \text{ mm}\cdot\text{s}^{-1}$, $p < 0.0001$, MWU, $n = 1450$) and had a lower stop time ($p < 0.0001$, MWU). Females also spent less time walking in the upwind cone (median 22.6%) than males (22.7%; $p < 0.0001$, MWU). Angular velocity was also lower for females (median $23.8^\circ\cdot\text{s}^{-1}$) compared to males (median $24.2^\circ\cdot\text{s}^{-1}$; $p < 0.0001$, MWU) due to the greater size and higher speed of females.

The influence of the age of adult ticks (one month to 13 months after moult) on the way they walked was also analysed. Only speed was significantly ($p < 0.0001$, Spearman test) affected, as ticks walked slower with increasing age (correlation coefficient - 0.399; $n = 1928$). Over one year, the average speed of ticks decreased by 30%.

Release rate of test chemicals in air over the servosphere

Analysis of the headspace from a 50 ml flask containing ONP and MS showed no difference in the rate of volatilisation between these two compounds of similar vapour pressure when added either together or

separately (coefficient of variation within the most differing pair was 25%). An interval experiment where the release rates during the first and last 30 s of the 1 min test period at the specified flow rate were compared, showed only a slight reduction (ca. 10%) in the recovery of volatiles in the second half minute compared to the first. Some 7-8% of the 100 µg source was recovered from the headspace of the 50 ml flask in 1 min. Extrapolating from this figure to the experimental conditions on the sphere, a 1 µg source dose would release an estimated 4×10^{10} molecules·ml⁻¹ of either ONP or MS in air flowing over the tick at the apex of the sphere, taking into account the dilution of the gas-wash effluent in the main air stream passing over the servosphere.

Attraction of A. variegatum to pheromone components

Vapours from the two main components of the aggregation-attachment pheromone of *A. variegatum* were tested individually and together. ONP, the predominant AAP constituent, was found attractive at source doses from 1 pg to 1 µg, but the response was variable at any of these doses. By contrast, MS was always attractive in a range from 100 pg to 1 µg (lower doses not tested, Fig. 3). A binary 1:1 mixture of equal source doses of MS and ONP was significantly more attractive than the natural ratio (3:1) of these compounds present over feeding males when delivered at either 1 µg ONP: 0.3 µg MS or at a thousand times higher (Fig. 3). However, even the best binary mix (Fig. 3 and Fig. 4) was not more attractive than MS presented on its own at this dose (Fig. 3). The 1:1 binary mix was attractive in four tests spanning a ten-thousand-fold concentration range. Moreover, the degree of attraction between the 10 ng and the 10 µg source doses was not significantly different (Fig. 5). However, there was a significant trend towards reduced attraction as the source dose of the 1:1 binary mix was

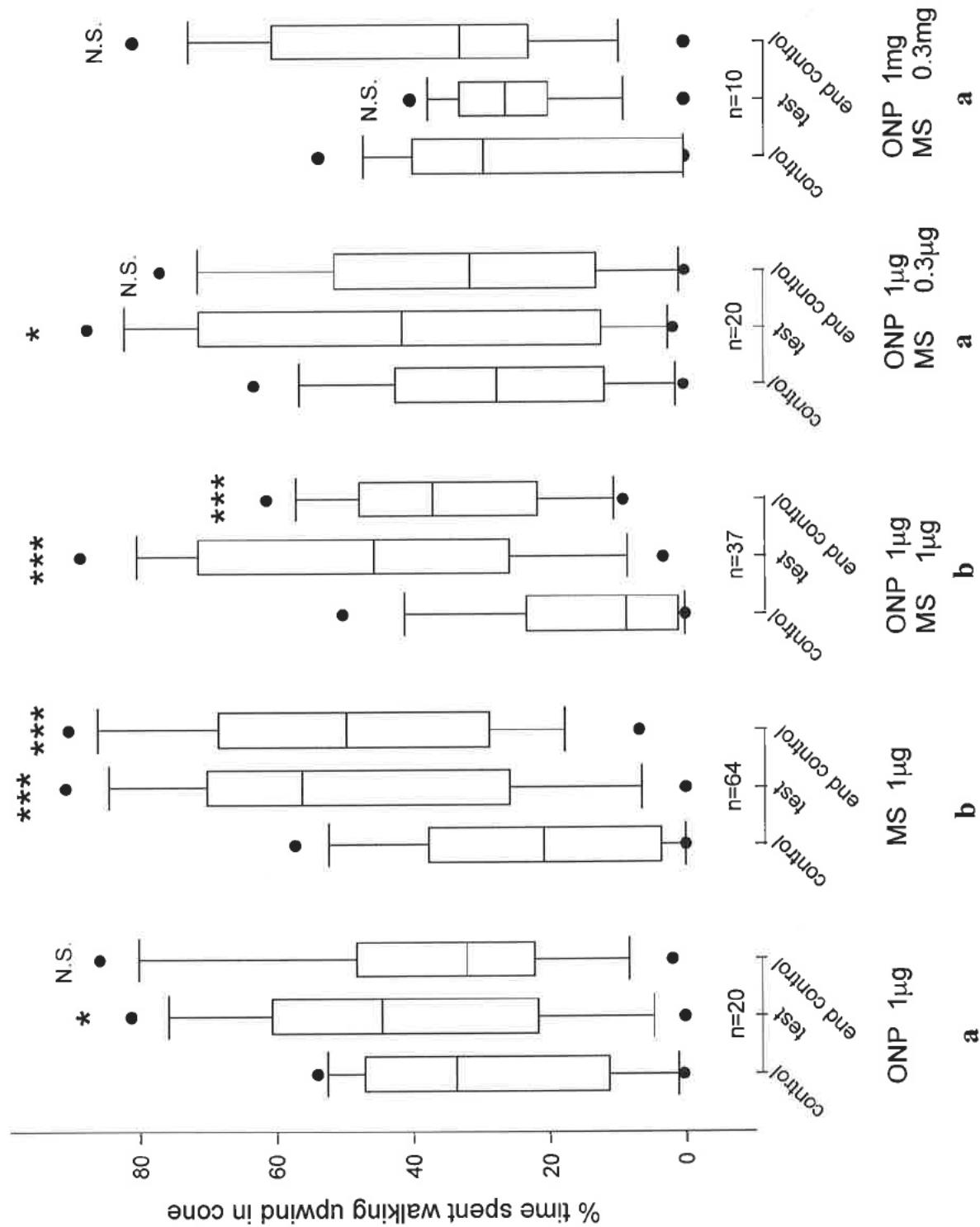


Figure 3. Box plots of the time spent walking in the upwind cone 60° either side of due upwind as a percentage of the total for the period by *A. variegatum* adults in response to delivery of vapours of the aggregation-attachment pheromone components o-nitrophenol (ONP) and methyl salicylate (MS) on their own or as mixtures in the air stream. Source doses are indicated under box plots. Responses to each treatment are preceded and followed by control periods, respectively, left and right of the central box. The line within a box marks the median, the lower and upper boundaries of a box indicate the 25th and 75th percentiles, error bars below and above a box indicate the 10th and 90th percentiles, and the 5th and 95th percentiles are shown as circles. Asterisks above a treatment indicate a significantly higher time spent walking upwind in the test or end-control period compared to the control period (* p < 0.05; ** p < 0.01; Wilcoxon's signed rank test). Treatments with different letters are significantly different (p < 0.05, Wilcoxon-Mann-Whitney test).

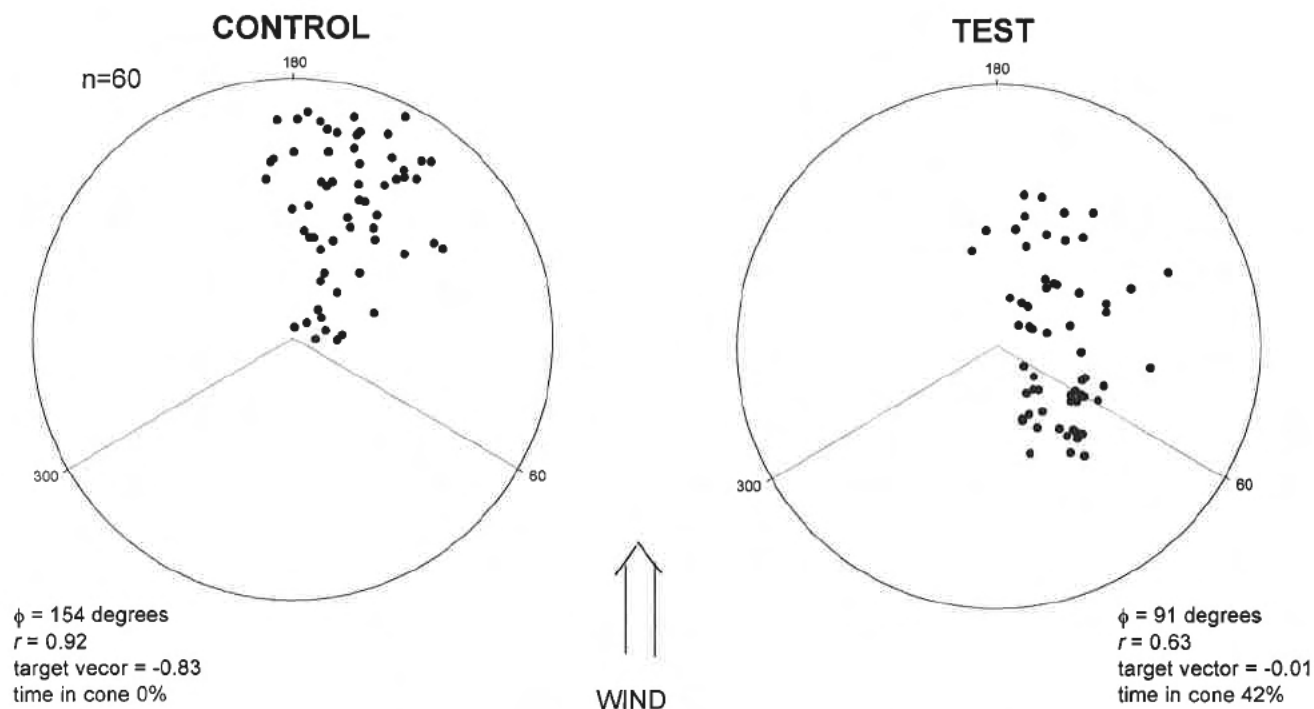


Figure 4a. Polar plot of the distribution of vectors ($n = 60$) sampled by the servosphere for a single *A. variegatum* adult tick walking for 1 min in the control followed by a second min where the tick was exposed to odours of methyl salicylate and *o*-nitrophenol (source dose $1 \mu\text{g}$ each). The distance from the origin represents the length of a vector. For graphical representation, vectors shown were collected using a sampling rate of 1 s. The outer circle represents a displacement of 30 mm. Note the reduced length of vectors collected in the test period compared to the control period as the animal slowed down, and the greater number of vectors in the upwind cone in test.

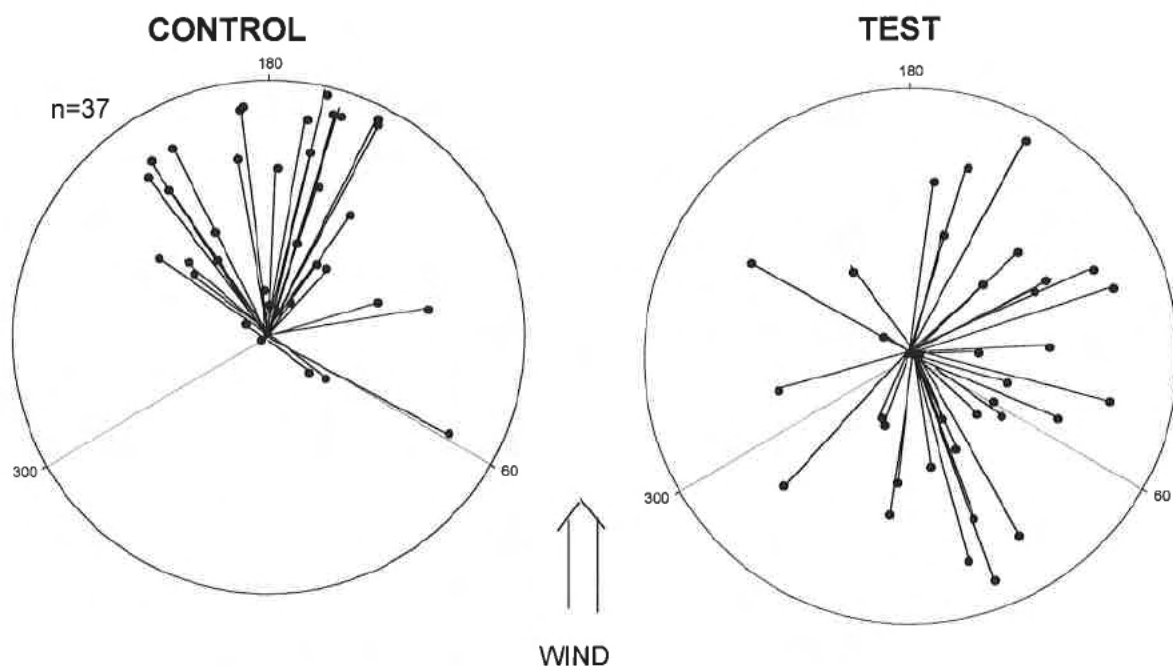


Figure 4b. Polar plot of the distribution of the mean direction (ϕ) of the walk of individual ticks ($n = 37$) on the servosphere for a 1 min control period followed by a 1 min test period where the same ticks were presented with vapours of *o*-nitrophenol and methyl salicylate (source dose $1 \mu\text{g}$ of each). The nearer the vector weighting (r) to the perimeter, the straighter the walk in that direction (circular statistics, after Batschelet, 1981). Note the increased numbers of mean vectors in the upwind cone in the test period.

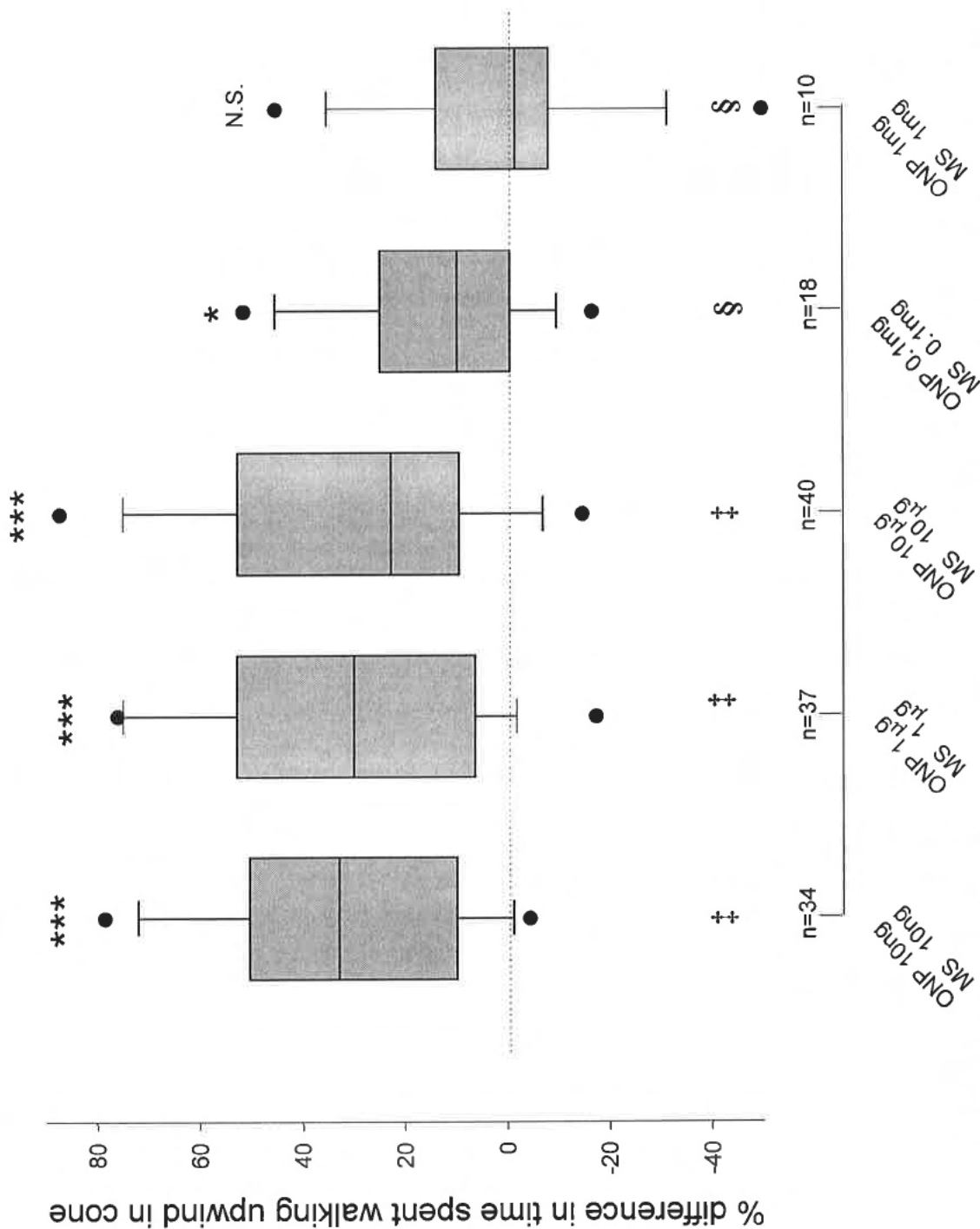


Figure 5. Box plots of the change in time spent walking upwind on the servosphere by *A. variegatum* adults in the presence of vapours from increasing source doses of a 1:1 mixture of o-nitrophenol and methyl salicylate. Asterisks above a treatment indicate a significantly higher time spent walking upwind in the test period compared to the preceding control period (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Wilcoxon signed rank test). Treatments sharing the same letter (§ or †) are not significantly different (Wilcoxon-Mann-Whitney test). Source doses are indicated under box plots. The trend towards reduced attraction at higher source doses of the binary mix was significant ($p < 0.001$, Jonckheere test). The 1:1 mixture was clearly overdosed at 1 mg.

increased ($p < 0.001$, Jonckheere test, after Siegel and Castellan, 1988). *A. variegatum* was less attracted to the 100 μg source dose of the 1:1 mixture. Vapours from 1 mg of the 1:1 binary mix and the natural ratio, 1 mg ONP: 0.3 mg MS, represent an overdose for although ticks turned upwind towards the source this did not lead to upwind walks.

The response of ticks to the binary mixtures (whether attractive or not) and components presented singly was always the same: an increase in angular velocity and a corresponding reduction in speed, often immediately (Figs. 6, 7). The effects on angular velocity and speed due to the attractant were independent of the increased upwind walk and of the dose, but were not always significant. No difference was found in the ticks' response to the binary mixture when presented in gradual increments over 1 min (0-150 $\text{ml}\cdot\text{min}^{-1}$ from the gas-wash bottle flask containing 1 μg of the binary 1:1 mix), or when presented at different air stream humidities (70% and 95%). Neither did gender, time of day, age (between one and nine months) or the number of generations the colony was reared in captivity influence the response. Moreover, the strain of ticks reared in captivity since 1981 and the more recent (1997) colony originating from a wild tick population of the Ivory Coast did not differ in their response to the binary 1:1 mix.

Response of the ticks to the binary mixture at different doses in terms of angular velocity and upwind walk in the cone were also calculated using distance-based sampling (0.1 mm) of the tracks. This analysis of 40 tests (10-20 ticks for each test) provided no increase in discrimination between test and control for angular velocity and a decrease in discrimination for the relative displacement in the cone as ticks frequently slowed down significantly in the presence of the attractant.

