AGGREGATION PHEROMONES OF THE BONT TICK *AMBLYOMMA HEBRAEUM:* IDENTIFICATION OF CANDIDATES FOR BIOASSAY

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ABSTRACT

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Volatiles emitted by male *Amblyomma hebraeum* while feeding on a rabbit were quantitatively sampled by the dynamic solvent effect and analysed by capillary gas-liquid chromatography and mass spectrometry. Changes in emissions of 2-methyl propanoic acid, benzaldehyde and 2-nitrophenol paralleled reported increases in the attractiveness of males to conspecific ticks after 4–5 days of feeding.

These compounds are therefore candidates for the bioassay of pheromonal activity.

INTRODUCTION

The use of phenolic compounds as attractants of males to feeding females appears to be general among ixodid ticks (Wood, Leahy, Galun, Prestwich, Meinwald, Purnell & Payne, 1975; Gothe, 1987). In 3 species; *Amblyomma hebraeum, Amblyomma maculatum* and *Amblyomma variegatum* feeding males also produce a chemical signal which attracts other males, females and nymphs (Gladney, Grabbe, Ernst & Oehler, 1974; Rechav, Parolis, Whitehead & Knight, 1977; Schöni, Hess, Blum & Ramstein, 1984). Such an attractant has obvious potential as a component of a tick control formulation. Rechav & Whitehead (1978) report the successful use of mixtures of acaricide with extracts of several hundred male ticks for control of *A. hebraeum* on cattle.

Using sophisticated on- and off-host bioassays Rechav, Whitehead & Knight (1976) and Rechav *et al.* (1977) established that the attractant emitted by *A. hebraeum* is volatile and that the attractiveness of males increases after 4–5 days feeding, reaching a plateau after 8–9 days. The chemical identity of the signal was not established.

The approach used in the present study was to exploit the quantitative precision of dynamic solvent effect sampling (Apps, Pretorius, Lawson, Rohwer, Centner, Viljoen & Hulse, 1987) and high resolution gas-liquid chromatography by sampling volatile compounds emitted by male *A. hebraeum* while actually feeding on a host. Compounds whose temporal patterns of emission paralleled changes in the attractiveness of male ticks were identified as candidates for bioassay. This paper records the identity of 3 such compounds. Their bioassay is beyond the scope of this study.

MATERIALS AND METHODS

Two adult, male, California white rabbits were used as hosts. On both rabbits a circular patch of hair about 25 mm in diameter was clipped very short on each flank. A glass cup 20 mm across and 20 mm deep with a screw top which held a perforated polytetrafluorethylene (PTFE) disc was glued over each clipped patch with a cyanoacrylate adhesive (Fig. 1). The following day 6 adult, male *A. hebraeum* were introduced into 1 cup on each rabbit. The other cup served as a control. Removal of the glass cups and the ticks by the rabbits' grooming was limited (though usually not prevented) by fitting the rabbits with cloth jackets. Water and rabbit pellets were available *ad lib.*

Each day samples of volatiles emitted by the ticks and from the control cups were obtained as follows: one end of a dimethyldichlorosilane[1] (DMDCS)-deactivated glass

tube was inserted through a hole in the lid of the cup and the other end was connected to a dynamic solvent effect concentrator with n-hexane as solvent (Apps *et al.*, 1987). Air was drawn from the cups and through the concentrators at a flow rate of 15 mℓ min^{-1} for 15 min for each sample (Fig. 1). Condensed water was evaporated from the concentrator by a 15 mℓ min^{-1} flow of palladium-purified hydrogen for 5 min.
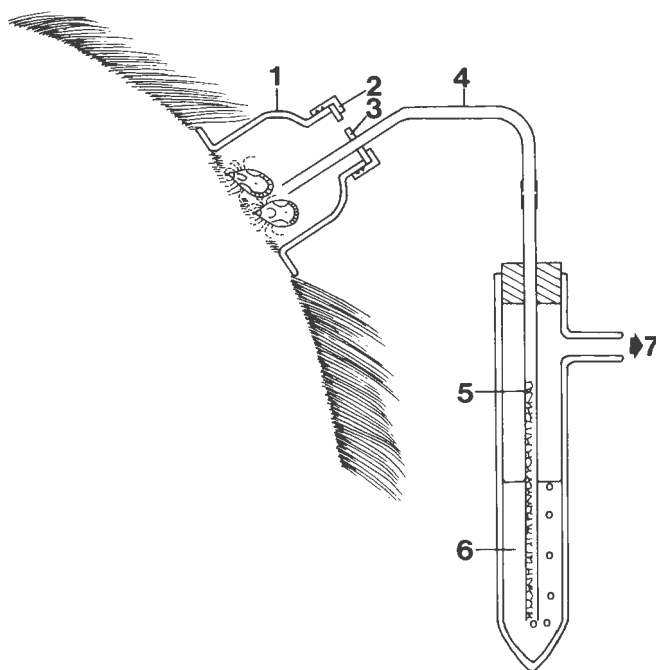

