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Rumen metabolites serve ticks to exploit large mammals

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Summary

Hard ticks spend most of their life isolated from passing vertebrates but require a blood meal to proceed to the next life stage (larva, nymph or adult). These opportunist ectoparasites must be capable of anticipating signals that render suitable hosts apparent. Large ungulates that tolerate a high ectoparasite burden are the favoured hosts of adult hard ticks. Ruminants, comprising the majority of ungulate species, must regularly eruct gases from the foregut to relieve excess pressure and maintain a chemical equilibrium. Through eructations from individuals, and particularly herds, ruminants inadvertently signal their presence to hard ticks. Here, we report that all adult hard tick species we tested are attracted to cud and demonstrate that these acarines possess olfactory receptor cells for the carboxylic acid, phenol and indole end-products of the rumen bioreactor. Compounds from each of these classes of volatiles attract ticks on their own, and mixtures of these volatiles based on rumen composition also attract. Appetence for rumen metabolites represents a fundamental resource-tracking adaptation by hard ticks for large roaming mammals.

Key words: tick, ectoparasite, rumen, behaviour, neurophysiology, rumen metabolite, *Amblyomma*, *Ixodes*.

Introduction

Terrestrial ungulates, especially ruminants, occupy a dominant position as eutherian herbivores whose eco- and morpho-physiological diversification permits use of all types of plants. These mammals subdivide mostly into either foregut fermenters (ruminants, 197 species) or hindgut fermenters (30 species; Wilson and Reeder, 1993; Eisenberg, 1983) that can exploit wide-ranging areas of younger and older vegetation, respectively (Feldhamer et al., 1999). Ruminants can reach high body masses, the reason why they have acquired a primordial role in human economics. The particular ability of these mammals to maintain and profit from large bioreactors in the gut has enabled them to thrive on nutrient-poor food, thus facilitating their global dispersion. Their roaming habit has led to continent-wide distributions of their haematophagous ectoparasites, especially hard ticks (Acari: Ixodidae). Hard ticks feeding upon wild ruminants represent significant foci for tick-borne diseases that afflict man and his domestic animals. For this reason, these ectoparasites have been the focus of several control programmes (Jaenson et al., 1991).

In contrast to soft ticks (Acari: Argasidae), which have successfully exploited the nidicolous habitat, hard ticks predominate in open range (Klompen et al., 1996). Hard ticks typically spend most of their life cycle on the ground, where they must survive prevailing environmental conditions until host encounter. For this reason, detection of a host at a distance is paramount. Host-emitted odours readily arouse ticks that ambush from questing sites, such as *Ixodes* adults and immature stages of all hard tick species, or provoke orientation in ticks that walk towards their prey, i.e. *Amblyomma* adults. Rumen fluid odour is the product of a stable bioreactor whose major chemical constituents do not vary greatly between ruminant species (Garcia et al., 1994). Given the affiliation of hard ticks for large wandering ungulates such as ruminants, we hypothesised that chemicals eructed from the rumen to the exterior might be significant in mediating their recruitment to suitable hosts.

Materials and methods

Tick species, locomotion compensator and track analysis

Five tick species of different continental origin and phylogeny were used in this study. They were the sub-Saharan bont ticks, *Amblyomma variegatum* Fabricius and *Amblyomma hebraeum* Koch (Ixodidae: Metastriata), the European sheep tick, *Ixodes ricinus* L., the N. Asian taiga tick, *I. persulcatus* Schulze, and the N. American black-legged tick, *I. scapularis* Say (Ixodidae: Prostriata). We recorded the behavioural responses of these ticks to the odour of rumen fluid presented in an air stream on a locomotion compensator. This apparatus has already been employed in the study of arthropod attractants including ticks (McMahon and Guerin, 2002). Parameters of tick locomotion were computed from coordinates supplied by pulse generators recording the displacement of the tick on the compensator (resolution 0.1 mm/0.1 s). Attraction was defined as the % change in time spent walking in a cone 60° either side of due upwind in the test period compared with that of a control period of equal duration (McMahon and Guerin, 2002). Change in speed (in %) was the increase (or decrease) in walking speed in the test compared with the control period (McMahon and Guerin, 2002). The mean walking direction with respect to the air stream (0°) was calculated from the vectors described by each tick using circular statistics (Batschelet, 1981). Simultaneous video recordings of the ticks walking on the compensator allowed records of local search responses of *A. variegatum*. These occur when the tick makes small circles or abruptly turns downwind within 10 s of removal of the test stimulus from the air stream, events that never occur in controls (McMahon and Guerin, 2002).