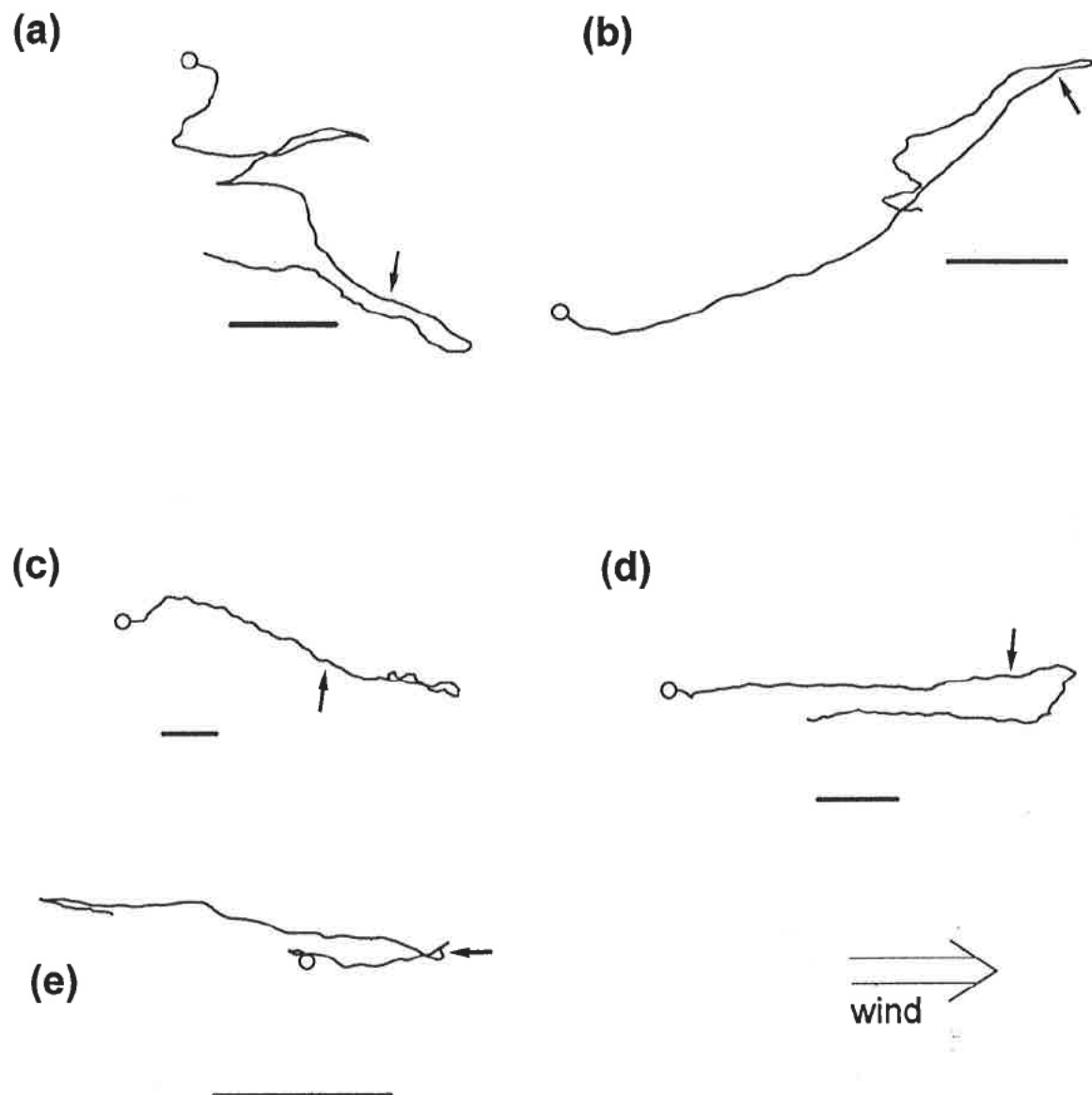


Figure 6. Tracks described by *A. variegatum* adults on the servosphere in response to vapours from a binary mixture of *o*-nitrophenol and methyl salicylate each at a 1 μ g source dose (a,b,c,d) and to vapours from 10 ng source dose of *o*-nitrophenol in the presence of steer hair odour (e; see text). The tracks started (o) with the ticks walking downwind (open arrow) in the control period. The bold arrows on the tracks indicate odour on. Bars beneath each track represent a displacement of 20 cm.

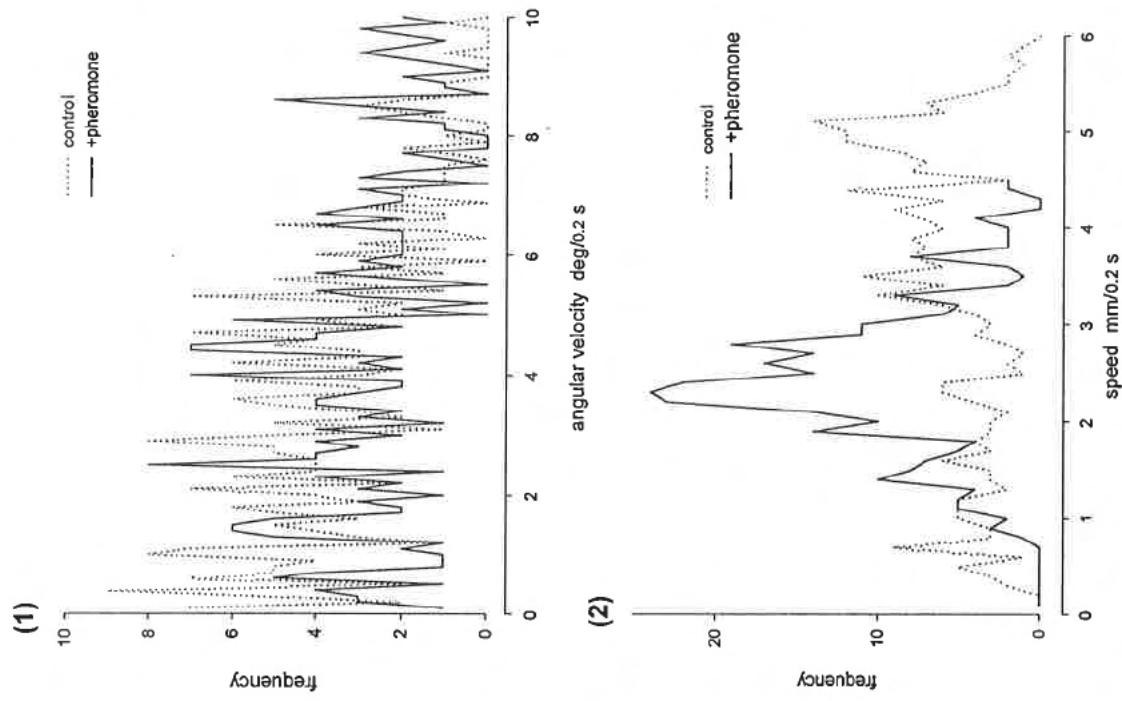


Figure 7b. Frequency distribution of the instantaneous (0.2 s) angular velocity (1) and speed (2) of data in Fig. 7a. Note the reduced speed and increased frequency of large angles in the presence of the pheromone mix. The distributions of the angular velocity and speed were significantly different ($p < 0.001$ Kolmogorov-Smirnov two-sample test) between the control (first 60 s) and the test (+pheromone, second 60 s) periods.

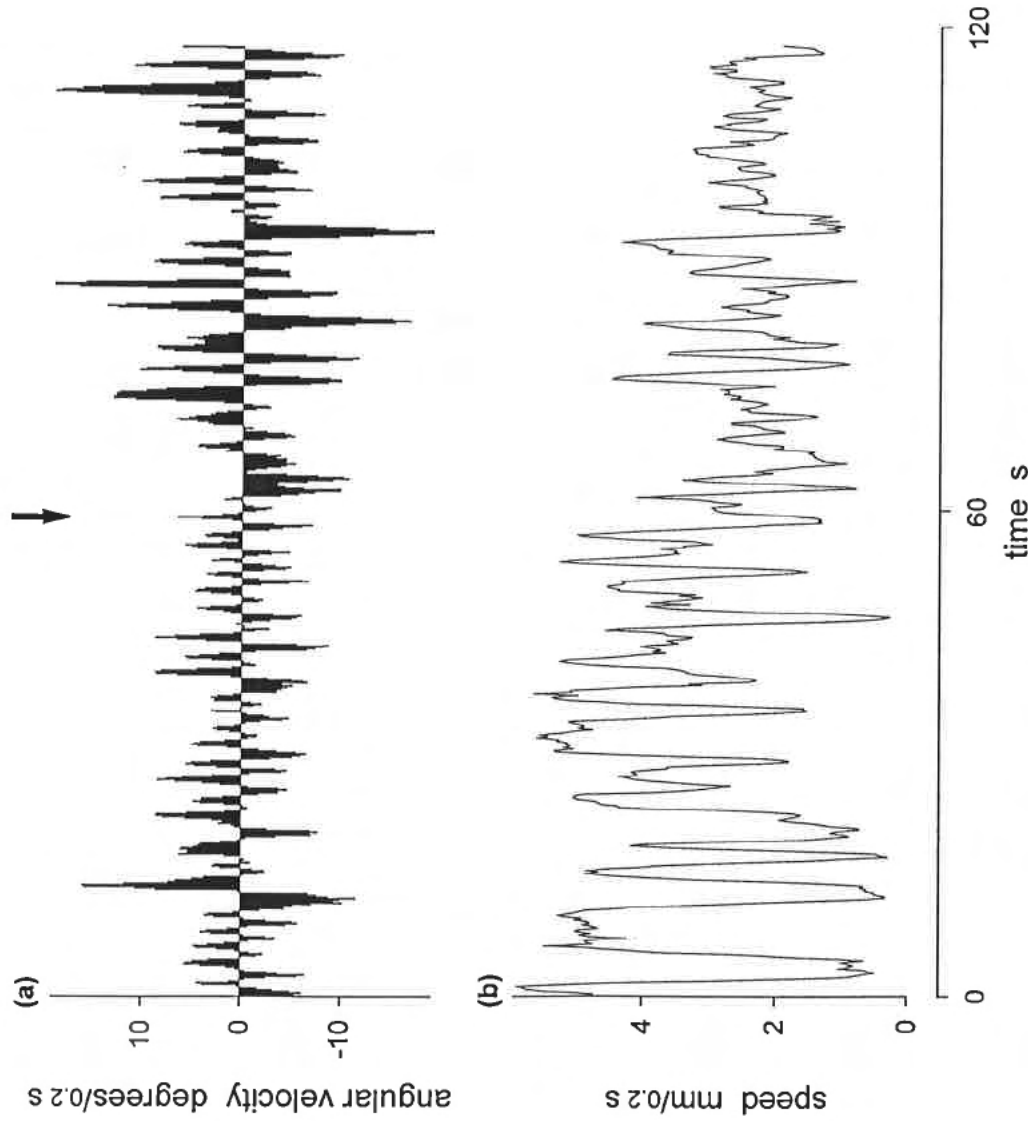


Figure 7a. The effect of vapours from the binary mixture of the aggregation-attachment pheromone components *o*-nitrophenol and methyl salicylate each at 1 μ g source dose on the angular velocity (a) and speed (b) of an *A. variegatum* adult. Running means were applied as detailed in the legend to Fig. 2a. Speed dropped and angular velocity increased significantly upon adding the *o*-nitrophenol and methyl salicylate odours (arrow at 60 s).

Generalities on attraction of A. variegatum to test chemicals on the servosphere

A. variegatum generally responded to the presentation of attractive binary mixtures of ONP and MS, or single components, in the air stream within the first 10 s by a sudden decrease in speed. The time taken to actually turn upwind was variable, but normally happened within the first 30 s of the 1 min test period. A general increase in the six-legged walk and associated reaching in the air with the forelegs was observed in responding ticks. Angular velocity increased and speed decreased (Fig. 6) as the tick responded to an attractant, effects that were independent of the increased upwind walk.

Ticks varied in the manner they approached the source: a small majority maintained an upwind approach from one side, or very rarely, walked straight upwind. Other ticks, however, crossed the axis of the air stream, and while veering downwind often made abrupt turns to re-orientate towards the source. This behaviour never occurred in the controls. The upwind walk to an attractant usually (ca. 80% of ticks) persisted long into the end control, significantly so in half of the experiments with attractants (Fig. 3). In the other cases (ca. 20%), ticks responded to the loss of chemical either by describing small circles, abruptly turning downwind, or both, behaviours that were also absent in controls. If a tick happened to be walking consistently downwind at the end of the test period, it too usually maintained this direction in the end control, turning upwind after loss of the chemical only in a minority of cases (ca. 20%). Ticks could not be induced to respond to presentation of pheromone components in the air when in the resting pose with legs folded beneath the abdomen on the servosphere.

Response to o-nitrophenol in the presence of host odour

Although steer hair odour itself was not attractive ($p < 0.6$, $n = 33$), the addition of ONP to steer hair was attractive on each of three days ($p < 0.0001$, $n = 33$). In contrast, ONP presented alone at this dose attracted on only one of three other days. The nature of the response to ONP in the presence of steer hair odour was also markedly different to that seen for either of the pheromone components and their binary mixtures, in that the ticks did not show any significant change in speed or angular velocity (Fig. 8). Ticks responded to ONP plus steer hair odour with a significantly ($p < 0.05$) greater increase in the upwind walk (median 20.1%, $n = 33$) compared to ticks presented ONP alone (median 3.9%; $n = 47$). The CO₂ concentration in the gas-wash bottle containing steer hair was not sufficient to cause the CO₂ indicator tube to respond.

Discussion

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

The air stream velocity had no discernible effect on the walk of *A. variegatum*. This contrasts with studies by Bell and Kramer (1979) and Heinzel and Böhm (1989) who noted that although cockroaches and

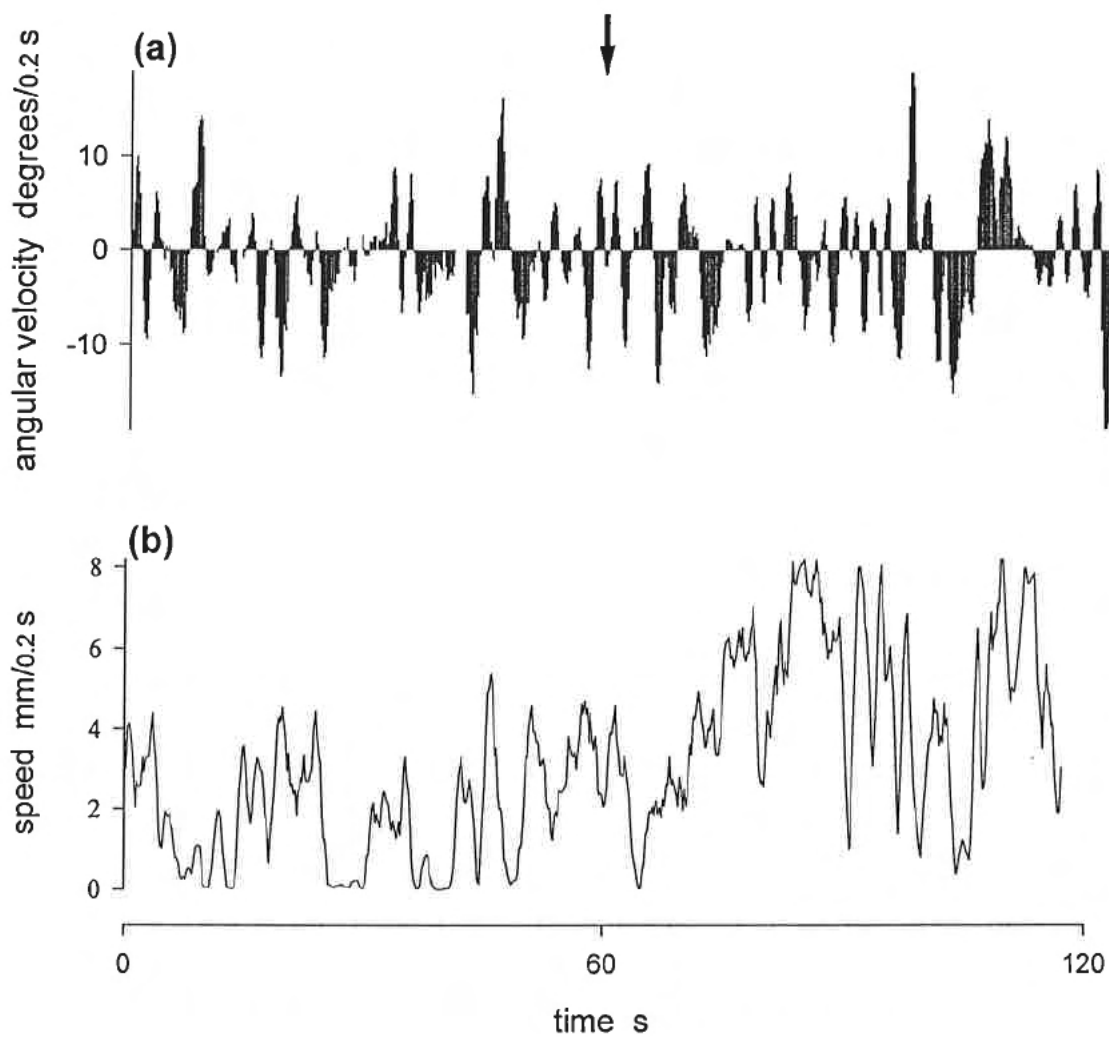


Figure 8. The effect of adding vapours from a 10 ng source dose of *o*-nitrophenol (arrow at 60 s) on angular velocity (a) and speed (b) on a walking *A. variegatum* adult on the servosphere already walking in steer hair odour on the servosphere. Running means were applied as detailed in the legend to Fig. 2a. In contrast to the change in angular velocity and speed induced by the binary pheromone mix (Fig. 7) no such change was observed here although the ONP plus steer hair odour was more attractive than steer hair odour alone.

carrion beetles, respectively, described circles in still air, these were modified to ever decreasing arcs as wind speed increased. For *A. variegatum*, however, wind seemed to act as a simple switch that initiated reaching behaviour, and consequently angular velocity increased and walking speed decreased. This wind induced ortho- and klinokinesis was further enhanced by the presentation of ONP (where attractive), MS, and ONP plus MS. As such it may represent a search strategy that enables the tick to sample the air stream vigorously while still maintaining an upwind course towards the source. An increased angular velocity might also aid the tick to stay in contact with the odour plume. Wolf and Heisenberg (1991) working with tethered *Drosophila* found that when the flies were stimulated with host odour in an open-loop design the increase in angular velocity recorded could explain the animal staying three times longer in the odour field.

A 1:1 binary mixture of ONP and MS was a robust attractant at source doses of less than 1 mg. Based on the known release rates of ONP from feeding adult males (Diehl et al., 1991), the 1 mg source dose released an equivalent amount to the tick on the servosphere as the quantity released from 50 feeding males. As the average population of *A. variegatum* adults on cattle rarely exceeds 200 per host (Pegram et al., 1986), it might be that the presence of one quarter of this population as males only at a predilection site would deter further males from arriving there and account for the lack of progress upwind to 1 mg source doses. High doses of AAP components have been shown to attract in the field when delivered with CO₂: Norval et al. (1991) reported that a mixture of 20 mg ONP and 200 µl MS attracted more than 40% of all ticks released, and Barré et al. (1997) found a synthetic pheromone mix equivalent to the extracts from 100 feeding males to be attractive, and suggested that the dose could be increased. In the open-loop design of experiments done here on the sphere, the tick was required to

respond in a confined system, whereas the concentration will vary widely in the field. Another possible reason for the discrepancy between results obtained in our laboratory study and the field may have been the manner in which ticks were activated. On the servosphere, it was wind that stimulated the tick to keep walking, whereas in field experiments, either breath (Hess and De Castro, 1986) or CO₂ (Norval et al., 1991; Barré et al., 1997) was required.

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

It was unexpected that a naturally occurring 3:1 ratio of ONP to MS would be less attractive than a 1:1 binary mix of both compounds at the same dose. This provides evidence that ratios of attractant components are critical in evoking an upwind response in this tick. Extracts from fed males have provided several candidate compounds that may be integral to the AAP of *A. variegatum* such as nonanoic acid (Schöni et al., 1984; Pavis and Barré, 1993) and benzaldehyde (Lusby et al., 1991). Also, feeding *A. hebraeum* males release several short-chain fatty acids, especially isobutyric acid (Apps et al., 1988). Interestingly, many of these substances are, like ONP, host compounds for which *A. variegatum* has chemoreceptors (Steullet and Guerin, 1994a). Therefore, what constitutes a host odour or an AAP

component may be somewhat arbitrary since some may occur in both the host odour and AAP blends, modulating the response to ONP and MS.