FIG. 1 Apparatus used for sampling volatiles emitted by feeding ticks. 1. glass cap attached to host's skin with cyanoacrylate adhesive, 2. screw cap, 3. perforated PTFE disc, 4. deactivated glass tube, 5. dynamic solvent effect concentrator, 6. n-hexane solvent, 7. vacuum mass-flow controlled at 15 mℓ min^{-1}, 8. ticks.

Volatiles collected by the concentrators were separated by high resolution, gas-liquid chromatography using the technique described by Apps *et al.* (1987). Analyses were carried out on a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet, with a 25 m × 0,3 mm × 0,4 μm methyl silicone capillary column. The carrier gas was hydrogen with a linear velocity of 55 cm s^{-1}. The starting temperature of both inlet and column was 40 °C, the inlet was heated ballistically to

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[1] Pierce, Rockford, Illinois

220 °C after 2,5 min and the column temperature was programmed at 5 °C min^{-1} after 6 min. The flame ionisation detector sensitivity was 4×10^{-11} A mv^{-1} full scale deflection.

If a peak appeared on chromatograms of tick samples and not on chromatograms from controls, and increased in area after 4 or more days feeding by the ticks it was identified by gas chromatography-mass spectrometry (GC-MS) using a Micromass 16F mass spectrometer with an electron energy of 70 eV and a source temperature of 200 °C. Identities based on mass spectrum library searches were confirmed by comparison of retention times with those of reference compounds.
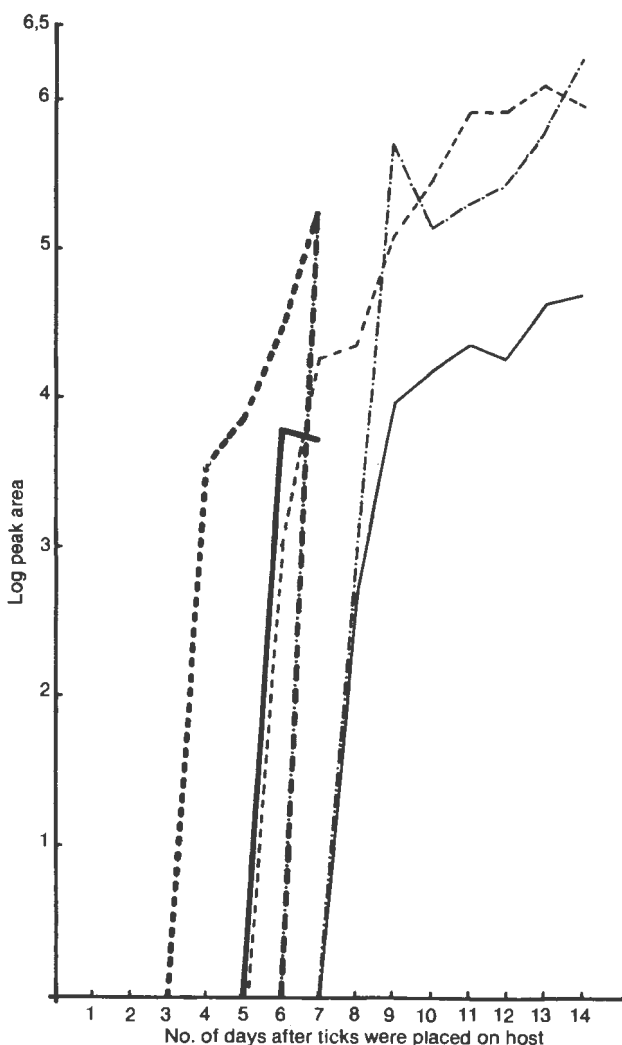

FIG. 2 Time courses of emission of volatiles by feeding, male *Amblyomma hebraeum*. The heavy and light lines represent 2 different series. The dashed lines plot 2-nitrophenol emissions, the solid lines plot benzaldehyde emissions and the dashed/dotted lines plot 2-methyl propanoic acid emissions in each series.