Odour delivery

Rumen fluid collected from freshly slaughtered cattle was conserved under N_2 at 5°C (<2 weeks) for behavioural tests. This fluid (0.4–1 ml) was applied to a filter paper disk in a borosilicate 500 ml gas-wash flask. Vapours from the flask were evacuated into an air stream (0.18 m s^{-1}; dilution 25×) flowing over an adult tick (male or female) walking on the locomotion compensator (McMahon and Guerin, 2002). No change in the CO_2 concentration in the air stream was detected after presentation of rumen fluid odour using an infrared gas analyser (detection limit ±2 p.p.m.; BINOS1; Leybold-Heraeus, Hanau, Germany). Behavioural responses were recorded for *A. variegatum* or *I. scapularis* to entrained rumen odour, acetic, propionic, butanoic and isobutanoic acid, 3-methylindole and 4-methylphenol, all diluted in dichloromethane and applied to filter paper. After evaporation of the solvent, the filter papers were placed in 500 ml gas-wash flasks from which the test odours are evacuated into an air stream (as above). *A. variegatum* adults were also presented with methane from a pressurised gas cylinder (2.5% v/v in synthetic air) injected into the air stream. The response of each tick was tested only once.

Statistics

Paired tests eliminated most of the biological variation among the behaviours of the individual ticks tested. The significance of the difference between test and control for a given treatment in terms of attraction and % change in speed (not normally distributed) was analysed using the Wilcoxon signed rank test (two-tailed). Differences between treatments were compared with the Wilcoxon–Mann–Whitney test (two-tailed). Comparisons of local search behaviours made by *A. variegatum* in response to removal of different treatments from the air stream were made with the Fisher exact test (two-tailed). These statistical tests were run on S-Plus (v.3.3 release 1, 1995).

Collection of rumen volatiles

The odour of rumen fluid was entrained immediately after collection from a steer (*Bos taurus*) or a roe deer (*Capreolus capreolus*) on a porous polymer (500 mg of Soxhlet-extracted Porapak Q®; Waters, Milford, MA, USA; 50–80 mesh, packed into a 6 cm×6.23 mm-diameter glass tube and stoppered with glass wool plugs). Before use, the cartridge was ventilated for 1.5 h with pure N_2 at 180°C. Pure N_2 (150 ml min^{-1}) was bubbled through the cud to the porous polymer cartridge for 2–5 h. The entrained volatiles were eluted with dichloromethane, and the first 3–5 drops (40–60 µl) were transferred to a glass ampoule and flame sealed for conservation at –20°C until use.

Gas chromatography linked single sensillum recordings

In ticks, odours are perceived *via* ~20 multiporous olfactory sensilla borne dorsally on each foreleg tarsus, with each sensillum housing between 4 and 12 receptor cells (Hess and Vlimant, 1986). Specific volatiles present in rumen fluid odour that excite olfactory receptors in *A. variegatum* and *I. ricinus* were identified by coupling recordings of action potentials from tick olfactory receptor cells to components of the entrained rumen odour eluting in succession from a high-resolution capillary column in a gas chromatograph (Steullet and Guerin, 1994). 2 µl aliquots of the porous polymer-extracted volatiles were injected on-column on a 30 m DB-WAX column (J&W Scientific, Folsom, CA, USA) in a Carlo-Erba 5160 gas chromatograph (Carlo Erba Instruments, Milan, Italy) for the single chemosensillum electrophysiology recordings coupled with flame ionisation detection (FID). The column was held at 40°C for 5 min and then programmed at 5 deg. min^{-1} to 250°C with hydrogen as carrier gas. The column effluent was split (glass Y-splitter) so that 60% was directed to the FID (280°C) and 40% was directed from a heated transfer line (240°C) in the wall of the chromatograph to the electrophysiological preparation in such a way that the column effluent was simultaneously monitored by the FID and the single sensillum preparation. To facilitate electrical contact, the tip of the sensillum was cut under the microscope by prising its tip between two blades held on holders mounted on micromanipulators.