The attraction to ONP in the presence of steer hair odour (the latter unattractive) was not associated with a change in turning angle or speed. This may suggest that ticks do not need to reach extensively when orienting to the source of a more complete blend of what constitutes an adequate attractant. This phenomenon of a straighter upwind track to the source has also been demonstrated for the flight of the European grape berry moth (*Lobesia botrana*) males to calling females as compared to a synthetic mix (Witzgall and Arn, 1990). Moreover, the constancy of the attraction to ONP plus steer hair odour compared to the variability of the response to ONP presented alone is consistent with release of this principal pheromone component of *A. variegatum* in the context of host odours. Olfactory receptor cells in Haller's organ sensilla have been characterised as responding to a wide range of host chemicals such as CO₂, H₂S, mercaptans, NH₃, branched and unbranched short-chain fatty acids, saturated and unsaturated aliphatic aldehydes, lactones, furfural, benzaldehyde and related compounds, and nitrophenols such as ONP (Steullet and Guerin, 1992a; 1992b). Steer hair may contain some or all of these products. Steullet and Guerin (1994a) postulated that components of the AAP, especially ONP, have been selected in the evolution of this pheromone system for the purpose of amplifying specific host chemical cues to which this tick is particularly sensitive (Steullet and Guerin, 1994a). This would suggest a virtuous circle for host seeking by this ectoparasite between host odour and the AAP. Our findings that the ratios of the attractive components are important in the attraction of *A. variegatum* adults, and that the response to the main pheromone component is both enhanced and modified in the presence of host odour are consistent with their hypothesis.

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Chapter 2. Responses of the tropical bont tick, *Amblyomma variegatum* (Fabricius), to vertebrate odours on a servosphere.

(for submission)

Abstract

The behavioural responses of the tropical bont tick *Amblyomma variegatum* were recorded on a servosphere to twenty-one host compounds presented in an air stream. Breath (4%) and sixteen compounds (fourteen volatiles and two gases) were found to attract the tick. Thirteen of the synthetic compounds tested had previously been shown to activate specific olfactory receptors in the olfactory sensilla of this species. Three binary mixtures and two ternary mixtures of host volatiles (i.e., (1) isoamylamine plus isobutyric acid; (2) γ -valerolactone plus E-2-heptenal; (3) nonanal plus isobutyric acid; (4) γ -valerolactone plus E-2-heptenal plus isobutyric acid; (5) isoamylamine plus isobutyric acid plus 1-octen-3-ol) also attracted. No lower concentration limit for behaviour was established, yet it appeared that the gases, CO₂ and NO were near the upper limit for attraction. NO, isoamylamine, acetone and 1-octen-3-ol, for which chemoreceptors had not been identified in *A. variegatum*, were also attractive for the species. The walking behaviour followed a continuum: (A) less attractive single compounds elicited a weak upwind response but no change in walking behaviour; (B) more attractive single compounds and some mixtures were generally more attractive than in (A) and evoked vigorous sampling left and right of the track by ticks approaching the source, a behaviour that continued long after odour off; (C) very attractive volatiles (of 1-octen-3-ol and two binary mixtures, isoamylamine plus isobutyric acid and γ -valerolactone plus E-2-heptenal) also evoked vigorous left and right sampling but also an off-response on removal of the odour; and (D) attraction to breath with a

smoother walk upwind without the left and right sampling and also with an off-response to loss of odour. These off-responses consisted of a vigorous local search, a behaviour that was seldom seen in the presence of vapours of single synthetics other than 1-octen-3-ol.

Introduction

Host finding by ticks is based on several sensory modalities including heat (Lees, 1948), sound (Webb et al., 1977), light (Kaltenrieder et al., 1989) and odour. Host odour has been demonstrated to attract ticks to vertebrates (Lees, 1948; Hafez and Bishara, 1982; Downes, 1984).

Although olfactory stimuli derived from host odour have been studied for blood-sucking arthropods as diverse as triatomines (Taneja and Guerin, 1995, 1997), tsetse flies (Torr et al, 1995) and mosquitoes (Cork and Park, 1996), ticks have been largely neglected, and no attractant based on host odour components has been developed for this group. Compared to many other blood-feeding arthropods, host odour perception by ticks is confined to less than 100 chemoreceptors on each foreleg (Hess and Vlimant, 1986), half of which are contained in a compound sensory organ named after Haller (1881). Steullet and Guerin (1992a; 1994a,b) identified host compounds that stimulate half of the olfactory receptors in Haller's organ of the tropical bont tick, *Amblyomma variegatum* (Fabricius). These chemostimulants were identified by passing host odour components over chemosensilla from a high-resolution gas-chromatography capillary column linked to the single-sensillum electrophysiological recordings. Olfactory cells were identified as responding to a wide range of host chemicals such as CO₂, H₂S, mercaptans, NH₃, branched and unbranched short-chain fatty acids, saturated and unsaturated aliphatic aldehydes, lactones, furfural, benzaldehyde and related compounds, and nitrophenols such as *o*-nitrophenol.

Previously, behavioural studies to host compounds by ticks had been limited to CO₂, a general attractant for haematophagous arthropods, which also attracts several tick species (e.g., Sauer et al., 1974; Adeyeye and Butler, 1991) and which has been employed in field trapping of ticks (Gray, 1985; Barré et al., 1997). This study aimed to investigate the behavioural responses of *A. variegatum* to vapours of host compounds for which Steullet and Guerin (1992a; 1994a,b) found specific olfactory receptors. Ticks were presented with the odours while walking in an air stream on a servosphere apparatus (Kramer, 1976), and the responses of the ticks to the chemostimuli were analysed from the tracks recorded and video analysis of the behaviour.

Materials and Methods

Ticks

Ticks were procured and prepared as described in chapter 1.

Stimuli and stimulus delivery for behavioural recordings

Vapours from individual host volatiles identified and found active by Steullet and Guerin (1994a,b) were presented to walking ticks as outlined in chapter 1. These compounds were: 3-pentanone (Fluka), γ -valerolactone (Fluka), hexanal (Aldrich), heptanal (Aldrich), E-2-heptenal (Aldrich), nonanal (Fluka), benzaldehyde (Merck), 2-hydroxybenzaldehyde (Fluka), butanoic acid (Fluka), isobutyric acid (Merck), pentanoic acid (Fluka), nonanoic acid (Merck), *o*-nitrophenol (Supelco) and 4-methyl-*o*-nitrophenol (Aldrich). Vapours from compounds for which chemoreceptors are not known in this species, i.e., isoamylamine (Sigma), 1-octen-3-ol (racemic mixture, Merck) and squalene (Sigma), were also presented to ticks. All compounds were > 98% pure as indicated by GC. For compounds in the

liquid state at room temperature, 50 μ l of each were dissolved in 2 ml dichloromethane (Merck, analytical grade) henceforth called 25 mg/ml stock solutions. 25 mg/ml solutions of the solids *o*-nitrophenol and 4-methyl-*o*-nitrophenol were also prepared in dichloromethane. Serial dilutions and mixtures of stock solutions were then made up to the desired concentration with the same solvent. Mixtures of these host compounds with solutions of the 1:1 methyl salicylate: *o*-nitrophenol aggregation-attachment pheromone binary mix (chapter 1) were also prepared. Acetone (50 μ l; Merck, residue analysis grade), another volatile for which chemoreceptors are not described in *A. variegatum*, was dissolved in 2 ml of paraffin oil (Merck, spectroscopy grade) to give a 25 mg/ml stock solution. NH₃ vapours were supplied from a 25% (g/v) aqueous solution of NH₄OH. Dilutions of acetone and NH₄OH solutions were made with paraffin oil and distilled water, respectively, and were not mixed with other compounds. For behavioural tests, human breath was provided by an adult male and CO₂ (2% v/v in O₂), methane (CH₄; 2.5% v/v in synthetic air) and nitric oxide (NO; 1 ppm in N₂) were from pressurised gas cylinders.

To test the response of ticks to individual volatiles and their mixtures diluted in dichloromethane, 0.4 ml of a test solution was placed on filter paper (7 cm diameter), left to dry for some seconds until the solvent had evaporated and then placed upright at the bottom a 500 ml gas-wash bottle. After the addition of treated filter paper, gas-wash bottles were left to equilibrate for at least 5 min before presenting the evaporated vapours to the ticks. Treated filter papers for both the control and test gas-wash bottles were replaced after every two tests. The same procedure was followed for the control using the same amount of solvent. Test solutions of acetone (0.1 ml) and NH₄OH (0.4 ml) were dispensed onto filter papers as above but were not left to evaporate before inserting them into the gas-wash bottle.

Separate filter papers were used for water and paraffin oil solutions. Ticks did not show a response to water, paraffin oil or any residual dichloromethane vapour from control gas-wash bottles.

The source doses tested for the volatiles were in the range 10 ng-1 µg, as vapours from these doses of the aggregation-attachment pheromone (AAP) had been shown to reliably attract *A. variegatum* adults on the servosphere (chapter 1). These doses also provided levels of chemostimuli in the air stream that were within the electrophysiological response range of chemoreceptors found active by Steullet and Guerin (1994a,b) to host volatiles.

For the NH₃ vapours which rise, the gas-wash bottle was inverted to ensure a constant flow of NH₃ over a 1 min period. This was quantified by delivering air (150 ml/min) through a gas-wash bottle containing 0.4 ml of an NH₄OH solution to a NH₃ receptor on an antenna of *Triatoma infestans* (Taneja and Guerin, 1997). As the spike discharge rate of the receptors is proportional to the ammonia concentration delivered, it could be shown that NH₃ vapours exiting from the gas-wash bottle in air at 150 ml/min had a stable concentration for at least two minutes. Breath was blown into a 500 ml gas-wash bottle for 30 s, shut, and left to stand upright for at least 20 min before the start of a test. Charcoal-filtered air was then passed through the gas-wash bottle at the rate of 150 ml/min into the main air stream (3.5 l/min). This provided a stable concentration (0.15%) of CO₂ at the apex of the sphere for 1 min as measured by an infrared gas analyser (below). 20 ml of distilled water was introduced into the control gas-wash bottle to preclude a shift in humidity during the test period.

Vapours were dispensed from the gas-wash bottles as in chapter 1. Computer-controlled solenoid valves switched the charcoal-filtered air flow (150 ml•min⁻¹) between control and test gas-wash bottles (or gas cylinder

effluents) for consecutive 1 min control, test and end-control periods, voiding 30% of the volume of the gas-wash bottle in one minute.

Servosphere and recording of behaviour

A servosphere (Kramer, 1976) was used to record the responses of *A. variegatum* adults to volatile chemostimuli. This apparatus functions in such a way as to keep the arthropod at the same position in space, i.e., at the apex of a perspex sphere (50 cm diameter) to which the stimulus delivery tube is directed. The detail of the equipment and procedure followed was after Taneja and Guerin (1995), with some modifications (chapter 1). The CO₂ concentration was measured at the apex of the sphere to within 10 ppm using an IR gas analyser (BINOS1, Leybold-Heraeus, Germany), and the NH₃ concentration delivered to the apex of the sphere was estimated using data from Taneja and Guerin (1997). The concentration of the other gases tested (NO, CH₄) at the sphere apex was estimated using the dilution factor of the gas cylinder effluent into the main air stream. Ticks were observed using two black and white infrared-sensitive video cameras as detailed in chapter 1.

Data analysis

The co-ordinates of the sphere's displacement while compensating for the displacement of the tick were collected by computer, and the vectors obtained were used to reconstruct the tracks described by the tick (Taneja and Guerin, 1995). These tracks were analysed and the upwind responses quantified using the relative time spent walking in an upwind cone as described in chapter 1. The paired differences in the time spent walking in the upwind cone between test and control were closely correlated with the

paired differences in target vector between test and control as in chapter 1 (Spearman correlation coefficient 0.89, $p < 0.0001$, $n > 1200$, two-tailed). Distance based analysis of the angular velocity ($^{\circ}/0.1$ mm) and attraction (the relative displacement in the upwind cone) did not improve the discrimination between test and control (also for chapter 1).

Results

Responses to host compounds presented singly

Out of a total of twenty-one host compounds tested, sixteen (fourteen volatiles and two gases) were found to attract ticks (Table 1). Steullet and Guerin (1992a; 1994a,b) had identified olfactory receptors for thirteen of these attractants (Table 1). 1-Octen-3-ol, γ -valerolactone, 3-pentanone, hexanal, E-2-heptenal, nonanal, benzaldehyde, butanoic acid, isobutyric acid and nonanoic acid all attracted at a source dose of 10 ng (Table 1, Fig.1). Acetone was attractive at a source dose of 250 ng (the only dose tested; Table 1, Fig. 1), and 4-methyl-*o*-nitrophenol and isoamylamine both attracted at source doses of 1 μ g but not at doses of 10 ng. The response to 2-hydroxybenzaldehyde was variable in the source dose range of 10 ng to 1 mg. Contrastingly, CO₂ and NO were only attractive at lower concentrations (57 ppm and 2.2 ppb respectively; Table 1). The responses of ticks to two doses of isobutyric acid (source dose 10 ng and 1 μ g) were not different. Male and female responses to 1-octen-3-ol (source dose 10ng), isobutyric acid (source dose 1 μ g) and to CO₂ (0.07%) did not differ. The five other compounds tested, i.e., squalene, CH₄, NH₃, heptanal and pentanoic acid (the latter three for which chemoreceptors were identified by Steullet and Guerin, 1994a,b) did not provoke any directed or other behavioural response in

Table 1. Upwind responses to naturally occurring volatiles and gases by *A. variegatum* on the Kramer locomotion compensator.

Compound	Attraction *	Source dose of volatile (g), ppb at sphere apex, or % (v/v above ambient) at sphere apex	p < (Wilcoxon's signed rank test)
Host odours for which receptor cells occur in <i>A. variegatum</i> ***			
heptanal	6.0	1 µg	N.S. (n=21)
pentanoic acid	6.9	10 ng	N.S. (n=32)
3-pentanone	14.7	10 ng	0.05 (n=19)
hexanal	14.8	10 ng	0.01 (n=17)
E-2-heptenal	15.7	10 ng	0.05 (n=17)
nonanoic acid	23.2	10 ng	0.05 (n=19)
nonanal	23.4	10 ng	0.01 (n=17)
benzaldehyde	29.8	10 ng	0.001 (n=18)
γ-valerolactone	30.7	10 ng	0.01 (n=19)
Isobutyric acid	32.3	10 ng	0.001 (n=29)
butanoic acid	34.1	10 ng	0 (n=16)
2-hydroxybenzaldehyde **	18.5	1 µg	0.01 (n=17)
4-methyl-o-nitrophenol	19.6	1 µg	0.01 (n=20)
isobutyric acid	20.0	1 µg	0.05 (n=20)
NH ₃	-0.4	0.04 ppb	N.S. (n=12)
NH ₃	5.6	4.2 ppb	N.S. (n=16)
NH ₃	5.2	420 ppb	N.S. (n=10)
CO ₂	18.3	0.006%	0.01 (n=18)
CO ₂	17.5	0.015%	0.05 (n=23)
CO ₂	-15.8	0.035%	N.S. (n=27)
CO ₂	-5.2	0.1%	N.S. (n=10)
CO ₂	-6.0	0.15%	N.S. (n=10)
NO	4.1	2.2 ppb	0.05 (n=21)
NO	11.4	11 ppb	N.S. (n=10)
NO	-2.1	17 ppb	N.S. (n=10)
Other volatiles			
1-octen-3-ol	47.2	10 ng	0.001 (n=21)
isoamylamine **	9.7	1 µg	0.01 (n=18)
acetone	34.6	250 ng	0.001 (n=16)
squalene	0.0	1 µg	N.S. (n=20)
methane	1.0	70 ppm	N.S. (n=13)

* Median of the % increase over the control in the time spent walking upwind, i.e., in a cone 60° either side of wind (0°).

** Chemicals found to be unattractive at a source dose of 10ng; note: the response to 2-hydroxybenzaldehyde was variable.

*** Steullet and Guerin (1992a,1994a,b)

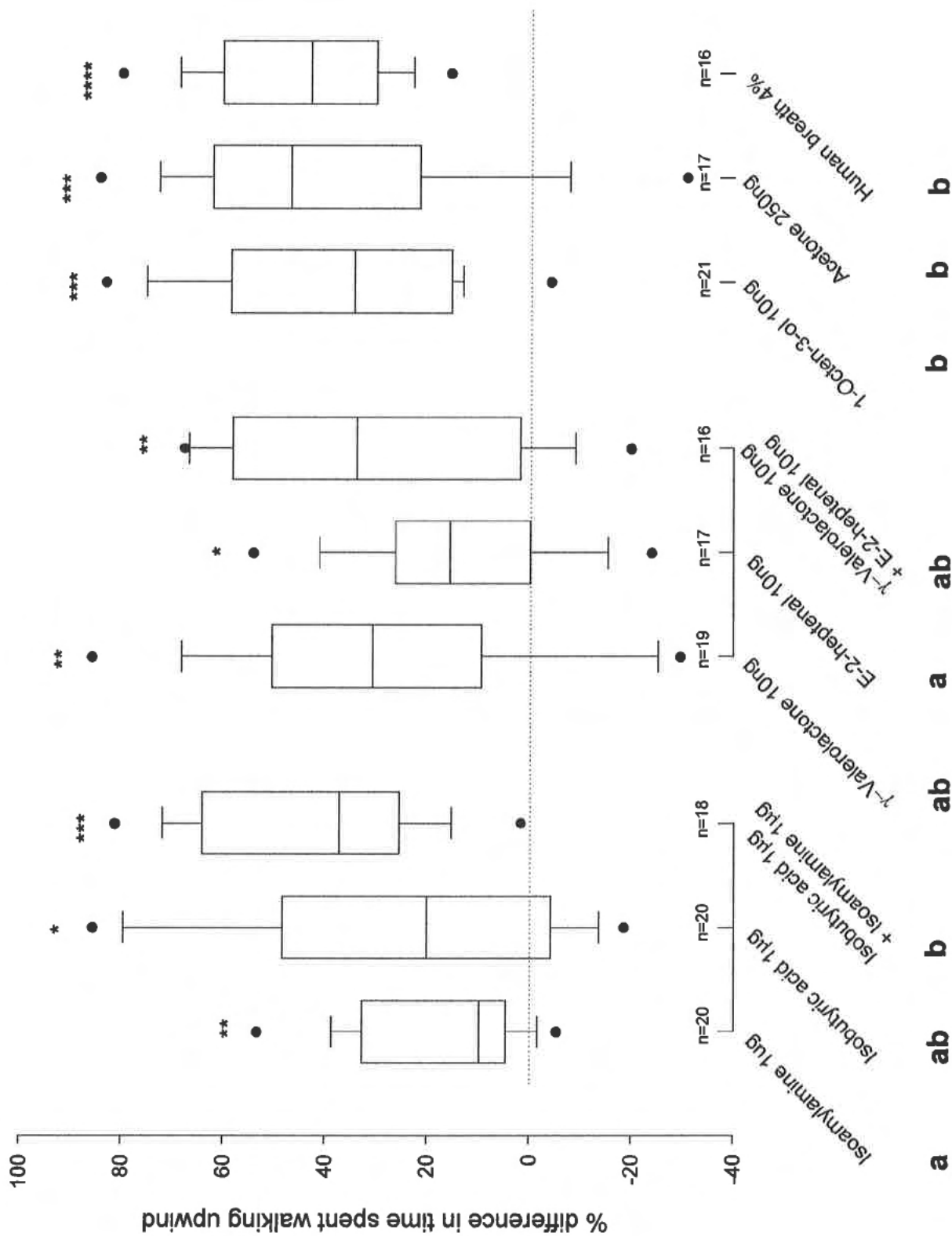


Figure 1. Box plots of the change in time spent walking upwind on the servosphere by *A. variegatum* adults in the presence of vapours from isobutyric acid, isoamylamine, γ -valerolactone, E-2-heptenal, 1-octen-3-ol, acetone and breath on their own or as mixtures in the air stream. Source doses are indicated under box plots. The line within a box marks the median, the lower and upper boundaries of a box indicate the 25th and 75th percentiles, error bars below and above a box indicate the 10th and 90th percentiles, and the 5th and 95th percentiles are shown as circles. Asterisks above a treatment indicate a significantly higher time spent walking upwind in the test or end-control period compared to the control period (* $p < 0.05$; ** $p < 0.01$; Wilcoxon signed rank test). Treatments with different letters are significantly different ($p < 0.05$, Wilcoxon-Mann-Whitney test).

walking *A. variegatum* adults. No attempt was made to establish a lower concentration limit of activity for any compound. Assuming an average molecular weight of 120, and 100 % volatilisation, a 10 ng source dose releases 4×10^9 molecules/ml of air passing over the ticks. However, the lower threshold was far beneath this, as flasks which had contained attractants were frequently active even after several water washes. A ranking of attractants (Table 2) showed that the breath-associated volatiles, 1-octen-3-ol and acetone, were particularly attractive, breath itself proving the strongest attractant (Table 2; Fig. 2).