RESULTS

In only 2 cases did the glass cups and the ticks survive the rabbits' grooming long enough for the ticks to begin signalling. Three peaks showed a marked, progressive increase in area on the chromatograms of volatiles emitted by feeding ticks 4–6 days after the introduction of the ticks to the host (Fig. 2). These peaks were identified by GC-MS as; 2-methylpropanoic acid [m/z(intensity) 27(27 %), 29(6 %), 39(13 %), 41(40 %), 42(10 %), 43(100 %), 45(10 %), 55(4 %), 73(25 %),88(8 %)], benzaldehyde [m/z(intensity) 50(16 %), 51(33 %),

52(8 %), 74(6 %), 77(90 %), 78(15 %), 105(100 %), 106(94 %)] and 2- nitrophenol [m/z(intensity) 30(7 %), 51(10 %), 53(16 %), 63(28 %), 64(26 %), 65(43 %), 81(19 %), 92(9 %), 93(13 %), 94(6 %), 109(24 %), 122(7 %), 139(100 %)]. Other peaks which appeared only in chromatograms from tick infested skin, and which showed transient increases, were identified as a series of short to medium chain fatty acids (Table 1).

TABLE 1 Fatty acids emitted intermittently by six male *Amblyomma hebraeum* while feeding on a rabbit and the days after attachment when they were detectable by dynamic solvent effect sampling and capillary gas-liquid chromatography

Acid	Days after attachment on which fatty acids were detectable
n-butanoic	9, 11, 14
2-methyl butanoic	11, 14
n-pentanoic	11, 12, 13
methyl pentanoic	13, 14
n-hexanoic	13, 14
n-heptanoic	14
n-octanoic	14
n-nonanoic	14

DISCUSSION

Some of the compounds accumulated from areas of skin infested with ticks could have originated from the skin rather than being produced by the ticks themselves. Even if this was the case such compounds could still be attractive to ticks. Nevertheless the 3 compounds identified as potential pheromones must have come from the ticks because they disappeared soon after the ticks had been removed, while the inflamed appearance of the skin remained the same. Additionally none of the 3 candidate signal compounds were collected from an area of skin to which 3 ticks remained attached for 6 days after they had died.

The time between placing the ticks on the host and the first detection of 2-nitrophenol was 4 days in one series and 6 days in the other, 2-methylpropanoic acid was first detected after 6 and 8 days and benzaldehyde after 7 and 8 days respectively. Rechav *et al.* (1977) first detected aggregation around males after they had fed for 5 days. The apparent delay in the detection of volatiles in one of the present series may have been due to slow attachment of the ticks. In preliminary experiments some ticks had still not attached 24 h after they were placed on the host.

There was no definite plateau in the emission of the 3 candidate signal compounds, while bioassays show a levelling off in aggregation after 8 days (Rechav *et al.*, 1977). This may have been due to a plateau of response to increasing signal strength, to a logarithmic relationship of response to signal intensity or to the failure of some ticks (approximately 10 %) to respond to signals of any strength.

Rechav (1978) suggested that the aggregation signal in *A. hebraeum* was a mixture of 2 or more components and Rechav, Norval & Oliver (1982) demonstrated the existence of species-specific and -non-specific aspects of the aggregation response in *A. hebraeum* and *A. variegatum*. Using extracts of 100 fed males Schöni *et al.* (1984) identified and bioassayed the volatile aggregation signal of *A. variegatum*. The latter tick employs 2-nitrophenol as a relatively long-range (100–200 mm) attractant. Mounting and clasping are stimulated by nonanoic acid and methyl salicylate in *A. variegatum* while 2-methyl propanoic acid and benzaldehyde have been found in *A. hebraeum*. The *A. variegatum* aggregation signal thus

has a non-species-specific, long-range component and a species-specific short-range and contact component. In addition male *A. hebraeum* intermittently produce small amounts of nonanoic, and other medium chain, acids. This situation is compatible with Norval & Rechav's (1979) report of a weak response by *A. variegatum* to fed, male *A. hebraeum* in a bioassay based on attachment. It may also account for the occurrence of sterile, interspecific matings between these two species when no conspecific partners are available (Rechav *et al.*, 1982). An analogous case of generalised long-range attraction coupled with species-specific short-range signals has been described for *Hyalomma dromedarii* and *Hyalomma anatolicum excavatum* by Khalil, Sonenshine, Sallam & Homsher (1983).

Gladney *et al.* (1974) have attracted female *A. maculatum* to acaricides by using extracts of fed males, and the similar use of extracts of fed, male *A. hebraeum* has been demonstrated by Rechav & Whitehead (1978). Synthetic, rather than natural, attractants must be available for such an approach to the control of ticks to be commercially viable. The identification provided here of 3 potential components of such a synthetic attractant is an additional step towards its formulation and possible large-scale application.

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