Gas chromatography–mass spectrometry (GC–MS)

The Carbowax column (see above) was installed in a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Meyrin, Geneva, Switzerland) with helium as carrier gas and connected *via* 1 m deactivated fused-silica capillary (0.25 mm i.d.) to the mass selective detector (Hewlett-Packard 5971, ionisation chamber temperature 160°C; ionisation energy 70 eV). The mass selective detector (EI mode) scanned for masses of 20 to 300. The eluting volatiles were identified by comparing their mass spectra with those in a library of the Hewlett Packard Chemstation software and by matching retention times and mass spectra of synthetic analogues.

Quantification of rumen carboxylic acids and aromatics

Rumen supernatant was first brought to pH 14 by the addition of 2 mol l^{-1} NaOH, after which amines plus neutral molecules were extracted into ether. The aqueous layer was brought to pH 1 with 5 mol l^{-1} HCl, and the remaining organic compounds extracted into ether (fraction F1). This ether

Table 1. *Responses of adults of five hard tick species to odour being released from rumen fluid*

	Amblyomma variegatum[b]	*Amblyomma hebraeum*	*Ixodes scapularis*	*Ixodes persulcatus*	*Ixodes ricinus*
N	18	15	20	13	16
Attraction (%)[a]	+44***	+26***	+21***	+13*	+4**
Change in speed (%)	+23**	+5	+1	+28**	+4*

[a] Median % change compared with control: **P*<0.05, ***P*<0.01, ****P*<0.001; Wilcoxon signed rank test.

[b] Eight of the 18 *A. variegatum* adults tested undertook significant local search behaviours (see Fig. 1) upon removal of rumen odour from the air stream (Fisher exact test, *P*<0.01, two-tailed).

fraction was then washed with saturated $NaHCO_3$ solution so that phenols were retained in the organic phase (fraction F2) and carboxylic acids were retained in the aqueous phase. These acids were subsequently extracted into ether after acidification as above (fraction F3). Fractions F2 and F3 were washed with saturated NaCl and desiccated using Na_2SO_4 before analysis by GC–MS. The ratio of butanoic acid:isobutanoic acid:4-methylphenol:3-methylindole recovered was 138:12:4:1.

Results

Behavioural responses to rumen fluid odour

All five tick species were attracted to the odour of rumen fluid collected from freshly slaughtered cattle (Table 1). Over 80% of ticks from each species showed a change in walking direction from mostly downwind in clean air to an upwind response in the presence of the rumen fluid odour (Fig. 1). Three species – *A. variegatum*, *I. persulcatus* and *I. ricinus* – showed a significant increase in speed (Table 1). The most mobile species, *A. variegatum*, responded to removal of rumen fluid odour from the air stream with an intense local search behaviour (Table 1; Fig. 1). This agile species was chosen as the model on which to test ruminal compounds and their mixtures. Rumen fluid odour entrained on a porous polymer was as attractive to *A. variegatum* as the rumen fluid, causing a median increase in upwind displacement of 42% (Table 2).

Tick sensory responses to rumen metabolites

Olfactory receptor cells of both *I. ricinus* and *A. variegatum* were stimulated by the same fractions of rumen fluid odour (Fig. 2). These compounds are from four sources: (1) straight-chain carboxylic acids derived from plant carbohydrates and aliphatic amino acids, i.e. acetic, propionic, butanoic, pentanoic and hexanoic acids; (2) a branched carboxylic acid derived from the branched aliphatic amino acids leucine or valine, i.e. isopentanoic acid; (3) phenols derived from tyrosine, i.e. phenol, 2-nitrophenol, 4-methyl-2-nitrophenol-2- and 4-methylphenol and (4) indoles derived from tryptophan, i.e. indole and 3-methylindole (Fig. 3). This complements a previous study where olfactory receptor cells responding to another branched amino acid catabolite present in the rumen, isobutanoic acid, were identified in *A. variegatum* (Steullet and Guerin, 1994). These four classes of rumen volatiles for which