Ticks generally responded to attractive compounds with a decrease in speed and an increase in angular velocity (Fig. 3), although these effects were not always significant. This change in behaviour, also seen in the response to the AAP binary mix (*o*-nitrophenol and methyl salicylate, at source doses of 1 μ g, chapter 1) can be described as an increased tortuous walk as the tick proceeded upwind towards the source, frequently reaching with the front leg-pair to vigorously sample the air above (chapter 1). Excepting NO, (which induced a significant decrease in speed ($p < 0.00$; 2.2 ppb), neither CO₂ (0.006%) nor weak attractants such as isoamylamine and 4-methyl-*o*-nitrophenol nor the unattractive compounds (CH₄, NH₃, heptanal and pentanoic acid) evoked any ortho- or klinokinetic effects.

Table 2. Percentage change in relative displacement in the upwind cone 60° either side of the wind direction between test and control for host compounds, and mixtures containing host compounds, that evoked an upwind response on the servosphere. The values given in parentheses represent the 25% and 75% percentiles. Significance between the test and control for any given treatment was determined using the Wilcoxon's signed rank test. Treatments were compared by applying the Wilcoxon-Mann-Whitney test to paired differences in the relative displacement in the upwind cone between test and control. Treatments followed by the same letter are not significantly different.

Treatment	Source dose of volatile (g); ppb at sphere apex, or % (v/v above ambient) at sphere apex	Median (interquartile range)	Wilcoxon signed rank test for a treatment p <	n =	Significance of differences between treatments (Wilcoxon-Mann-Whitney test)
NO*		4.1 (0) (35)	0.0312	21	abc
CO ₂ + NO*	57 ppm; 2.2 ppb	8.9 (-1) (26)	0.0229	16	abc
†Isoamylamine*	1 µg	9.7 (5) (28)	0.0012	18	abc
†Isoamylamine* + isobutyric acid + 1-octen-3-ol*	1 µg; 1 µg; 10 ng	10.7 (1) (45)	0.010	20	abi
E-2- heptenal	10 ng	15.7 (2) (24)	0.0312	17	abe
Hexanal	10 ng	14.7 (9) (23)	0.0019	17	abg
3-Pentanone	10 ng	14.7 (-1) (40)	0.0141	19	abi
Nonanal	100 pg	17.7 (6) (38)	0.0084	18	abi
†4-Methyl- <i>o</i> -nitrophenol	1 µg	19.6 (0) (35)	0.0055	20	abi
Nonanoic acid	10 ng	23.2 (-2) (38)	0.0123	19	abi
Methyl salicylate + 2-hydroxybenzaldehyde	10 ng; 10 ng	23.3 (16) (37)	0.0043	18	abi
CO ₂	57 ppm	18.3 (3) (47)	0.0057	18	abj
Benzaldehyde	10 ng	29.8 (9) (43)	0.0007	18	abk
Nonanal	10 ng	23.5 (1) (37)	0.0075	17	abi
Isobutyric acid	1 µg	20.0 (-4) (46)	0.0228	20	am
<i>o</i> -Nitrophenol + methyl salicylate + isobutyric acid	1 µg; 1 µg; 10 ng	23.4 (8) (49)	0.0284	11	am
<i>o</i> -Nitrophenol + methyl salicylate + NO	1 µg; 1 µg; 2.2 ppb	29.7 (18) (43)	0.0262	11	am
γ-Valerolactone	10 ng	30.7 (10) (48)	0.0038	19	am
γ-Valerolactone + E-2- heptenal	10 ng; 10 ng	34.1 (4) (58)	0.0052	16	am
Isobutyric acid	10 ng	32.3 (4) (50)	0.0001	29	defghijklm
Acetone* + CO ₂	250 ng; 57 ppm	33.2 (14) (55)	0.0014	17	defghijklm
<i>o</i> -Nitrophenol + methyl salicylate	1 µg; 1 µg	29.9 (7) (51)	0.00001	37	ighijlm
<i>o</i> -Nitrophenol + methyl salicylate	10 ng; 10 ng	32.9 (10) (50)	0.00001	34	ghim
Isobutyric acid + E-2-heptenal + γ-Valerolactone	10 ng; 10 ng	38.7 (5) (59)	0.0013	17	gim
Butanoic acid	10 ng	34.1 (16) (59)	0.00001	16	lm
<i>o</i> -Nitrophenol + methyl salicylate + 1-octen-3-ol*	10 ng; 10 ng; 10 ng	35.6 (3) (52)	0.0028	20	lm
<i>o</i> -Nitrophenol + 1-octen-3-ol*	10 ng; 10 ng	35.6 (3) (49)	0.0040	20	lm
Acetone*	250 ng	34.6 (16) (57)	0.0004	17	lm
<i>o</i> -Nitrophenol + methyl salicylate + isobutyric acid	1 µg; 1 µg; 1 µg	47.1 (23) (67)	0.0044	11	lm
Isobutyric acid + isoamylamine*	1 µg; 1 µg	37.3 (26) (61)	0.0002	18	ilm
1-Octen-3-ol*	10 ng	47.2 (22) (62)	0.0004	21	klm
Isobutyric acid + nonanal	10 ng; 10 ng	45.4 (30) (67)	0.0009	18	lm
Breath	4%	43.3 (32) (60)	0.00001	16	m

* compounds for which chemoreceptors have not been identified in *A. variegatum*

† compounds which did not attract at a 10 ng source dose.

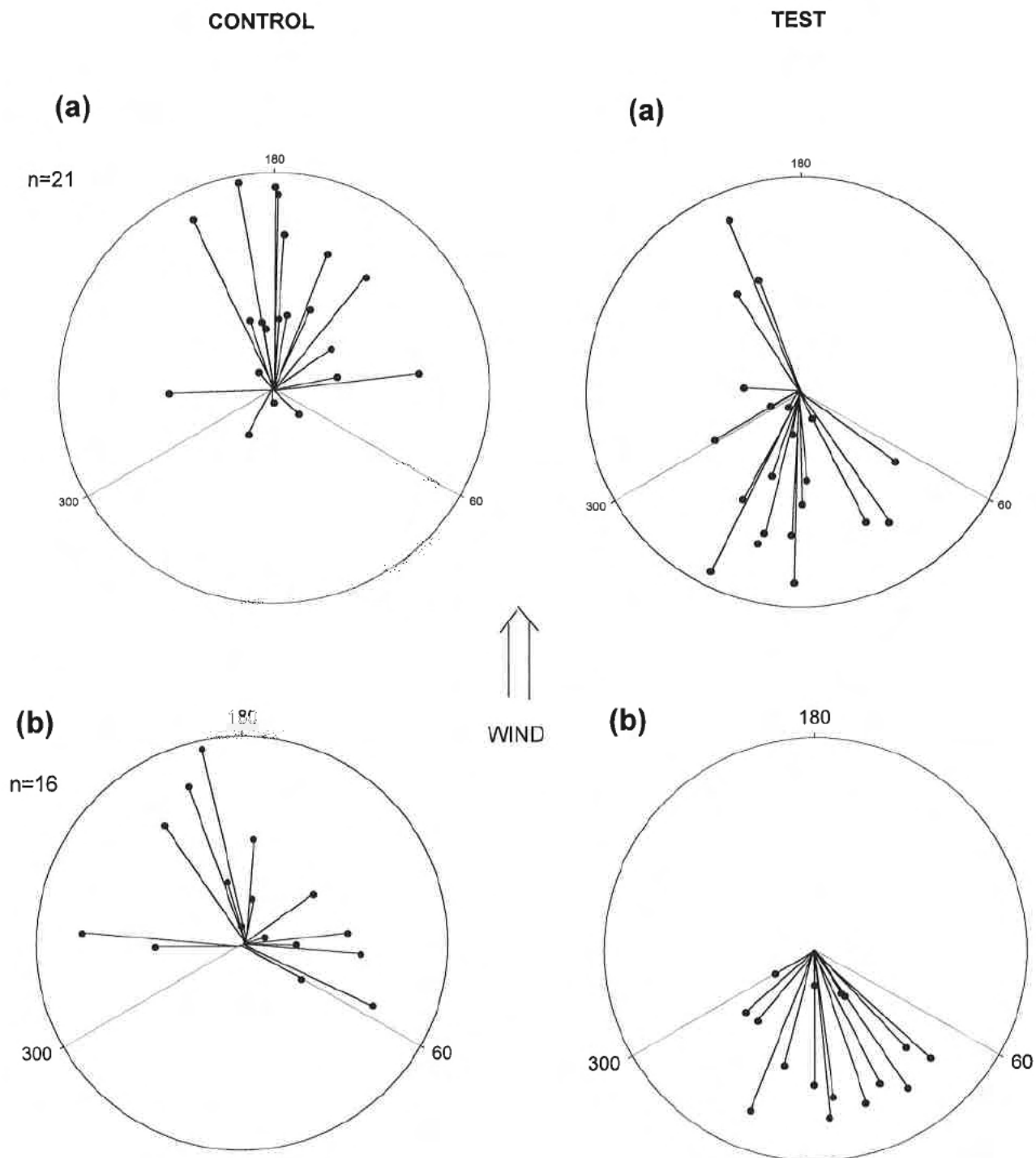


Figure 2. Polar plot of the distribution of the mean direction (ϕ) of the walk of individual ticks on the servosphere for a 1 min control period followed by a 1 min test period where the same ticks were presented with **(a)** vapours of 1-octen-3-ol (source dose 10 ng) and **(b)** human breath (diluted 24 times at the apex of the sphere) in separate experiments. The nearer the vector weighting (r) to the perimeter the straighter the walk in that direction (circular statistics, after Batschelet, 1981).

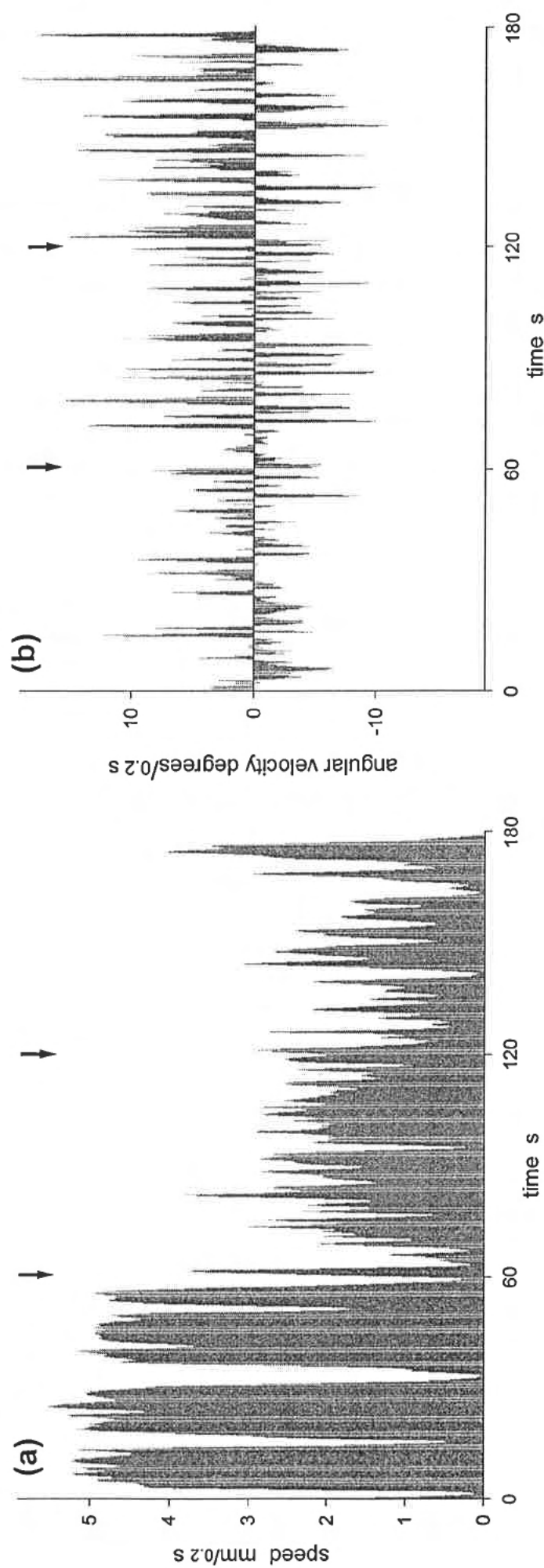


Figure 3. The effect of vapours from a binary mixture of isoamylamine and isobutyric acid each at a 1 μ g source dose on the angular velocity (a) and speed (b) of an *A. variegatum* adult. A running mean over 5 vectors was applied once to speed and twice to angular velocity for graphical representation. Speed dropped and angular velocity increased significantly upon adding the isobutyric acid and isoamylamine odours (arrow at 60 s) and continued after odour off (arrow at 120 s).

Responses to synthetic mixtures of host compounds

The following binary mixtures (Table 3) were attractive: NO plus CO₂ (2.2 ppb and 57 ppm respectively); acetone plus CO₂ (delivered in a ratio equivalent to that present in human breath of 250 ng source dose and 57 ppm respectively); γ -valerolactone plus E-2-heptenal (source dose, 10 ng each; Fig. 1); isobutyric acid plus nonanal (source dose 10 ng of each; Fig. 1), and isobutyric acid plus isoamylamine (source dose 1 μ g of each; Fig. 1). One ternary mixture, γ -valerolactone plus E-2-heptenal plus isobutyric acid (source dose 10 ng each) also elicited a strong upwind response (Fig. 1, Table 3). By contrast, the addition of 1-octen-3-ol (source dose 10 ng) to the isoamylamine plus isobutyric acid mixture significantly reduced the attraction (Table 2) and the admixture of acetone to isoamylamine plus isobutyric acid mixture was not attractive: the ticks, although responding to the initial presentation of the vapours in both these experiments with a sudden reduction in speed, did not maintain an upwind walk.

Ticks responded to mixtures of the volatiles presented in the same manner as they responded to most single compounds (above), i.e., with a reduction in speed and an increase in angular velocity (Fig. 3). Although no mixture was significantly more attractive than its most active component, mixtures of volatiles are prominent in the ranking of attractants (Table 2) and 'off-responses' were induced on withdrawal of two of the binary mixtures (below).

The response to CO₂ and breath

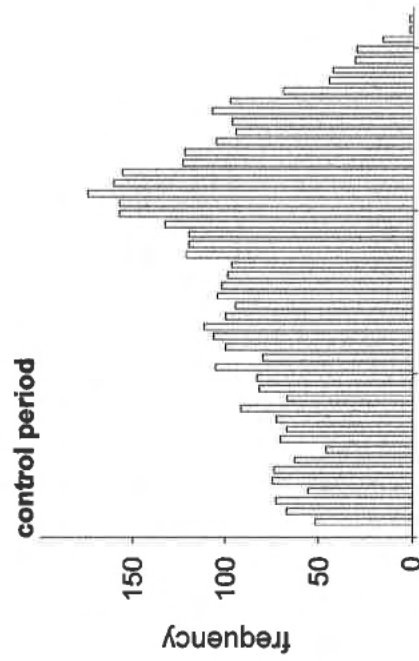
More than half of the ticks tested showed a unique response to human breath (0.15% CO₂ at the sphere apex; Fig. 4). To breath, the ticks

Table 3. Upwind responses of *A. variegatum* to mixtures of host odours and admixtures of host odours to components of the aggregation-attachment pheromone on the Kramer locomotion compensator.

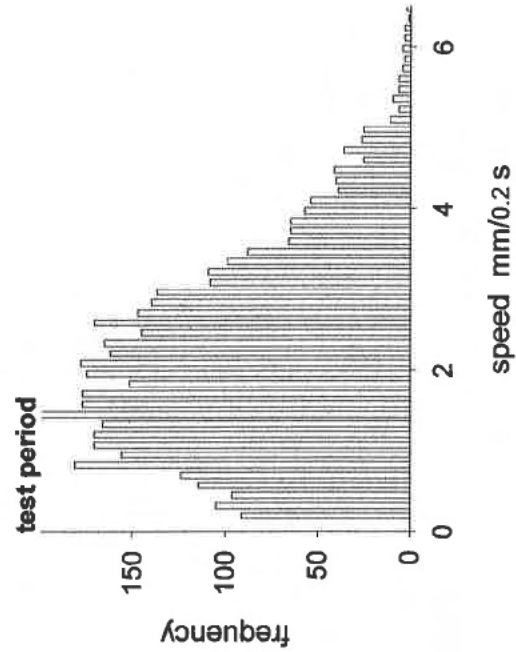
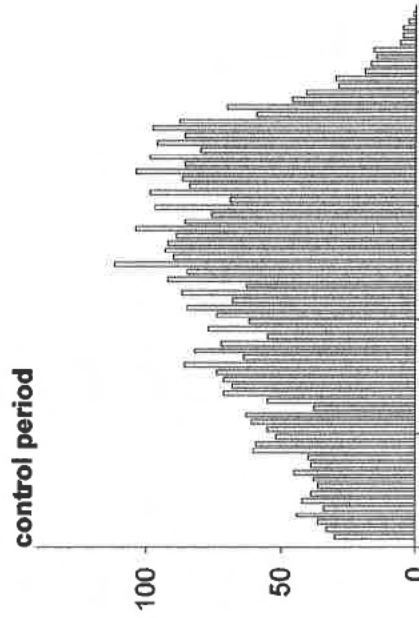
Treatment	Attraction *	Source dose of volatile (g); ppb at sphere apex, or % (v/v above ambient) at sphere apex	p < (Wilcoxon's signed rank test)
Mixtures of host odours			
γ -Valerolactone + E-2-heptenal	34.1	10 ng each	0.01 (n=16)
Isobutyric acid + nonanal	45.4	10 ng each	0.01 (n=17)
Isobutyric acid + isoamylamine	37.3	1 μ g each	0.001 (n=18)
γ -Valerolactone + E-2-heptenal + isobutyric acid	38.7	10 ng each	0.01 (n=17)
Isobutyric acid + isoamylamine + acetone	5.7	1 μ g, each: 250 ng	N.S. (n=17)
Isobutyric acid + isoamylamine + 1-octen-3-ol	10.7	1 μ g, each: 10 ng	0.010 (n=20)
CO ₂ + NO	8.9	0.006% + 2.2 ppb	0.05 (n=16)
CO ₂ + NO	-24.6	0.1% + 2.2 ppb	N.S. (n=10)
CO ₂ + acetone	33.2	0.006% + 250 ng	0.01 (n=17)
Aggregation-Attachment pheromone components + host odours			
<i>o</i> -Nitrophenol+methyl salicylate	29.9	1 μ g each	0.0001 (n=37)
<i>o</i> -Nitrophenol+methyl salicylate + 1-octen-3-ol	34.1	10 ng each	0.0040 (n=20)
<i>o</i> -Nitrophenol + 1-octen-3-ol	35.6	10 ng each	0.0044 (n=20)
Methyl salicylate + 2-hydroxybenzaldehyde	23.3	10 ng each	0.0001 (n=18)
<i>o</i> -Nitrophenol+methyl salicylate + isobutyric acid	23.4	1 μ g, 10 ng	0.03 (n=11)
<i>o</i> -Nitrophenol+methyl salicylate + isobutyric acid	47.1	1 μ g each	0.0001 (n=11)
<i>o</i> -Nitrophenol+methyl salicylate + isobutyric acid + NO	29.7	1 μ g +2.2 ppb	0.03 (n=11)
Methyl salicylate + nonanal	-2.4	10 ng each	N.S. (n=19)
<i>o</i> -Nitrophenol + methyl salicylate + CO ₂	-0.4	1 μ g each +0.006%	N.S. (n=10)

* Median of the % increase over the control in the time spent walking upwind, i.e., in a cone 60° either side of wind (0°).

isobutyric acid + isoamylamine



breath



test period

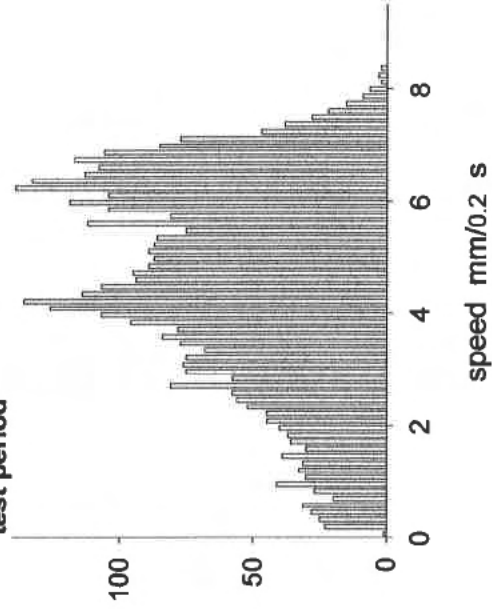


Figure 4. A frequency plot of the instantaneous (0.2 s) vector lengths of *A. variegatum* adults exposed in one experiment to vapours from isobutyric acid plus isoamylamine (source dose 1 μ g each) and in another experiment, to human breath diluted 24 times ($n = 17$ ticks in each experiment). Note the general reduction in speed from the control period in response to delivery of vapours of isobutyric acid and isoamylamine in the test period. This contrasts significantly ($p < 0.025$;

Wilcoxon-Mann-Whitney test) with a lack of such a reduction relative to the control when the ticks were exposed to breath, even though both treatments were equally attractive (Table 2).

did not reach while running upwind or lift the body in the air, but orientated upwind by shifting the body axis left and right of the path at up to 1 half turn every two seconds ($< 30^\circ$ per half-turn). The regular left and right shifts could not be described by the vectors collected by the servosphere as the reflector was positioned on the rotational axis of the swivel executed by the tick. This “swivelling” type of locomotion was also seen occasionally ($< 10\%$) in the presence of high ($> 0.1\%$) concentrations of CO_2 .