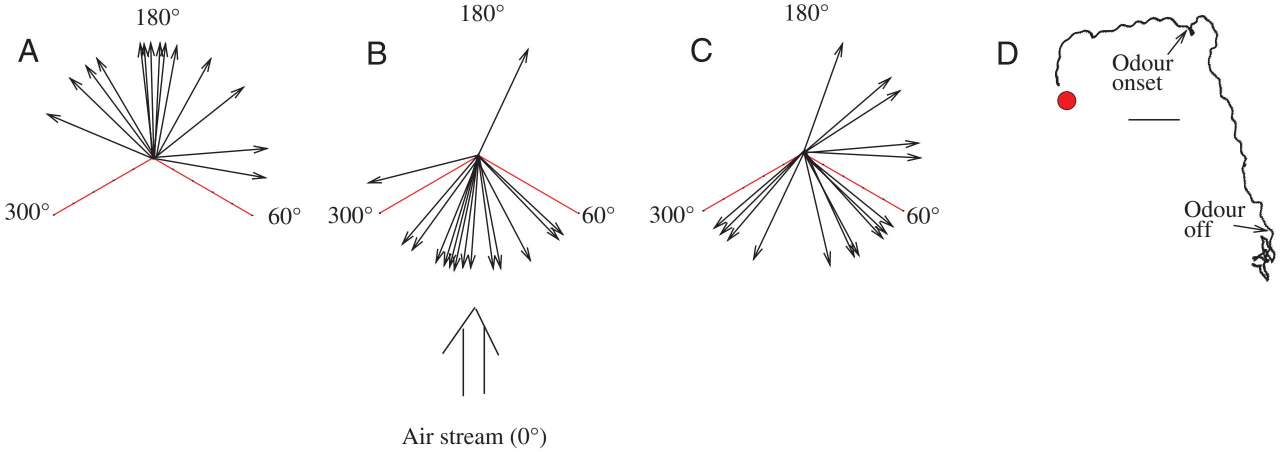

Fig. 1. Attraction of *A. variegatum* to rumen fluid odour. Polar plots of the mean walking direction of 18 *A. variegatum* adults presented in three successive 1 min intervals with (A) a control air stream (0°), (B) rumen fluid odour in the air stream and (C) the control air stream (without rumen fluid) again. Ticks that spent more time walking in the cone delimited by 60° either side of upwind (in red) after presentation of the rumen odour were considered to be attracted. (D) A track described by an *A. variegatum* adult in the same experiment; the track started (filled circle) with the tick walking down or across wind in the control period; the arrows on the track indicate stimulus on and off; the bar represents a displacement of 20 cm. Note the local search behaviour induced in *A. variegatum* by withdrawal of the rumen odour from the air stream. Open arrow at bottom indicates airstream direction (0°) for A,B,C and D.

Table 2. *Responses of adult* A. variegatum *to entrained rumen odour and to individual chemostimulants of the rumen fluid odour and their mixtures*

Treatment	Ticks tested (*N*)	Source dose	% Attraction[1]	% Change in speed[1]
Entrained rumen fluid odour	17	1/10 dilution	$+42^{***,a}$	$+4^{e,f}$
Single components				
Butanoic acid	16	10 ng	$+34^{***,a,b}$	$-34^{**,f,g}$
Isobutanoic acid	29	10 ng–1 μg	$+32^{***,a,b}$	$-9^{*,e,f}$
4-Methylphenol	11	10 ng	$+24^{**,b,c}$	-2^{e}
3-Methylindole	20	10 ng	$+12^{*,b,c}$	$-7^{e,f}$
Acetic acid	14	1 μg	$+15^{*,b,c}$	$+3^{e}$
Propanoic acid	20	10 ng	$+22^{***,a,b}$	$-12^{***,f}$
Mixtures				
Butanoic acid:isobutanoic acid:4-methylphenol: 3-methylindole	18	1 μg:100 ng:10 ng:10 ng	$+25^{***,a,b}$	$-3^{e,f}$
Butanoic acid:isobutanoic acid:4-methylphenol: 3-methylindole	11	1 μg:10 ng:10 ng:10 ng	$+3^{c}$	$-5^{e,f}$
Butanoic acid:isobutanoic acid:4-methylphenol: 3-methylindole	20	10 ng:10 ng:10 ng:10 ng	$+2^{c}$	$+6^{e}$
[2]Methane	13	70 ppm	$+1^{c}$	$-6^{e,f}$

[1] Median % change compared with control: *$P<0.05$, **$P<0.01$, ***$P<0.001$; Wilcoxon signed rank test. Treatments sharing the same letter are not significantly different; ($P>0.05$; Wilcoxon–Mann–Whitney test.

[2] Estimated concentration in the air stream.

receptors have been identified in ticks arise, not as intermediates, but as anticipated stable end-products of different pathways of ruminal fermentation. Short-chain carboxylic acids such as butanoic and isobutanoic acid are absorbed by the host and enter amphibolic pathways whereas toxic aromatic catabolites such as 4-methylphenol and 3-methylindole are excreted in urine (Martin, 1982; Yang and Carlson, 1972).

Tick behavioural responses to rumen metabolites

We then proceeded to measure the behavioural responses of *A. variegatum* to the rumen volatiles that excite its receptor cells. The compounds were chosen from the four classes of chemostimulants. Butanoic and isobutanoic acid, 4-methylphenol and 3-methylindole each attracted *A. variegatum* on their own (Table 2). However, a mixture of these products where butanoic acid, isobutanoic acid, 4-methylphenol and 3-methylindole were presented together at an identical source dose (1:1:1:1) failed to induce attraction in *A. variegatum* (median increase=2%, $P>0.4$, $N=20$; Table 2). As straight-chain carboxylic acids predominate among the organic compounds of rumen fluid (Clarke and Bauchop, 1977), we increased the dose of butanoic acid to 100 times the dose of the other three constituents, i.e. at 100:1:1:1, but this mixture also failed to attract (median increase=3%, $P>0.5$, $N=11$; Table 2). The relative amounts of each component of the mixture were then adjusted according to their approximate proportions ($\mathrm{g\,ml^{-1}}$) in rumen fluid to give a 100:10:1:1 mixture of butanoic acid:isobutanoic acid:4-methylphenol:3-methylindole. This four-component mixture induced attraction at approximately half that recorded to rumen fluid odour (median increase=25%, $P<0.001$, $N=18$; Table 2). This same synthetic mixture (at a 10× higher dose) also induced attraction in *I. scapularis*, similarly at half that induced by the natural odour (median increase=12%, $P<0.01$, $N=16$). These results indicate that both the nature and proportions at which rumen volatiles are present in an odour are important to ticks. The discrepancy recorded here between the behavioural responses to the synthetic mixtures and the natural rumen odour may be in part due to the absence in our test mixtures of gaseous rumen fluid components (NH_3, H_2S) for which ticks have receptor cells (Steullet and Guerin, 1992, 1994). It is also the case that ticks respond to other constituents of natural rumen odour absent in the test mixtures such as acetic and propionic acid (Table 2). Methane, a major end-product of rumen metabolism (Clarke and Bauchop, 1977), failed to induce any behavioural response (Table 2).

Discussion

Our finding that odours arising from gut fermentation attract a range of hard tick species corresponds to their association with ungulates and their particular life history. Systematic analysis based on structural and developmental characters suggests an Australian origin for hard ticks in the Cretaceous (Klompen et al., 1997). Nevertheless, the earliest record of this group occurs in the northern hemisphere, dating from the Oligocene (Weidner, 1964). In this era, ungulates were already established as dominant herbivores (Sloan et al., 1986), and hard ticks have proved adept at exploiting this resource. Ungulates, however, do not hibernate and must continually forage, presenting a difficult target for the free-living non-