CO_2 did not evoke a change in walking behaviour in terms of either angular velocity or speed at the attractive concentration (57 ppm) tested. However, *A. variegatum* adults were seen to pause significantly more (for ca. 2 s of the 60 s test period) in the presence of either CO_2 (57 ppm) or breath (4%), holding their legs vertically against the air stream. Breath and CO_2 were the only treatments to elicit this behaviour, which was more pronounced in the presence of higher concentrations of CO_2 (0.1%). At the level of 0.15% (the amount of CO_2 present in diluted breath), pausing accounted for some one third of the one minute test period, indicating that *A. variegatum*'s pausing response to CO_2 was modulated by other chemicals present in breath. Unlike the other chemicals and mixtures tested, both CO_2 at > 350 ppm and breath caused over a 50% increase in speed for about one-third of the animals. Although this change in speed was not always significant, it was different ($p < 0.05$; Wilcoxon-Mann-Whitney test) to that evoked by two thirds of the other attractants tested (Fig. 4). The bimodal distribution clearly visible in the frequency plot of instantaneous speed in the presence of breath (Fig. 4) is probably related to the slight shifts to a lower speed at the tips of the sinusoidal waves described in the walks. As breath did not evoke a reduction in speed (Fig. 3) or the vigorous foreleg sampling associated with a higher angular velocity, it may be that this host odour is the more complete.

Admixture of host odours to constituents of the aggregation- attachment pheromone components

Ticks were not attracted to methyl salicylate delivered in the presence of nonanal (source dose, 10 ng each; Table 3), whereas admixture of NO (2.2 ppb), 1-octen-3-ol (source dose, 10ng) or isobutyric acid (source doses of 10 ng and 1 µg) to the AAP mixture (methyl salicylate plus *o*-nitrophenol; source dose of 1 µg each, chapter 1) did not influence the attraction of ticks. However, the addition of CO₂ (57 ppm) to the binary pheromone mixture (above) destroyed attraction (Table 3), as the ticks did not maintain an upwind walk on perceiving the mix. However this gas did not diminish the attraction to acetone (Table 3) when presented in ratios approximating to what occur in breath, a potent attractant on its own (Table 2 and Fig. 1). This suggests a specificity on the part of *A. variegatum* for the dose or ratio of the mixture of attractants.

Off-responses

The walk of *A. variegatum* adults was more upwind in the period following the presentation of an attractant as compared to the initial control period significantly so for two thirds of the treatments. In addition, some treatments provoked an 'off-response' at the end of the test period. Here the ticks reacted to the loss of the attractant either by describing small circles, abruptly turning downwind, or both. These behaviours were never seen in controls. In four treatments the percentage of off-responses was over 60%, i.e., for 1-octen-3-ol, breath, and for the binary mixtures E-2-heptenal plus γ-valerolactone, and isoamylamine plus isobutyric acid (Fig. 5). The percentage of off-responses for both the binary mixtures was greater than the sum of the percentage off-responses shown to their individual components

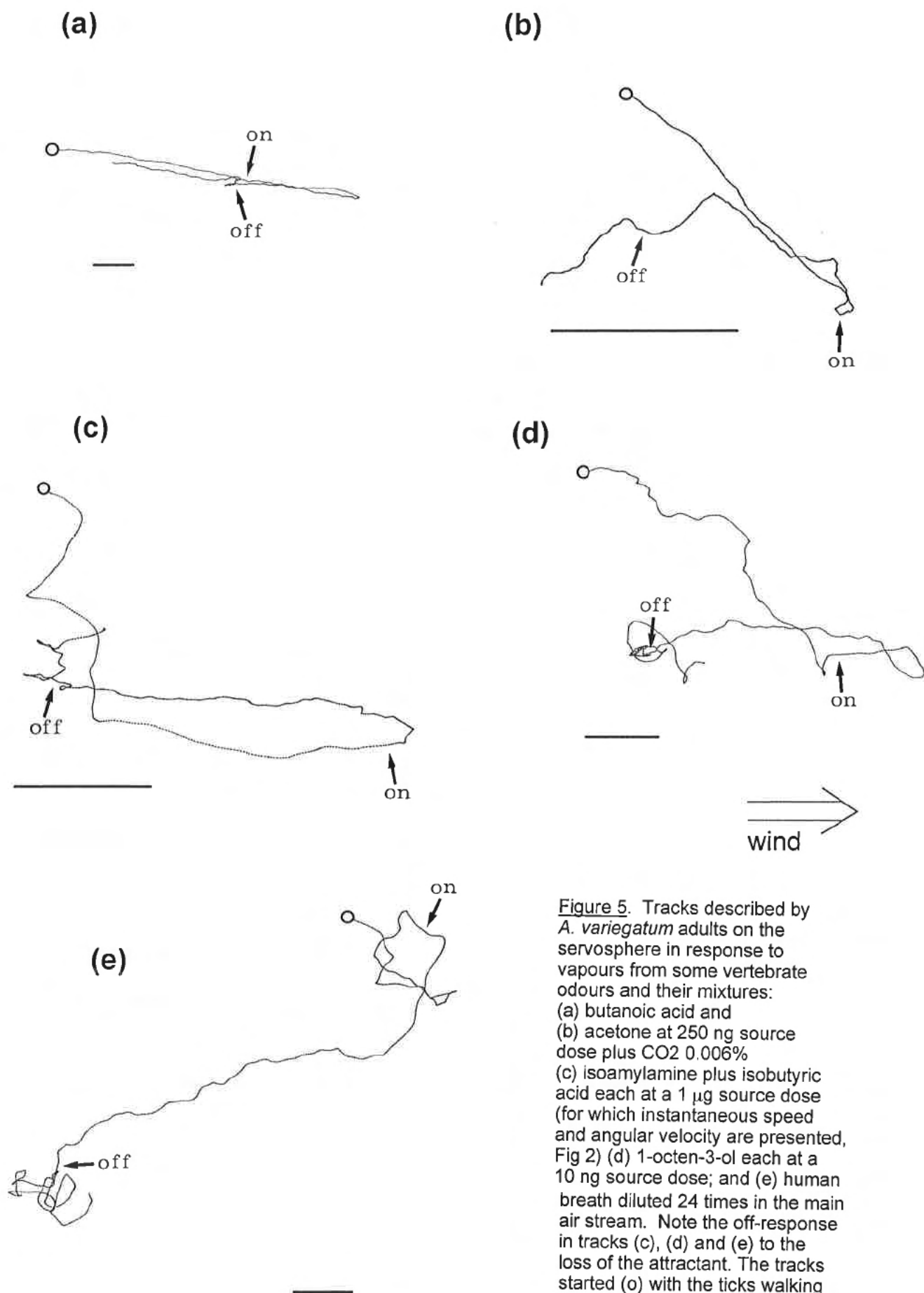


Figure 5. Tracks described by *A. variegatum* adults on the servosphere in response to vapours from some vertebrate odours and their mixtures: (a) butanoic acid and acetone at 250 ng source dose plus CO₂ 0.006% (c) isoamylamine plus isobutyric acid each at a 1 µg source dose (for which instantaneous speed and angular velocity are presented, Fig 2) (d) 1-octen-3-ol each at a 10 ng source dose; and (e) human breath diluted 24 times in the main air stream. Note the off-response in tracks (c), (d) and (e) to the loss of the attractant. The tracks started (o) with the ticks walking downwind (open arrow) in the control period. The bold arrows on the tracks indicate odour on and odour off. Bars beneath each track represent a displacement of 20 cm.

where it was always less than 20%. These four treatments were also amongst the most attractive (Table 2).

Discussion

Sixteen host compounds, three binary mixtures, two ternary mixtures and human breath attract walking *A. variegatum* adults on the servosphere by odour-mediated anaemotaxis. Thirteen of these compounds were identified by Steullet and Guerin (1992a, 1994a,b) as eliciting responses in olfactory receptors in the Haller's organ sensilla of *A. variegatum*. The success of these chemicals in attracting *A. variegatum* on the servosphere proves that these ticks do, in fact, respond to selected volatiles, mostly present at low concentrations, amongst the welter of compounds which make up host odour (Steullet, 1993). Three other attractants were identified. Two of these are acetone and 1-octen-3-ol, well known as attractants for blood-feeding arthropods. The other, isoamylamine, has not previously been shown to attract arthropods.

The off-responses executed by *A. variegatum* to just four treatments which are among the most attractive, suggest a hierarchy in efficacy of attractants with complete host odours proving to be the strongest. No ortho- and klinokinetic responses are evoked by weak attractants such as isoamylamine and 4-methyl-*o*-nitrophenol. More attractive single volatiles elicit a vigorous left and right sampling of the air on either side of the track as the tick proceeds upwind. Breath, however, elicits an energetically efficient upwind displacement during which the tick shifts the body axis minimally from the chosen direction. During this walk, the ticks maintain their body parallel to the walking surface, i.e., minimal reaching in the air. A more complete attractant causing an efficient walk upwind has

also been found with the AAP component *o*-nitrophenol in the presence of steer hair odour (chapter 1). Increase in tortuosity of the upwind path evoked by a single compound has been reported for tsetse flies (Paynter and Brady, 1993) stable flies (Schofield and Brady, 1997) in the presence of the attractive vapours of either 1-octen-3-ol or acetone, whereas straighter upwind paths have been detailed for the upwind flight of males of the grapevine moth, *Lobesia botrana*, to calling females in comparison with a synthetic mixture of the female's pheromone components (Witzgall and Arn, 1990).

Breath and single host volatiles differ in yet another respect. For single attractive compounds (excepting 1-octen-3-ol) the animals usually continue to reach and quest after the chemical has been removed, but upon the withdrawal of breath or 1-octen-3-ol, ticks undertake a 'local search', presumably in an effort to re-contact the lost odour. Such an off-response recalls the classic cross-wind casting response of gypsy moths (*Lymantria dispar*) to loss of pheromone in a wind tunnel (David and Kennedy, 1987). *A. variegatum* adults are also sensitive to the loss of attractive vapours from two binary mixtures (γ -valerolactone plus E-2-heptenal, and isobutyric acid plus isoamylamine), where the sum of the percentages of the off-response to their individual components is less than that observed to either binary mix. This is clear evidence that it is the blend of odours, as signals integrated centrally from the olfactory receptors, that evokes the off-response. Such an off-response disappears upon admixture of 1-octen-3-ol to the isobutyric acid plus isoamylamine mixture and upon admixture of isobutyric acid to the second of these mixtures (γ -valerolactone plus E-2-heptenal). Although this latter ternary mixture is highly attractive, the absence of off-responses to loss of the attractant underscores the high specificity of the blend of individual constituents required, and the probability of concocting a sub-optimal ratio with three compounds is higher than with two. This point is

reinforced by the significant reduction in attraction upon admixture of an extra compound to four attractants (nonanal to methyl salicylate; 1-octen-3-ol to isoamylamine plus isobutyric acid; acetone to isoamylamine plus isobutyric acid; CO₂ to ONP plus MS). Ticks have therefore, evidently long adapted to respond to the unique mixture that constitutes breath.

If the off-responses reflect sensory coding of blends and is thus intrinsically useful to the animal, the question can be asked the other way: what purpose does it serve to navigate to the source of a single compound or a sub-optimal mixture? A possible explanation is that the ticks are walking in clean air in this experimental design where it is the wind, not odour, that provokes the animal to keep walking (chapter 1). In this design, once activated, the ticks may navigate to the source of a single compound and sample vigorously in an effort to contact a more complete stimulus.

CO₂ was not an attractant at levels greater than 100 ppm above ambient, which is somewhat surprising in view of the success achieved by blocks of dry ice in trapping adults of this and other tick species in the field (Barré et al., 1997; Gray, 1985). Similarly, Steullet and Guerin (1992a) reported that a 0.1% concentration of CO₂ evoked an upwind response in 80% of *A. variegatum* adults tested in a wind tunnel. However, in the experimental design of the servosphere, ticks do not have the freedom to orientate to another concentration other than what is delivered (open-loop design), whereas in the field and wind-tunnel, CO₂, or any other attractant, is present at varying levels in the air through which the tick passes as it draws nearer the source (closed-loop design). Furthermore, it may be that while the ticks are sensitive to lower levels of chemicals delivered in the experimental design used here, they may simultaneously be intolerant of higher levels. What serves as an overdose of the mixture of the AAP on the sphere is attractive to ticks in the field (chapter 1). Perritt et al. (1993) did

find that a concentration of 9 ppm CO₂ above ambient was sufficient to induce questing behaviour in *Dermacentor variabilis* and *A. americanum*, and Steullet and Guerin (1992a) have demonstrated the existence of a CO₂ receptor in Haller's organ of *A. variegatum* that is sensitive to shifts in such levels of CO₂ around ambient. As an activator of adult ticks (Barré et al., 1997), CO₂ may be a primordial attractant, in whose presence other chemicals exert their effect. Ticks may thus respond to this chemical using chemotaxis as the gas diffuses to the litter zone where ticks shelter. The activating effect of H₂S (Steullet and Guerin, 1992b) suggests that this chemical too may excite ticks in a similar manner.

1-Octen-3-ol was found to be a strong attractant and the only single host volatile to evoke an off-response, indicating that this chemical may be particularly important in mediating an approach to a host based on olfactory cues. 1-Octen-3-ol has been widely employed in traps for mosquitoes (Kline et al. 1991), tsetse flies (Hall, 1990) and biting midges (Blackwell et al., 1996). It is possible that this chemical may have contributed to the off-response elicited by breath of which it is a constituent. However, breath is a complex host emanation containing more than 100 volatile compounds (Krotoszynski et al., 1977) as well as NO, acetone, CO₂ and NH₃, all of which may be implicated in the unique behavioural responses of *A. variegatum* ticks to this host odour. Also, only breath from humans (which are not a host for *A. variegatum* adults) was used in this study, and it may be that the response to bovid breath might have been greater. The concentration of 1-octen-3-ol in human breath is not known, but the levels of the synthetic compound tested here were equivalent to about one twentieth of bovid breath (Vale and Hall, 1985), and within the range of concentrations found attractive to the tsetse fly *Glossina morsitans morsitans* (Paynter and Brady, 1993) and the stable fly *Stomoxys calcitrans* (Schofield and Brady, 1997) in wind-tunnel tests. This is the first report of

1-octen-3-ol being attractive to ticks. Norval et al. (1987) found no response by *A. hebraeum* in the field to an open vial containing acetone or another containing 1-octen-3-ol. However, in a later study, this group showed that the simultaneous presentation of CO₂ (an activator and attractant of *Amblyomma* spp., Barré et al., 1997) was a prerequisite to obtaining catches to known attractants such as the AAP components *o*-nitrophenol and methyl salicylate (Norval et al., 1992). Adult *A. variegatum* are ordinarily inactive in the field (Barré et al., 1997). On the sphere, it is wind that stimulates the ticks to keep walking once activated.

Three further compounds, NO, isoamylamine and acetone, for which no specific chemoreceptors have been identified in ticks, were attractive. NO is present in human breath but also produced by the chemical reduction of sweat nitrite by surface bacteria (Weller et al., 1996). It was only weakly attractive, perhaps because the dose delivered at 2 ppb, (equivalent to the concentration in human breath, Leone et al., 1994) was too near the upper limit for attraction. As a second messenger within cells and a neurotransmitter, it can easily diffuse across cell membranes, so this compound might not need a specific receptor within olfactory sensilla to exert its effect. Isoamylamine, a suggested pheromone component of rodents present in the urine of male mice (Nishimura et al., 1989), has not previously been reported as an attractant for haematophagous arthropods. Acetone is a well known attractant for haematophagous insects employed by tsetse (Torr et al., 1995) and stable flies (Schofield and Brady, 1997) and attracted *A. variegatum* at a level ca. 1% that present in human breath, and also in the presence of CO₂.

The other host compounds found attractive here for *A. variegatum* belong to fatty acid, ketone, aldehyde and aromatic functional classes. All attracted at source doses between 10 ng to 1 µg when presented individually. The fatty acids, nonanoic acid, butanoic acid and isobutyric acid were

attractive, and this class of compounds has long been suspected as chemicals that might play a large part in imparting individual host odour specificity. For instance, the great variety of fatty acids (>200) on skin may contribute directly to an individual odour profile, but may also do so indirectly by providing an environment favourable to specific microflora (Nicolaidis, 1974) which themselves contribute to the fatty acid profile (Kloos et al., 1975; Korting et al., 1988) and release several other compounds such as NO (Weller et al., 1996). Similarly, ruminants expire fatty acids during eructation that vary over time (Funk et al., 1987). Relative to the few olfactory receptors *A. variegatum* possesses, it can monitor a wide range of fatty acids (Steullet and Guerin, 1994a,b), so these ticks are well equipped to monitor such differences in odour quality. Other compounds can contribute to host specificity. The output of CO₂ is closely correlated with body mass (Linstedt and Thomas, 1994), and the level of ketone bodies in breath is a reflection of the fed state with acetone alone increasing in concentration by up to fifteen times in the starved state (Skrupskii, 1995).