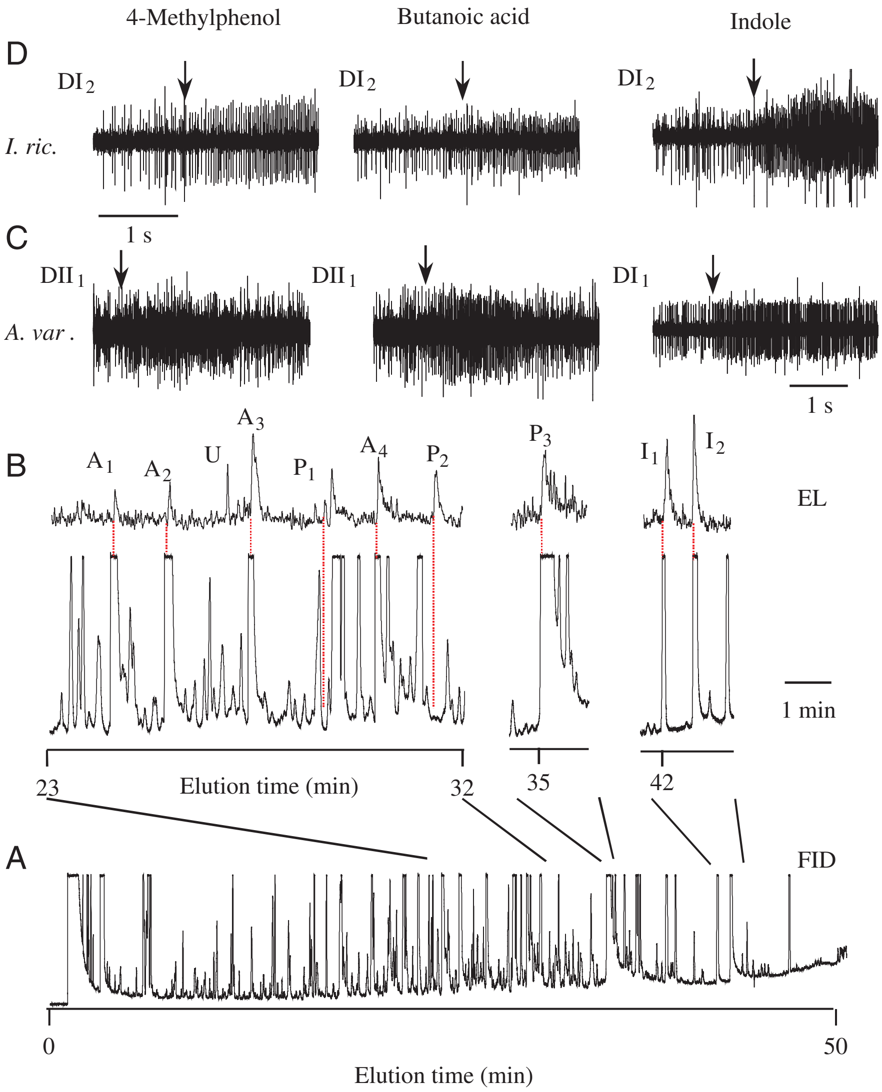

Fig. 2. Identification of rumen volatiles that stimulate olfactory receptor cells of ticks. Cattle rumen volatiles were analysed after collection on a porous polymer by gas chromatography coupled with electrophysiology recordings from olfactory receptor cells in wall-pore olfactory sensilla on the first leg tarsus of *Amblyomma variegatum* and *Ixodes ricinus*. (A) Flame ionisation detector (FID) response of the gas chromatograph to the separated rumen volatiles. (B) Enlarged sections of A where butanoic (A_1), isopentanoic (A_2), pentanoic (A_3) and hexanoic (A_4) acids, 2-nitrophenol (P_1), 4-methyl-2-nitrophenol (P_2), 4-methylphenol (P_3), indole (I_1) and 3-methylindole (I_2) eluted and induced electrophysiological (EL) responses from olfactory receptor cells of *A. variegatum* and *I. ricinus* (trace for *A. variegatum* presented). The receptor cell response profiles in B were generated by summing the frequencies of the action potentials of the responding sensory cells (frequency to voltage conversion). U indicates an unidentified stimulus. (C,D) The spike trains generated in responding (from the arrows) *A. variegatum* (C) and *I. ricinus* (D) receptor cells to 4-methylphenol, butanoic acid and indole eluting from the gas chromatographic column. Receptor cells from both tick species also responded to acetic acid, propionic acid, phenol and 2-methylphenol (data not presented). The same carboxylic acid and phenolic volatiles identified in rumen odour of cattle were also recovered in the odour of roe deer rumen. Sensillum identity (DI and DII types) is according to a classification by Hess and Vlimant (1982).