Three of the attractants, nonanoic acid, isobutyric acid and benzaldehyde, are also identified as compounds implicated in the AAP from solvent extraction of whole ticks and associated with volatiles present over feeding males of both *A. variegatum* and *A. hebraeum*. Nonanoic acid was found to attract *A. variegatum* in this study. In the investigation by Schöni et al. (1994), nonanoic acid was not attractive although compounds were tested in still air, thus excluding anaemotaxis as an orientation mechanism. Isobutyric acid and benzaldehyde are both released by feeding *A. hebraeum* males along with *o*-nitrophenol, and are the predominant components for the AAP for that species (Apps et al., 1988). Therefore, it appears that both *A. hebraeum* and *A. variegatum* secrete products which are present in host odour, and that these products are also attractive on their own (Norval et al., 1991). It is thus likely that ticks do indeed secrete attractive host volatiles to

attract conspecifics, as proposed by Steullet and Guerin for *o*-nitrophenol (Steullet and Guerin, 1994b). Such amplification of a specific host volatile would allow the ticks to mark a host as suitable for infestation (Norval et al., 1989) as males only secrete the volatiles from the dermal glands type II after having successfully attached to the host for three days (Diehl et al., 1991). Of note is the attraction of *A. variegatum* to 4-methyl-*o*-nitrophenol described here, a compound known to activate receptors sensitive to *o*-nitrophenol in *A. variegatum* (Steullet and Guerin, 1994b). These authors have suggested that its presence in bovid odour at a level greater than 100 times that of *o*-nitrophenol may make it an important compound for males of *A. variegatum* who are the pioneering colonisers of vertebrate hosts.

Three other compounds for which Steullet and Guerin (1994a,b) found receptors were not attractive: NH₃, pentanoic acid and heptanal, suggesting that these chemicals do not elicit odour-mediated anaemotaxis in *A. variegatum* when presented alone. Pentanoic acid, a sweat component, causes an electrophysiological response on the antennae of mosquitoes and the screwworm (Cork, 1994; Cork and Park, 1996), but no behavioural response by *A. variegatum* was found here. NH₃, a constituent of vertebrate breath and sweat, attracts flour mites (Levinson et al., 1991), evokes an upwind walk in triatomines (Taneja and Guerin, 1997) and is an attractant for tabanids (Hribar et al., 1992), but knowledge of its behavioural role in ticks is lacking. Haggart and Davis (1980) reported that sessile *Rhipicephalus sanguineus* adults were induced to extend their forelegs on top of a stalk when exposed to NH₃ in an air stream, but it is not clear if this activating effect would have been visible on the servosphere apparatus as the ticks were already stimulated by the presence of wind (chapter 1). Squalene was not found to influence the behaviour of *A. variegatum* in any way on the sphere, although Yoder et al. (1988) reported strong attraction to this compound for *Dermacentor variabilis* and *Amblyomma americanum*.

However, Yoder et al. (loc. cit.) and Schöni et al, (1984) tested compounds in the absence of wind making comparisons with the present study difficult.

Ticks have a relatively simple olfactory apparatus with a limited number of receptors, compared to *Stomoxys* which possess over 30,000 receptors on the antenna (McIver, 1987). Nonetheless, pro- and metastriate tick species share a considerable homology in the structure, number and morphology of their olfactory sensilla and have almost the same number of chemoreceptor cells (Hess and Vlimant, 1986). Recordings from homologous sensillae in different species suggest that the receptor cells they house may themselves be homologous in their specificity. This has been proven for a 2,6-dichlorophenol receptor in the d I and d II sensilla on the anterior knoll and pit, respectively, for the metastriate species *A. variegatum*, *Rhipicephalus appendiculatus* (Waladde, 1982) and *Boophilus microplus* (De Bruyne and Guerin 1994) and in the d I sensillum of the anterior knoll of *Ixodes ricinus* (Thonney, 1987). A methyl salicylate receptor also exists in the capsule of Haller's organ in both *A. variegatum* (Schöni, 1987) and *I. ricinus* (P.M. Guerin, pers. comm.). This lends support to the argument that the chemicals identified here as attractants on the servosphere for *A. variegatum* may have a general importance for ticks. To date, only half of the olfactory receptors of Haller's organ have been characterised, and less again of the ca. 50 chemoreceptor cells on the tarsus outside Haller's organ. Furthermore, the stimulants that have been identified are compounds that are easy to separate by gas-chromatography, or other analytical methods. These same methods are not so appropriate for the identification of amines and amides, leading to their neglect as host cues. This comment is valid for the whole area of research on attractants for haematophagous arthropods. What might be said, however, is that none of the host volatile attractants thus far identified are specific to vertebrates. Even microbial cultures have been

demonstrated to attract blood-feeding arthropods such as triatomines (Guerenstein et al., 1995) and mosquitoes (Knols and de Jong, 1996).

Although a large number of host-associated volatiles may successfully attract *A. variegatum* and indeed, other haematophagous arthropods, field data collected from catches of tsetse flies (Hall, 1984) and mosquitoes (Kline et al., 1991) have indicated that ratios of chemicals applied in lures are important in trapping, implying an underlying odour blend specificity that is employed by these animals when orientating towards a host. Although the behavioural mechanisms involved are not known for these insects, we have demonstrated that *A. variegatum* can discriminate in its responses to single compounds, mixtures and host odours.

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Chapter 3. Identification of tick-associated volatiles and behavioural responses of *Amblyomma variegatum* adults to some of them.

Abstract

The tropical bont tick, *Amblyomma variegatum*, exudes droplets and an odour when it is disturbed. An analysis was undertaken of the headspace over undisturbed and disturbed unfed adults using solid-phase microextraction linked to gas chromatography–mass spectrometry (GC-MS). Furthermore, the headspaces over disturbed and engorged female *Amblyomma hebraeum* and *A. variegatum* were analysed by GC-MS after collecting the odour on a charcoal trap. An octenol isomer was identified as being a major constituent of the headspace over most of the tick material sampled, although it was not found over unmoulted ticks and exuviae. This product was also associated with the disturbance of ticks and moulting adults, and was identified as R-(-) 1-octen-3-ol using the antenna of *Glossina brevipalpis* in gas chromatography linked antennogramme analysis of the extracts. Unfed *A. variegatum* adults walking on a servosphere were not attracted to the odours over engorged females, unmoulted ticks and newly moulted ticks, yet dead ticks and detritus associated with newly moulted ticks did attract. In addition to 1-octen-3-ol, the aliphatic products hexanol, hexanal, heptanal and nonanal were also identified over engorged females. All five are known host volatiles, and three, i.e., 1-octen-3-ol, hexanal and nonanal have been shown to attract *A. variegatum* on the servosphere. No response was found for the suspected tick pheromone, 2,6-dichlorophenol, which was only detected over female ticks cut into four pieces with a scalpel.

Introduction

Metastriate ticks, including engorged females after drop-off, are known to secrete droplets from the cuticle in response to physical disturbance. The content of the secretion was studied by Yoder et al. (1993a) who found that four metastriate tick species, *Dermacentor andersoni*, *D. variabilis*, *A. maculatum* and *A. americanum* release a pungent odour while secreting highly volatile droplets from dermal glands after being disturbed; these droplets disappear after some 20 seconds. Yoder et al. (1993b) demonstrated that this exudate gave significant protection against fireant predation in a petri dish arena for 1 day and also promoted clustering by conspecifics (Yoder, 1995). Pavis et al. (1994) also found that transfer of such secretions from *A. variegatum* adults to lepidopteran larvae protected them from fireant predation and inhibited bacterial and fungal growth. However, as these predation experiments were run over one day, it is likely that protection was afforded by the less volatile complex of products present, and not by the transient droplets or by the pungent odour associated with them i.e., the immediate response of the tick to physical disturbance.

Here I describe how I sought to analyse this 'pungent odour' released by *A. variegatum* rather than any less volatile material that may be present. Vapours in the headspace (the confined space over odorous material in which volatiles collect) were entrained on to charcoal and on to solid-phase microextraction fibres (SPME). The latter is a relatively new solvent free method of odour sampling where analytes in the vapour phase establish equilibria with a polymer coated on a fused silica fibre. The volatiles can be thermally desorbed from the fibre in the injection port of a chromatograph and passed directly to a gas-chromatography column. The servosphere experimental design (chapter 1) was used to examine the behavioural response of *A. variegatum* adults to the headspace over ticks and to synthetic analogues of some of the compounds identified in the headspace.

Materials and methods

Ticks

Ticks were procured as detailed in Chapter 1. *A. hebraeum* adults, and engorged *A. hebraeum* and *A. variegatum* females (ca. 1 week after drop-off) were also supplied by the Centre de Recherche Santé Animale S.A., Novartis, St. Aubin, Switzerland.

Odour sampling

Solid-phase microextraction: With this sampling technique volatiles are adsorbed on a fibre positioned at the end of a plunger which can be freely exposed (for sampling) or withdrawn into a hollow needle of a syringe (for injection) and re-exposed in the injection port of a chromatograph. The fibre is coated with a polymer (stationary phase) onto which volatiles can be adsorbed. The profile of the volatiles adsorbed depends on the affinity of the polymer and concentration of analyte.

Two SPME stationary phases were used in this study, a Carbowax/Divinylbenzene (CW/DVB) one which can collect volatiles such as alcohols relatively quickly, and a Carboxen/Polydimethylsiloxane (Carboxen/PMDS) one which has a greater affinity for hydrocarbons but which requires several hours to achieve equilibration with the analyte. Despite this, the latter phase was more suitable for sampling in humid environments because it had a lower affinity for water. After sampling, the fibre was withdrawn into the hollow needle of the syringe and re-exposed in a split/splitless injection port (injection temperature, 280°C) of a gas chromatograph (Hewlett-Packard 5890 series chromatograph) linked to a HP 5971A mass selective detector (MSD), in splitless mode (1 min) to desorb the trapped volatiles. The analyte was thereby introduced onto a DB-WAX

polar high-resolution fused-silica capillary column (30m, 0.25 mm ID, 0.25 μ m film thickness, J & W Scientific, California) equipped with a precolumn (1 m deactivated fused-silica) and connected via a 1 m deactivated fused-silica capillary (0.25 mm ID) to the mass spectrometer (ionisation chamber temperature 280°C; ionisation energy 70 eV). The MSD, in the EI mode, scanned for masses of 20 to 300. Helium was used as carrier gas exerting a constant 50 kPa head pressure on the top of the column. Components of the desorbed material were identified by comparing the mass spectra of unknowns in the analyte with those of standards in the computer-based library of the GC-MS associated HP chemstation.

To sample the headspace over resting or dead ticks, the Carboxen/PDMS fibre was inserted into a glass tube containing ca. 200 ticks (length 12 cm, diam 2 cm) which was sealed at both ends with plastic perforated stoppers. A control consisted of placing a fibre in an empty tube within 50 cm of the test tube. The collection fibres were left to stand overnight. The headspace over the following biological substrates was sampled: *A. variegatum* nymphs (> 9 months old), resting *A. variegatum* adults (> 9 months old), *A. hebraeum* adults (> 12 months old), freshly moulted *A. variegatum* adults (< 1 month old) and *A. variegatum* adults that had died naturally in the previous two months.

Volatile sampling over disturbed ticks: The CW/DVB fibre was used to sample the headspace over five disturbed unfed *A. variegatum* adults and four *A. variegatum* females killed by freezing and cut into 4 pieces.

To identify the volatiles released over disturbed ticks, 5 unfed adults were held in a 2 ml vial and disturbed by squeezing with forceps after which the vial was sealed with parafilm. A CW/DVB fibre was inserted through the parafilm seal and held over the ticks for one hour and the sampling was also repeated overnight above the ticks. The control for this test was to hold

the fibre above undisturbed ticks for the same time intervals.

Collection of odours on charcoal: Air under pressure from a membrane pump was passed (200 ml/min) over five disturbed engorged females of *A. hebraeum* held in a 500 ml gas-wash bottle onto a 5 mg charcoal trap held in a 60 mm x 4 mm diam. glass tube (from the closed-loop stripping apparatus of Grob and Zürcher, 1976). Charcoal was found by us to be a particularly efficient adsorbant for material containing compounds with a wide range of functional groups. As water condensation can drastically reduce the effectiveness of charcoal as an adsorbant, the charcoal trap was placed in a metal sleeve heated to 10⁰ above room temperature (Grob and Zürcher, loc.cit.) during odour entrainment. To the human nose, this odour is similar to that present over disturbed unfed adult ticks. After collection of volatiles for 10 min, the flask was gently shaken to provoke the release of more volatiles and air collected onto the charcoal trap for a further 10 min. The charcoal trap was then extracted with 12 µl dichloromethane of which 2 µl were introduced on-column to a high resolution capillary column linked to a mass spectrometer using the column (above) and conditions outlined above. Helium was used as carrier gas under constant flow (velocity 30 cm/s at 40⁰C). This procedure was subsequently repeated with engorged *A. variegatum* females.

Gas-chromatography linked antennogramme analysis

Headspace over disturbed unfed adults, exuviae (ca. 5 days old), unmoulted and newly (< 48 hours) moulted ticks were tested for the presence of R-(-) 1-octen-3-ol using the antenna of *Glossina brevipalpis* in GC linked antennogramme (GC-EAG) analysis. Some 40% of the effluent of the column was directed to the antenna, the remainder to a flame ionisation detector (FID). In this laboratory, *Glossina brevipalpis* has been

found to be sensitive to sub nanogramme levels of the compound eluting from a GC column (P.G. Guerenstein, pers. comm.). Furthermore, as the antenna of *G. brevipalis* is most sensitive to the R-(-) 1-octen-3-ol isomer (Ujváry and Guerin, 1998), this compound could be identified by determining the specific activity on the antenna of the product eluting from the column following injection of an extract. This was achieved by comparing the ratio of the EAG to FID peak heights for the extract and pure R-(-) 1-octen-3-ol.

The presence of 1-octen-3-ol over disturbed ticks were determined after shaking 200 adult ticks in a 50 ml gas-wash bottle. Odours from this bottle were collected on charcoal and extracted with dichloromethane (as above). A 2 µl aliquot of this extract was then introduced to a GC column (same model as used in the GC-MS analysis, above) which was temperature programmed to carry the vapours to the antenna and the FID. To determine if 1-octen-3-ol was present over exuviae (ca. 5 days old), 0.3 g were placed in a closed 5 ml vial. One day later, 2 ml were withdrawn from the headspace of this vial using a gas-tight syringe, and injected on to the column in splitless mode. The determination of the presence of R-(-) 1-octen-3-ol over ten unmoulted ticks was carried out in the same way. The influence of moulting on the presence of this compound was tested by sealing 10 unmoulted ticks in a 5 ml vial as before. After two animals had emerged, 2 ml of this headspace was analysed for the presence of the compound by GC-EAG as above.

Behaviour

Vapours from the following biological substrates were presented to walking *A. variegatum* adults on the servosphere from 500 ml gas-wash bottles as described in chapter 1: (1) air at 150 ml/min swept for 1 min over two hundred adult *A. variegatum* that had died naturally; (2) air at either 10 ml or 75 ml/min passed over 0.6 g of haematin pellets, faeces and exuviae of freshly moulted (< 2 weeks) *A. variegatum* adults. This material smelled of the odour of disturbed ticks; (3) air at 75 ml/min passed for 1 min over five engorged female *A. hebraeum* 1 week after drop-off ; (4) 5 engorged female *A. variegatum* (as per 3); (5) 20 unmoulted ticks (as per 3) and (6) 10 freshly moulted ticks (< 48 hours after emergence) (as per 3). After every two tests, the bottle was shaken gently to provoke the ticks to release more droplets from the dermal glands. The blank control for the above experiments consisted of air passing for 1 min through an empty gas-wash bottle at the same flow rate as used in the test.

Synthetic volatiles tested: Vapours of 2,5-dimethyl pyrazine (Aldrich), 2,6-dichloroanisole (Aldrich), heptanal (Aldrich) (source dose 10 ng and 1 µg) and from a racemic mixture of 1-octen-3-ol (Merck), hexanal, nonanal (Fluka) (each at source dose 10 ng) and 2,6-dichlorophenol (2,6-DCP; Supelco; source doses 100 fg - 10ng) were delivered separately to *A. variegatum* adults on the servosphere from gas-wash bottles at the rate of 150 ml/min following an appropriate control (described in Chapter 1). All compounds were > 97% pure as indicated by GC.

Results

Identification of volatile compounds in headspace

Several compounds, including short-chain aliphatic aldehydes and alcohols could be recovered from the charcoal trap over engorged females but fewer were recovered from the same substrate by SPME (Table 1). The MS has a detection limit for a full mass spectrum at ca. 5 ng, so using this criterion, relatively few of the products (Table 1) were recovered in sufficient quantities to permit identification.

2,5-Dimethyl pyrazine was only found over dead ticks, and 2,6-dichloroanisole was found only over old or dead ticks, indicating that both may be degradation products. 2,6-Dichlorophenol could not be detected in the headspace over whole ticks but was detected over *A. variegatum* females cut into pieces. The natural product matched the mass spectrum and retention time of the synthetic analogue. An isomer of octenol was found throughout most of the tick material sampled (*A. variegatum* and *A. hebraeum*) both by charcoal trapping and sampling by SPME (Table 1; Fig. 1). This product was identified as R-(-) 1-octen-3-ol (Fig. 2) using the antenna of *Glossina brevipalpis* in GC-EAG analysis of the vapours over disturbed ticks collected on charcoal (above) and vapours over newly moulted ticks. Volatiles from exuviae and unmoulted ticks failed to elicit a response from the antenna. Another alcohol, hexanol, was also identified in charcoal and SPME sampling of the headspace over engorged females of both species (Fig. 1). Three aldehydes found over engorged *A. variegatum* were not found over engorged *A. hebraeum*, but this difference was probably due to the overall lower amounts collected from engorged *A. hebraeum*.

Table 1. Compounds identified in the headspace over *A. hebraeum* and *A. variegatum*. Several compounds were not identified as they were below the detection limit of the mass spectrometer (< 5 ng) are not shown.