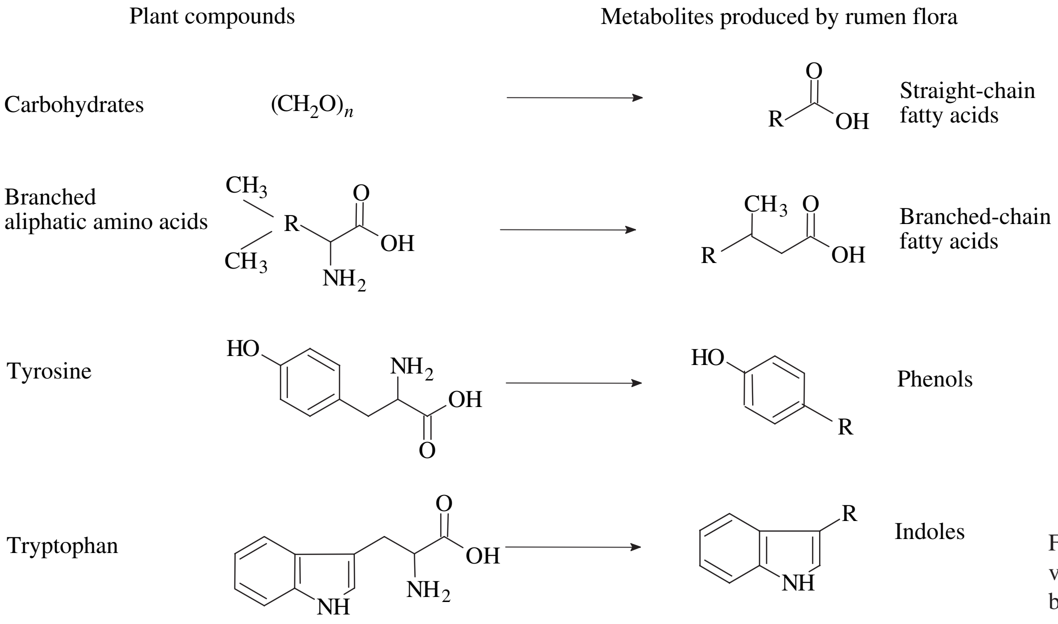

Fig. 3. Microbial production of volatile rumen products perceived by ticks.

volant hard ticks. It is generally true that complex multi-host life histories arise in parasites when encounters with the final host (where reproduction can take place) present a limiting factor. Hard ticks fit this scheme, enduring an arduous three-host life cycle (larva, nymph, adult), with each life stage requiring successively bigger blood meals to proceed to the next stage. The immature stages feed on almost any vertebrate, but adults require blood meals of more than 1 ml (Rechav et al., 1994) from animals that can better provide it, such as ungulates. Further, the transience of such hosts imposes semelparity, where reproduction depends entirely on the ingestion of a single blood meal by the female. In Metastriates, the more advanced class of hard ticks, the coupling of feeding to reproduction is still more pronounced, since fecundation only occurs on the final host and males even condition the host immune system so that females can feed to repletion (Wang et al., 1998).

The attraction of ticks to volatile rumen end-products explains how ticks track their ruminant hosts. The rumen fermenter generates a great variety of partially oxidized volatiles in which short-chain carboxylic acids (acetic, propionic, butanoic and isobutanoic) predominate (Garcia et al., 1994). Ruminants eruct at regular intervals (once every 2–3 min), voiding half the rumen gaseous contents into breath in 1 h (Waghorn and Stafford, 1993). Breath itself is a fundamental host cue for both ticks (McMahon and Guerin, 2002) and other haematophagous arthropods (Guerin et al., 2000; Stange, 1996). Nevertheless, anaerobic environments are not exclusive to the rumen, and hard ticks feed on a wide range of both mono- and digastric vertebrates (Cumming, 1999). Moreover, no compound formed in the rumen is specific to the foregut, and all occur to a lesser extent in the hindgut, particularly in the large bioreactors in the caecum and large intestine of other ungulates, although it is difficult to quantify volatiles released from such regions. Similar products may also be released from protected areas of the host pelage where oxygen may be limiting, such as the axillar and genital areas, preferred tick predilection sites. It is also interesting that these same classes of compounds feature prominently elsewhere in the biology of these ticks: 2-nitrophenol, derived from the anaerobic degradation of tyrosine and released from cattle (Steullet and Guerin, 1994), forms the major component of the aggregation–attachment pheromone secreted from dermal glands in at least two *Amblyomma* spp. (Diehl et al., 1991; Guerin et al., 2000), and isobutanoic acid, derived from valine or leucine, is a component of the aggregation–attachment pheromone of *A. hebraeum* (Apps et al., 1988). Such scents are secreted by feeding males to enhance the recruitment of conspecifics to the same host (Norval et al., 1989). Similarly, other phenols (2-, 3- as well as 4-methylphenol) derived from tyrosine are important attractants for tsetse flies (Saini and Hassanali, 1994). 3-Methylindole, derived from tryptophan, is a known oviposition stimulus for mosquitoes (Beehler et al., 1994), and the rumen products indole, acetic acid and propionic acid are also olfactory stimulants for mosquitoes (Meijerink et al., 2000). Recent investigations show that aliphatic acids present on human skin induce mosquito attraction (Bosch et al., 2000). The importance of products released from an anaerobic milieu may represent a general motif in the sensory ecology of haematophagous arthropods and, particularly, in their resource-tracking habit on vertebrates.

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