<u>Volatiles collected by SPME</u>	<i>A. variegatum</i>	<i>A. hebraeum</i>
Dead ticks	3-Methyl-1-butanol 2,5-Dimethyl pyrazine *Octenol 2,6-Dichloroanisol, 1,2 Benzisothiazole	Not tested
Dissected female <i>A. variegatum</i>	*Octenol 2,6-Dichlorophenol	Not tested
Old (> 9 months)	*Octenol *2,6-Dichloroanisol	2,5-Dimethyl pyrazine; *Octenol 2,6-Dichloroanisol
Nymphs	*Octenol	Not tested
Engorged females	Hexanol *Octenol	Hexanol *Octenol
Engorged females (water washings)	Hexanol *Octenol	Hexanol *Octenol
Disturbed unfed adults	*Octenol	Not tested
Undisturbed unfed adults	Blank	Not tested
<u>Volatiles collected on charcoal</u>		
Engorged females		
	Hexanol	Hexanol
	*Octenol	*Octenol
	Nonanol	
	Hexanal	
	Heptanal	
	Nonanal	
	Decanal	
	Linalool	Linalool oxide
	Borylene/1-limonene	
	2,5-Dimethyl 2,6heptadiene	
		α -terpinolene
<u>**Presence of R-(-) 1-octen-3-ol</u>		
disturbed tick	Present	
unmoulted ticks	Not found	
newly moulted ticks	Present (ca. 1 ng)	
exuviae	Not found	

* determined by matching mass spectra and retention times on a chromatographic column

** determined by GC-EAG using responses to the antenna of *Glossina brevipalpis*

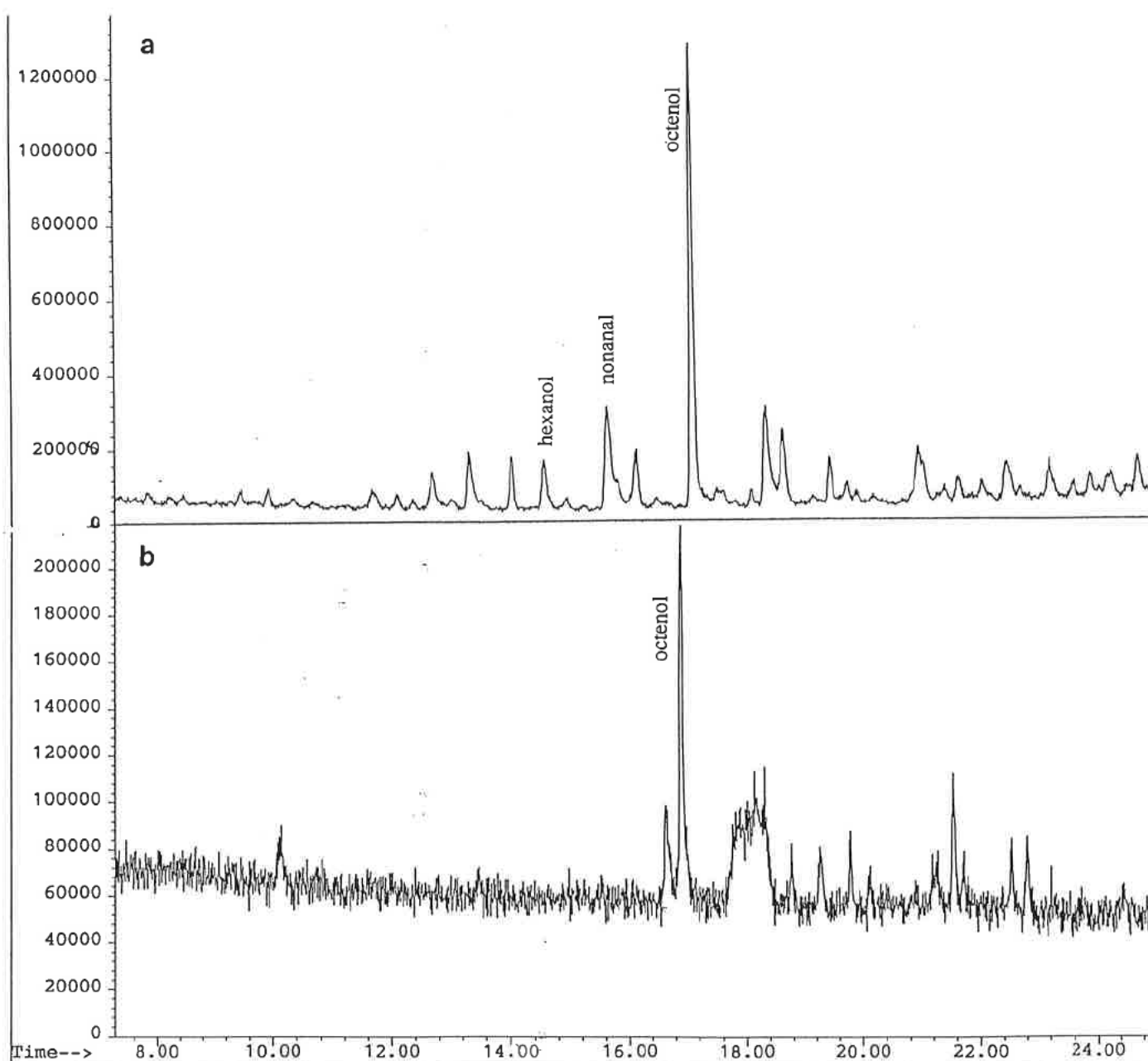
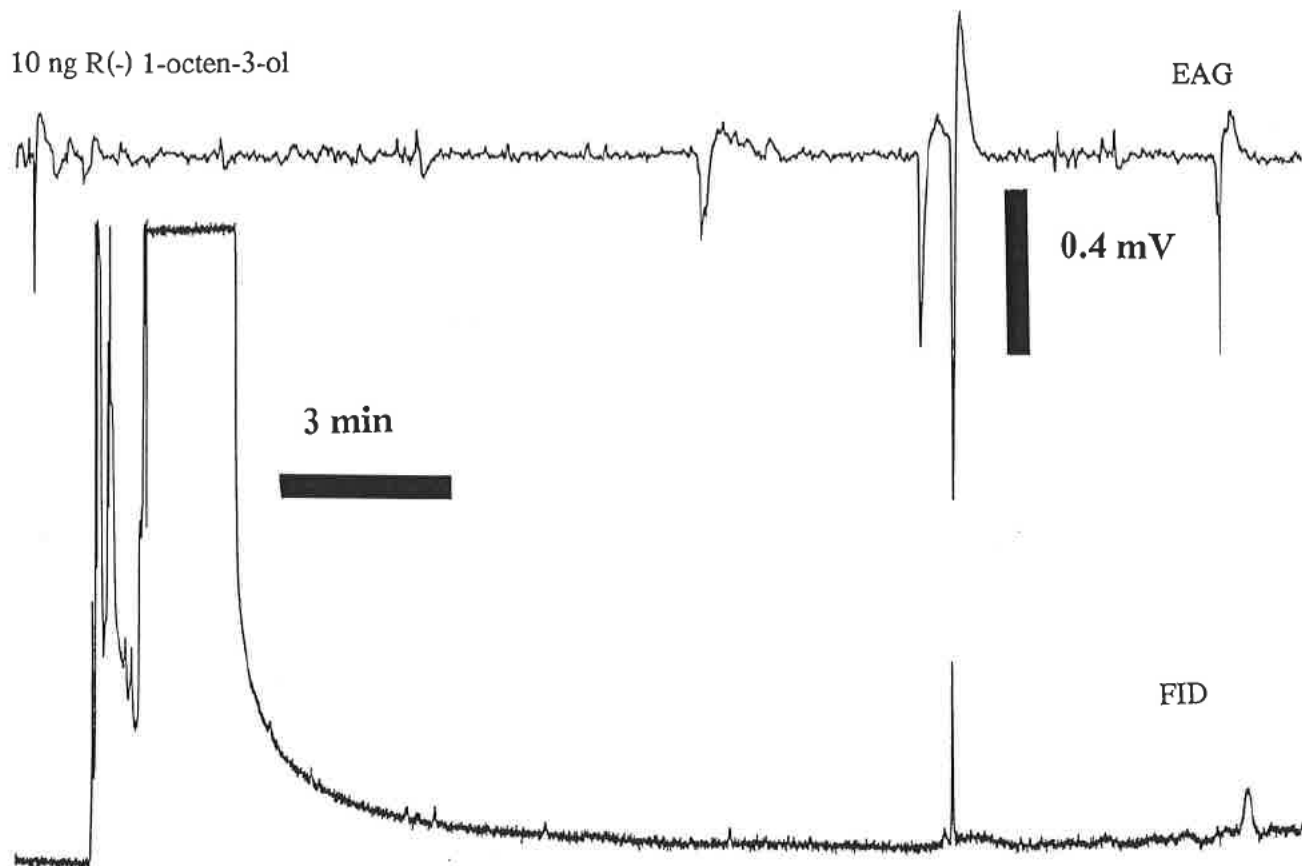


Figure 1. Total ion chromatograms of volatiles collected over **a**, disturbed engorged *A. variegatum* females using a charcoal trap and on-column injection of desorbed material and **b**, over disturbed *A. variegatum* adults (unfed) sampled by solid-phase microextraction with a Carbowax/Divinylbenzene coated silica fibre (split-splitless injection). The peaks that were identified (> 5ng) are presented in Table 1. Each 2 min interval (abscissa on the chromatogram) is equivalent to a 10° rise in the oven temperature, starting at 40° after 5 min. The octenol isomer is the largest peak present in both chromatograms occurring at a level of ca. 100 ng in **a** and ca. 10 ng in **b** in the 55° to 135° elution temperature range presented. The sensitivity scale in **b**, is higher than in **a**, and the shift in the retention time of the octenol isomer between the two chromatograms is due to a difference in pressure applied to the top of the column between the two injection methods. Two other marked peaks, nonanal and hexanol, were tentatively identified by GC-MS in this headspace.

10 ng R(-) 1-octen-3-ol



Volatiles from disturbed ticks

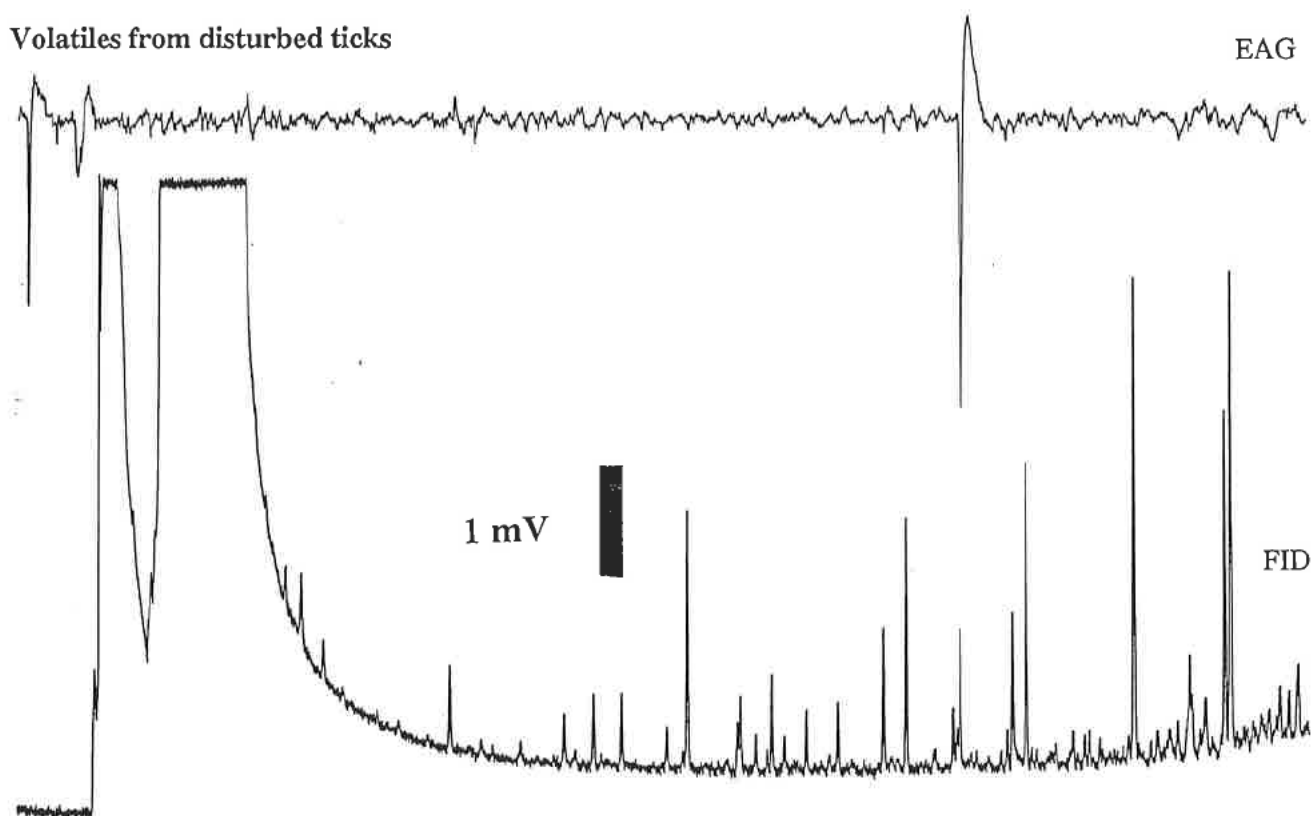


Figure 2. Analysis of volatiles released by *A. variegatum* using the antenna of the tsetse fly, *Glossina brevipalpis*, as a biosensor (electroantennogram coupled to gas chromatography). The amplitude of the electroantennogram response (top trace) to the amount of the synthetic R(-) 1-octen-3-ol injected matches the response of the amount of the biologically active product in the extract.

Analysis of the headspace over five unfed, undisturbed and disturbed *A. variegatum* adults using a CW/DVB fibre showed that release of the octenol isomer, among other compounds, was associated with the disturbance of the *A. variegatum* adults held in a 2 ml vial (1 hour sampling period; Table 1; Fig. 1). If the first hour was excluded and sampling continued for a further 13 hours, no compounds were detectable by SPME. No compounds were detected over five undisturbed ticks (Table 1).

Behaviour

Odour from disturbed engorged *A. hebraeum* or *A. variegatum* was not attractive to *A. variegatum* adults. Males and females did not differ in their response. However, *A. variegatum* adults responded to the odour of disturbed engorged *A. hebraeum* with a significant decrease in speed ($n=14$; $p < 0.0261$) and to disturbed engorged conspecifics with a decrease in angular velocity ($p < 0.004$).

Adult *A. variegatum* were attracted ($n = 13$; $p < 0.0071$) to the odour of dead conspecifics and also showed a decrease in speed ($p < 0.0071$). Odours from the exuviae and other detritus from freshly moulted ticks also attracted *A. variegatum* ($p < 0.0245$, $n = 18$) with a flow rate of just 10 ml/min through the flask where they were contained. When the flow rate through the flask was increased to 75 ml/min, the ticks were no longer attracted ($p < 0.41$, $n = 8$).

The odours of unmoulted and freshly moulted *A. variegatum* adults were not attractive and did not influence walking behaviour.

Vapours from 2,6-chloroanisole and 2,5-dimethyl pyrazine (source dose: 10 ng and 1 μ g) presented to *A. variegatum* males ($n=15$ and $n=16$ respectively) showed no significant effects on any of the ticks' behaviours. 2,6-DCP also had no effect on *A. variegatum* behaviour in several tests

spanning a source dose range of 10 fold increases from 100 fg to 10 ng ($n > 10$ for each dose). Hexanal, nonanal and a racemic mixture of 1-octen-3-ol (source dose, 10 ng) were found to attract (chapter 2). Heptanal was not attractive (chapter 2).

Discussion

Metastriate ticks are very sensitive to disturbance. Yoder et al. (1993b) reported that even application of bright light was sufficient to provoke the secretion of droplets by engorged *D. variabilis*, suggesting that the secretion of this exudate is a reaction to disturbance. Compounds from the exudate might serve to repel predators. Evidence for this was reported by Yoder et al. (1993b) who demonstrated that fireants probing *D. variabilis* provoked droplet secretion by the unfed tick, and that when such ticks were artificially exhausted of the secretion, they were more susceptible to predation. Prostriate species such as *Ixodes dammini* and argasid ticks such as *Ornithodoros moubata*, lacking dermal glands responsible for this, were killed more readily (Yoder et al., loc. cit.) Fireants are also known to be predators of *A. variegatum* (Barré et al., 1991), especially of engorged females, and such predation may have been the driving force in the development of the secretion. Yoder (1995) showed that while *D. variabilis* adults were ordinarily passive to the presence of other ticks, clustering of conspecifics occurred around a tick to which the exudate had been topically applied. It is not clear from their study if this clustering response was caused by olfactory stimuli in the exudate. Although a reduced speed and reduced angular velocity by *A. variegatum* adults in response to the odour of both engorged *A. hebraeum* and *A. variegatum* was found, the lack of any attractive or repellent response by *A. variegatum* on the servosphere to the volatiles released by engorged females and newly moulted ticks suggests

that adult ticks are rather insensitive to the strong odour released or that some essential components of it did not reach the ticks on the sphere. Apart from affecting conspecifics, it is possible that the strong odour functions as part of a multicomponent signalling complex interacting with the colour from the alloscutum of males as has been described for the development of the aversion of chicks to pyrazine released from insects for defence purposes (Rowe and Guilford, 1996), thus helping to reduce predation by tick-picking birds. Although oxpeckers are the only bird species that specialise on feeding on ticks on the host, several other birds will devour these Acari, particularly the engorged stages, when offered (Samish and Rehacek, 1999). The release of octenol found here as *A. variegatum* adults moult is consistent with a defensive role for this compound, as newly moulted adults remain extremely vulnerable for some days after emergence. Aliphatic alcohols are implicated in the defensive secretions of several arthropods, particularly Hymenoptera (reviewed by Blum, 1981).

Single compounds present in this exudate might, however, prove to be attractants to conspecifics. R-(-) 1-octen-3-ol was found in all headspace samples over ticks, except for unmoulted adults and exuviae. Vapours smelling of this compound from detritus of *A. variegatum* including exuviae, haematin pellets and faeces were attractive to *A. variegatum* when supplied at low levels (10 ml/min) on the servosphere. At a higher delivery rate (75 ml/min), the ticks did not respond, suggesting that the concentration of octenol and the other compounds from engorged and freshly moulted ticks presented to walking adults may have been too high to evoke a behavioural response. The presence of this octenol isomer in the headspace over aged, young and even dead material suggests that this odour is continually being released at low levels and it is tempting to suggest that it might be implicated in the clustering or aggregation response of unfed ticks.

Aponomma concolor exuviae have been reported as attractants of adults of

this species (Treverrow et al., 1977), and filter papers exposed to the prostriate tick *Ixodes holocyclus* and *I. echidna* also attract conspecifics, yet the compounds involved remain unidentified (Treverrow et al., 1977).

A. variegatum adults did not respond to the vapours of 2,5-dimethyl pyrazine or of 2,6-dichloroanisole that were associated with dead ticks, or to 2,6-DCP. The origin of the former two compounds is unknown, and as constituents of dead material, their role in the biology of *A. variegatum* may be limited. 2,6-DCP, however, is a suspected tick sex pheromone and has been identified in several species of metastriate ticks. It was only detected in this study after females were cut. The absence of 2,6-DCP recorded here in the headspace over whole ticks concurs with De Bruyne and Guerin (1994) in their investigation of *Boophilus microplus*. They recovered only 10 pg per feeding female tick after collecting the headspace on to an adsorbent for 20 min, but thirty times more by extraction of whole adult ticks in solvent. As in this study, De Bruyne and Guerin (1994) failed to show a behavioural response by *Boophilus microplus* to the chemical on the servosphere despite its presence in all life stages of the tick. Although the behavioural role of this compound remains controversial, Yunker et al. (1992) found strong attraction of *A. variegatum* to 2,6-DCP when delivered in a two choice olfactometer in an air stream. 2,6-DCP was also found to attract *A. variegatum* in the field in the presence of CO₂ (Norval et al., 1991). The reasons for the anomalous results between these two reports and this study are unclear. Nonetheless, 2,6-DCP is produced by innervated foveal glands in metastriate ticks (Sonenshine, 1991) and is present in the headspace over fed *A. variegatum* males detached from cattle (P.A. Diehl, pers. comm.). As the specific circumstances leading to the release of this chemical remain unresolved, a behavioural role cannot yet be assigned or rejected for this tick-associated volatile.

Some of the same compounds found as volatiles over engorged *A. variegatum* also occur in host odour, i.e., nonanal, hexanal and heptanal (Steullet and Guerin, 1994) and hexanol (Phillips, 1997). *A. variegatum* has olfactory receptors that respond to these, two of which, hexanal and nonanal, were found to attract this species on the servosphere in this study. Evidently hexanol should be tested. It serves to underline that once again a species with a limited olfactory repertoire can profit from the parsimonious use of infochemicals.

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3. General discussion

Single host compounds from a diverse range of functional groups were found to attract *A. variegatum* on the servosphere. Of sixteen compounds for which olfactory receptor cells are known, thirteen were shown to induce an upwind walk that was remarkably similar for most of the compounds tested. Such a strong behavioural effect caused by the activation of just one or two receptors is promising. Indeed, the responses of haematophagous arthropods to host volatiles, particularly to compounds such as CO₂ have proved to be extraordinarily unspecific: *Culex quinquefasciatus* has been shown to be attracted equally to cattle and a synthetic equivalent of CO₂ in the field, even though the sub-family studied never feeds on cattle (Mullens and Gerry, 1998). It appears that haematophagous arthropods, living a precarious existence, are readily attracted by the *suggestion* of a host caused by the increasing doses of CO₂ perceived and this may be why single compounds also attracted ticks in this study.

While much attention has been paid to CO₂ and acetone, being two vertebrate volatiles released in large amounts, haematophagous arthropods are evidently equipped to monitor several other host vapours. 1-Octen-3-ol, which has been shown to attract *A. variegatum* here, and also known as an attractant for other bloodfeeding arthropods, is a degradation product of polyunsaturated fatty acids as is hexanal. Other attractants such as the aliphatic fatty acids (isobutyric acid, nonanoic acid and butanoic acid) are produced from the degradation of amino acids and larger fatty acids by microflora of skin. Skin lipids present a particularly unique and individual substrate composed of a great variety of fatty acids synthesised by the host, have long been suspected of contributing to specific host odours (Nicolaidis, 1974). Many of these are branched and do not occur inside tissues. To date,

however, the study of host volatiles has been restricted to those compounds that were easy to collect and identify. It may be predicted that decarboxylated amino acids such as isoamylamine, found attractive here for *A. variegatum*, will also prove attractive to other haematophagous arthropods.

The aggregation-attachment pheromone (AAP) of *A. variegatum* and *A. hebraeum* are largely composed of host volatiles. This would not be the first time a host product was incorporated in a pheromone system in arthropods. Cholesteryl esters derived from the host are used by *Boophilus microplus* females to arrest males in courtship behaviour (De Bruyne and Guerin, 1998), and pioneering females of the western pine beetle (*Dendroctonus brevicomis*) release myrcene derived from the host to attract conspecifics (Vité and Francke, 1976). The response to the AAP, as to host compounds such as CO₂, may also be quite generalist: unfed adults of *A. variegatum* and *A. hebraeum* are attracted to the AAP of the other species at least on rabbits (Yunker et al., 1990), yet this study has shown highly specific responses depending on the mixture delivered for *A. variegatum*. It may be that the lack of specificity found on the rabbit by the latter authors may be due to some missing component, as this animal is not a known natural host for adults of either of these two tick species.

Ticks are considered simple arthropods, containing less than 100 olfactory receptor types, yet recent work has estimated that *Drosophila* possess less than 200 olfactory receptor types (Vosshall et al., 1999). Unlike ticks, it appears that insects modify their olfactory capabilities by the multiple expression of the same receptors in antennal sensilla, yet the underlying pattern remains the same. Since single compounds attract, apparently the link between activating one chemosensory channel and motor output has been maintained in ticks, as indeed is the case in *Bombyx mori*

(Kaissling and Priesner, 1970), where a hit of just a few molecules is sufficient to cause the male courtship dance. Haematophagous arthropods may be as sensitive: *o*-nitrophenol was found here to attract *A. variegatum* at such concentrations, and 1-octen-3-ol elicits electroantennogramme responses from the tsetse fly *Glossinia brevipalpis* (P.G. Guerenstein, pers. comm) when presented at a concentration of 10^6 molecules/ml of air. This is within an order of magnitude of the threshold known for moths (Kaissling, 1990). Single compounds found attractive for *A. variegatum* in this study have since been tested on other tick species. We have found NO to attract the soft tick *Ornithodoros moubata* using the servosphere, and CO₂ is known to attract *Ixodes ricinus* on its own (Riegler, 1995), on the servosphere. Breath was found here to also attract *I. ricinus*. It therefore appears that ticks are not only sensitive to single compounds, but that breath is an important host emanation that ticks recognise. In the future, dispensers containing lures for *A. variegatum* will most likely contain components of the aggregation-attachment pheromone, such as the *o*-nitrophenol: methyl salicylate (1:1) mixture found attractive here. This binary mixture was found to be extremely reliable in several tests and has been used by us to evaluate the responses of *A. variegatum* to repellents. However, in the ranking of the attractants (Table 1), this binary mixture was not particularly attractive compared to the more complete response to breath, or even to the single compound 1-octen-3-ol, suggesting that it could be improved, as *o*-nitrophenol is in the presence of host odour (chapter 1). The hierarchy of responses (Table 1) to attractants indicates a considerable degree of discrimination on the part of the tick, but the reduced attraction when compounds are mixed is at least as interesting. Tick associated volatiles (1-octen-3-ol and the pheromone components) appeared to be compatible with each other, as were the breath components (CO₂, acetone), yet 1-octen-3-ol and CO₂ each significantly reduced the attraction to host associated volatile

mixtures and pheromone mixtures, respectively. It may be that the discriminatory responses that the animal displays are not simply due to quantity or proportions of an attractant, but also to the chemical ecology in which these attractants function as infochemicals.

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Table 1a. Percentage change in relative displacement in the upwind cone 60° either side of the wind direction between test and control for host compounds, and mixtures containing host compounds, that evoked an upwind response on the servosphere. The values given in parentheses represent the 25% and 75% percentiles. Significance between the test and control for any given treatment was determined using the Wilcoxon's signed rank test. Treatments were compared by applying the Wilcoxon-Mann-Whitney test to paired differences in the relative displacement in the upwind cone between test and control. Treatments followed by the same letter are not significantly different.

Column 2: treatments denoted by the letter **A** are weakly attractive and do not cause any significant change in walking behaviour; treatments denoted by the letter **B** are generally more attractive than **A** and induce a reduction in speed and/or an increase in angular velocity.

Treatment		Source dose of volatile (g), ppb at the sphere apex or % (v/v) above ambient at the sphere apex	Median difference in time walked in upwind cone (interquartile range)	Wilcoxon signed rank test for a treatment $p <$	n =	Significance of differences between treatments (Wilcoxon-Mann-Whitney test)
CO ₂ + NO*	A	57 ppm; 2.2 ppb	8.9 (-1) (28)	0.0229	18	Abc
Isoamylamine	A	1 µg	9.7 (5) (29)	0.0012	18	abc
CO ₂	A	57 ppm	18.3 (3) (47)	0.0057	18	abj
*4-methyl- <i>o</i> -nitrophenol	A	1 µg	19.6 (0) (35)	0.0055	20	abi
NO*	B	2.2 ppb	4.1 (0) (35)	0.0312	21	abc
Isoamylamine + isobutyric acid + 1-octen-3-ol	B	1 µg; 1 µg; 10 ng	10.7 (1) (45)	0.010	20	abi
E-2- heptenal	B	10 ng	15.7 (2) (24)	0.0312	17	abe
Hexanal	B	10 ng	14.7 (9) (23)	0.0019	17	abg
3-pentanone	B	10 ng	14.7 (-1) (40)	0.0141	19	abi
Nonanal	B	100 pg	17.7 (8) (38)	0.0084	18	abi
Nonanoic acid	B	10 ng	23.2 (-2) (38)	0.0123	19	abi
Methyl salicylate + 2-hydroxybenzaldehyde	B	10 ng; 10 ng	23.3 (16) (37)	0.0043	18	abi
Benzaldehyde	B	10 ng	29.8 (9) (43)	0.0007	18	abk
Nonanal	B	10 ng	23.5 (1) (37)	0.0075	17	abi
Isobutyric acid	B	1 µg	20.0 (-4) (46)	0.0228	20	am
<i>o</i> -Nitrophenol + methyl salicylate + isobutyric acid	B	1 µg; 1 µg; 10 ng	23.4 (8) (49)	0.0284	11	am
<i>o</i> -Nitrophenol + methyl salicylate + NO	B	1 µg; 1 µg; 2.2 ppb	29.7 (18) (43)	0.0262	11	am
γ-Valerolactone	B	10 ng	30.7 (10) (48)	0.0038	19	am
Isobutyric acid	B	10 ng	32.3 (4) (50)	0.0001	29	defghijklm
Acetone* + CO ₂	B	250 ng; 57 ppm	33.2 (14) (55)	0.0014	17	defghijklm
<i>o</i> -Nitrophenol + methyl salicylate	B	1 µg; 1 µg	29.9 (7) (51)	0.00001	37	fghilm
<i>o</i> -Nitrophenol + methyl salicylate	B	10 ng; 10 ng	32.9 (10) (50)	0.00001	34	ghim
Isobutyric acid + E-2-heptenal + γ-Valerolactone	B	10 ng; 10 ng	38.7 (5) (59)	0.0013	17	gim
Butanoic acid	B	10 ng	34.1 (16) (59)	0.00001	16	lm
<i>o</i> -Nitrophenol + methyl salicylate + 1-octen-3-ol	B	10 ng; 10ng; 10 ng	35.56 (3) (52)	0.0028	20	lm
<i>o</i> -Nitrophenol + 1-octen-3-ol	B	10 ng; 10 ng	35.6 (3) (49)	0.0040	20	lm
Acetone*	B	250 ng	34.6 (16) (57)	0.0004	17	lm
<i>o</i> -Nitrophenol + methyl salicylate + isobutyric acid	B	1 µg; 1 µg; 1 µg	47.1 (23) (67)	0.0044	11	lm

* compounds for which chemoreceptors have not been identified in *A. variegatum*

‡ compounds which did not attract at a 10 ng source dose.

Table 1 (contd). Percentage change in relative displacement in the upwind cone 60° either side of the wind direction between test and control for host compounds, and mixtures containing host compounds, that evoked an upwind response on the servosphere. The values given in parentheses represent the 25% and 75% percentiles. Significance between the test and control for any given treatment was determined using the Wilcoxon's signed rank test. Treatments were compared by applying the Wilcoxon-Mann-Whitney test to paired differences in the relative displacement in the upwind cone between test and control. Treatments followed by the same letter are not significantly different. Column 2: treatments denoted by the letter **C** very attractive and cause a reduction in speed and an increase in angular velocity followed by an off-response as the odour is withdrawn; the treatment (breath) followed by the letter **D** is very attractive yet is not associated with a decrease in speed or a increase in angular velocity of the walk of *A. variegatum*. Ticks also display an off-response to the loss of breath.

Treatment		Source dose of volatile (g), or % (v/v) or ppb at sphere apex	Median difference in time walked in upwind cone (interquartile range)	Wilcoxon signed rank test for a treatment p <	n =	Significance of differences between treatments (Wilcoxon-Mann-Whitney test)
γ-Valerolactone + E-2- heptenal	C	10 ng; 10 ng	34.1 (4) (58)	0.0052	16	am
Isobutyric acid + †isoamyamine*	C	1 µg; 1 µg	37.3 (26) (61)	0.0002	18	jl m
1-Octen-3-ol*	C	10 ng	47.2 (22) (62)	0.0004	21	kl m
Breath	D	4%	43.3 (32) (60)	0.00001	16	m

* compounds for which chemoreceptors have not been identified in *A. variegatum*

† compounds which did not attract at a 10 ng source dose.

4a. Summary

1) The walking responses of adults of the tropical bont tick, *A. variegatum* were recorded using a servosphere in response to wind and wind plus odours from hosts and congeners.

2) In the absence of wind, *A. variegatum* walked on all eight legs, but with long halts. Wind provoked continuous walking and induced a reaching response where the first leg pair was used to actively sample the air. This increased the angular velocity and reduced walking speed.

3) The effects induced by wind were amplified in the presence of vapours from *o*-nitrophenol (ONP) and methyl salicylate (MS), the two principal components of the aggregation-attachment pheromone (released by feeding males to attract conspecifics) in the air flowing over the ticks. Vapours from a binary 1:1 mixture of ONP and MS was attractive over a 10^4 fold concentration range (source dose 10 ng to 100 μ g) providing an increase in upwind displacement of 20-40%, significantly higher than the natural ratio where ONP vapour predominates.

4) The attraction to ONP vapour was variable when presented on its own, but consistent when delivered with steer hair odour, a substrate that was unattractive on its own. Moreover, the upwind walk to this combination did not cause a change in speed or angular velocity. This modification of the response to ONP by host odour, which leads to a more efficient upwind displacement, supports the hypothesis that the pheromone is used to enhance the response of ticks to colonised hosts.

5) The response of *A. variegatum* to twenty-one host compounds was also examined. Breath and sixteen host odour compounds (fourteen volatiles and two gases) were found to attract the tick. Thirteen of these compounds

had previously been shown to activate specific olfactory receptors in the olfactory sensilla of this species.

6) Vapours from three binary mixtures and two ternary mixture of these attractive compounds also evoked an upwind walk (i.e., γ -valerolactone plus E-2-heptenal; isobutyric acid plus nonanal; isobutyric acid plus isoamylamine; isobutyric acid plus isoamylamine plus 1-octen-3-ol; γ -valerolactone plus E-2-heptenal plus isobutyric acid).

7) NO, isoamylamine, acetone and 1-octen-3-ol, for which olfactory receptors are not described in *A. variegatum*, were identified as new chemostimulants for this species as they also served as attractants.

8) The walking behaviour in the presence of attractants followed a continuum. (A) less attractive single compounds evoked a weak upwind response but no change in walking behaviour. (B) single compounds and some mixtures, generally more attractive than in (A), and which elicited vigorous sampling left and right of the track by ticks approaching the source, a behaviour that continued long after odour off (this type of response was also observed in the presence of the AAP binary mix of ONP and MS, above). (C) very attractive volatiles (of 1-octen-3-ol and two binary mixtures, isoamylamine plus isobutyric acid; γ -valerolactone plus E-2-heptenal) also elicited vigorous left and right sampling but also evoked an off-response on removal of the odour. (D) attraction to breath with a smoother walk upwind without the left and right sampling and also with an off-response to loss of odour.

9) The percentage (> 60%) of off-responses to the two binary mixtures (above) was higher than the sum of the off-responses recorded to individual components, clear evidence that it is the blend that evokes the local search.

10) These responses to mixtures and the modification of the response to ONP by host odour, indicate an interplay among chemicals in eliciting the orientation responses of *A. variegatum* to hosts.

11) As the tropical bont tick, *A. variegatum*, exudes odorous droplets when disturbed, an analysis was made of the headspace over undisturbed and disturbed ticks using solid-phase microextraction fibres and charcoal to adsorb volatiles. An octenol isomer was present over all tick material excepting exuviae and adult ticks shortly before their emergence from exuviae. This isomer was liberated by the disturbance of ticks or spontaneously upon their emergence as adults from their exuviae, suggesting that it is released as part of a defensive secretion. It was identified as R-(-) 1-octen-3-ol, the predominant isomer in bovid breath. Hexanol, hexanal, heptanal and nonanal were also identified over engorged female *A. variegatum*.

12) Although odours of engorged females and freshly moulted adults did not attract ticks on the servosphere, exuviae plus detritus from newly moulted ticks did.

13) Three volatiles i.e., 1-octen-3-ol, hexanal and nonanal, associated with ticks were shown to attract *A. variegatum* on the servosphere. As these compounds also occur in host odour, it is probable that even though ticks possess a relatively reduced olfactory system, they compensate for this by parsimonious use of products for purposes of aggregation and host attraction.

4b. Résumé

1) Les déplacements des adultes de la tique, *Amblyomma variegatum* (Fabricius), soumis à un flux d'air contrôlé puis au flux d'air additionné des odeurs de ses hôtes ou de ses congénères, ont été enregistrés à l'aide d'une servosphère.

2) En l'absence de flux d'air, *A. variegatum* utilisait huit pattes pour se déplacer et effectuait de longs arrêts. En présence de vent, la première paire de pattes était employée pour échantillonner activement l'air. Ceci a augmenté la vitesses angulaire et a réduit la vitesse linéaire.

3) Les effets induits par le flux d'air ont été amplifiés en présence des vapeurs de l'*o*-nitrophenol (ONP) et du salicylate de méthyl (MS), les deux composés principaux de la phéromone d'agrégation-attachement (AAP). Cette phéromone est libérée par les mâles qui se nourrissent afin d'attirer leurs congénères. Les vapeurs d'un mélange binaire de rapport 1:1 d'ONP et de MS étaient attractives dans un rayon de concentrations de 10^4 unités (doses 10 ng à 100 µg appliqués à la source). L'augmentation du déplacement contre le vent due à ces vapeurs était de 20-40% et significativement plus élevée que la réponse induite par le rapport naturel largué par les mâles où la vapeur d'ONP prédomine.

4) L'attraction à la vapeur d'ONP était variable lorsque celle-ci était présentée seule mais cette même vapeur livrée avec l'odeur des poils de bovin, un substrat inactif à lui seul, était uniformément attractive. De plus, le déplacement contre le flux d'air en présence de cette combinaison n'a pas causé un changement de la vitesse de marche ou de la vitesse angulaire. Cette modification de la réponse à l'ONP par l'odeur de l'hôte, qui cause un déplacement plus rectiligne contre le vent, supporte l'hypothèse que la phéromone d'agrégation-attachement est employée pour augmenter la réponse des tiques aux hôtes déjà colonisés.

5) Les réponses d'*A. variegatum* aux vapeurs de vingt et un composés des hôtes vertébrés ont été également examinées. L'haleine ainsi que seize composés purs (14 volatils et 2 gaz) ont attiré les tiques. Treize de ces composés ont précédemment démontré leur activité biologique comme chimiostimulants sur des récepteurs de sensilles olfactives de cette espèce.

6) Les vapeurs de trois mélanges binaires et de deux mélanges ternaires de ces composés volatils étaient aussi attractives: isoamylamine et acide isobutyrique; γ -valerolactone et (E)-2-heptenal; nonanal et acide isobutyric; isoamylamine et acide isobutyric et 1-octen-3-ol; γ -valerolactone et (E)-2-heptenal et acide isobutyrique.

7) Le monoxide d'azote, l'isoamylamine, l'acétone et le 1-octen-3-ol, pour lesquels aucun récepteur olfactif n'a été décrit chez *A. variegatum*, ont été nouvellement identifiés en tant qu'attractants pour cette espèce.

8) Le comportement de marche en présence des attractants identifiés peut être décrit comme un 'continuum'. (A) Les composés moins attractifs ont induit une faible réponse contre le vent et n'ont pas eu d'influence sur les caractéristiques du comportement de marche de la tique. (B) Les composés généralement plus attractifs que (A), ainsi que certains mélanges ont induit une réponse de marche accompagnée de vigoureux mouvements d'échantillonnage gauche-droite. Ce comportement continuait longtemps après l'arrêt de l'odeur. Le même type de réponse a été observé avec le mélange des composants d'AAP, l'*o*-nitrophenol et le salicylate de méthyl (ci-dessus). (C) Les volatils très attractifs (le 1-octen-3-ol et les deux mélanges binaires d'isoamylamine et d'isobutyric acid ainsi que de γ -valerolactone et de E-2-heptenal) ont induit une réponse gauche-droit comme en (B), ainsi qu'une réponse de recherche locale à l'arrêt de l'odeur. Ce comportement est comparable à celui des Noctuidés lors de la perte de phéromone dans le flux d'air. (D) En présence de l'haleine, les tiques ont

montré une forte attraction sans oscillation de gauche à droite ainsi qu'une réponse de recherche locale à l'arrêt de l'odeur.

9) Le pourcentage (>60%) des réponses suite à l'arrêt de l'odeur (ci-dessus) était plus élevé que la somme des réponses à l'arrêt de chacun de ses composés. Il est donc évident que c'est le mélange qui évoque la recherche locale.

10) Ces réponses aux mélanges de composés ainsi que la modification de la réponse envers l'ONP par l'odeur de l'hôte indiquent une interaction entre les produits chimiques dans le processus de recherche de l'hôte chez *A. variegatum*.

11) La tique, *A. variegatum*, exsude des gouttelettes odorantes quand elle est dérangée dans l'air autour de tiques calmes et de tiques dérangées. Nous avons analysé les volatils présents dans l'air en utilisant des fibres adsorbantes et du charbon actif pour extractions en phase solide. Un isomère d'octenol était présent dans tous les échantillons sauf ceux des tiques peu avant leur émergence et des exuvies. L'isomère d'octenol était libéré lors de la perturbation des tiques adultes et spontanément à l'apparition des adultes après la mue, ce qui suggère qu'il est libéré comme sécrétion défensive. Il a été identifié comme R-(-) 1-octen-3-ol, l'isomère prédominant dans l'haleine des bovins. L'hexanol, l'hexanal, l'heptanal et le nonanal ont également été identifiés au-dessus des tiques femelles gorgées.

12) Bien que les odeurs des femelles gorgées et de tiques nouvellement muées n'aient pas attiré *A. variegatum* adultes sur la servosphère, les exuvies et les excréments des tiques nouvellement muées étaient attractifs.

13) Trois substances volatiles, c-à-d le 1-octen-3-ol, l'hexanal et le nonanal qui sont associées aux tiques ont été attractives pour les *A. variegatum* adultes sur la servosphère. Comme ces composés sont également présents dans l'odeur de l'hôte, il apparaît que les tiques, qui

possèdent un système olfactif relativement réduit, compensent en utilisant les mêmes composés pour la reconnaissance de l'hôte et à des fins d'agrégation (principe de la parsimonie).

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