

## BIOSYNTHESIS, PRODUCTION SITE, AND EMISSION RATES OF AGGREGATION-ATTACHMENT PHEROMONE IN MALES OF TWO *Amblyomma* TICKS

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**Abstract**—The aggregation-attachment pheromone components *o*-nitrophenol (ONP) and methyl salicylate (MS) in male *Amblyomma variegatum* ticks appeared after three days of feeding on the host and reached high values after about six days. Variable quantities of 1.3–7.3  $\mu\text{g}$  ONP and about 0.6  $\mu\text{g}$  MS were present within ticks. ONP and MS were released at the high rates of 300–1800 ng/hr and 20–600 ng/hr per male tick, respectively. After a temporary decrease, males continued to emit at high rates after nearby attachment of females. In *A. hebraeum*, ONP showed a similar pattern, but with a delay of about a day. A male, which had fed during 14 days, contained about 2  $\mu\text{g}$  and released 225–280 ng/hr. Emission in forcibly detached males of both species dropped rapidly to low levels of less than 10 ng/hr per tick. Host skin and tick feces in the vicinity of feeding males were pheromone-impregnated. The very high emission rates are consistent with the observations that the pheromone is an important component of the host-location mechanism of conspecifics. ONP and MS are produced in the dermal glands type 2 associated with the ventrolateral cuticle.

**Key Words**—*Amblyomma variegatum*, *Amblyomma hebraeum*, Acari, aggregation-attachment pheromone, host location, *o*-nitrophenol, methyl salicylate, pheromone glands.

### INTRODUCTION

The partially fed females of at least 14 tick species from five genera of metastriate ticks attract fed males by using the volatile 2,6-dichlorophenol as sex

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attractant pheromone; this substance is produced by the foveal glands (Sonenshine, 1984, 1985, 1986, Sonenshine et al., 1982). After establishment of close contact, the following scenario occurs in *Dermacentor andersoni* and *D. variabilis*. A mounting sex pheromone induces the mounting and exploratory behavior that guides the male to the gonopore on the female venter (Hamilton et al., 1989). The male then probes the gonopore and inserts its chelicerae. After recognition of a genital sex pheromone, copulation is induced and a spermatophore placed into the vagina (Allan et al., 1988, 1989).

Unlike the situation in most metastriate species, the males of *Amblyomma gemma*, *A. hebraeum*, *A. maculatum*, *A. lepidum*, and *A. variegatum* begin to emit an aggregation-attachment pheromone after several days of feeding on the host. This pheromone attracts conspecific females and males to the feeding males, and in *A. hebraeum* even nymphs (Gladney, 1971; Gladney et al., 1974a,b; Norval and Rechav, 1979; Rechav, 1977; Rechav et al., 1976, 1977; Schöni, 1987; Schöni et al., 1984). The males clasp the attracted ticks very efficiently; this results in the formation of dense clusters at suitable feeding sites like the genitals or belly.

The pheromone is active over a considerable distance; steers infested with male *A. hebraeum* (Norval et al., 1989a), or artificial sources of pheromone for *A. hebraeum* and *A. variegatum* proved to be attractive over several meters downwind (Hess and De Castro, 1986; Norval et al., 1989b). In an extreme case, a few *A. hebraeum* males reached the steer within 13 min of first being sighted 9 m away (Norval et al., 1989a). The pheromone is thus an important component of the host-location bouquet of products, most probably together with other host volatiles such as CO<sub>2</sub>.

The aggregation-attachment pheromone of six-day-fed male *A. variegatum* is composed of *o*-nitrophenol (ONP), methyl salicylate (MS), and nonanoic acid in a ratio of 2:1:8 µg per tick (Schöni, 1987; Schöni et al., 1984). The long-range attractant ONP induced an incomplete aggregation; MS and nonanoic acid contribute to the complete activity, but do not induce aggregation when offered separately (Schöni, 1987; Schöni et al., 1984). The closely related *A. hebraeum* emitted ONP, benzaldehyde, and 2-methylpropanoic acid, along with some nonanoic and other medium chain fatty acids (Apps et al., 1988).

The sex attractant pheromone 2,6-dichlorophenol (2,6-DCP) has been reported in females of *A. variegatum* and in both sexes of *A. maculatum* (Kellum and Berger, 1977; Wood et al., 1975). In the latter species, this pheromone has been shown to attract fed males; but no behavioral response was observed in females to explain the presence of 2,6-DCP in males.

Males of both *A. variegatum* and *A. hebraeum* become attractive only after about three or five days of feeding, respectively (Norval and Rechav, 1979; Rechav, 1977; Schöni, 1987; Schöni et al., 1984). In this paper we report on the time course of biosynthesis and emission of the aggregation-attachment

pheromone components ONP and MS in *A. variegatum* males and, to a lesser extent, of ONP in *A. hebraeum* males, from the initiation of feeding on rabbits. We also looked for the possible site of pheromone production among the different exocrine glands of *A. variegatum*.

#### METHODS AND MATERIALS

*Ticks.* Adults of *Amblyomma variegatum* Fabricius and *A. hebraeum* Koch (Acari, Ixodidae) were supplied by Ciba-Geigy Ltd. (St. Aubin, Switzerland), where they are bred on Simmenthaler calves. During molting, ticks were held at 29°C and 90% relative humidity and thereafter in constant darkness at 28°C and 80–90% relative humidity for several months. Ticks were never older than about 9 months.

For on-host experiments, the ticks were placed inside a nylon bag glued to the shaved back of mature New Zealand female rabbits (weight ca. 2 kg). Before adding the unfed *A. variegatum* males, a synthetic pheromone mixture of 100 ng of ONP, 30 ng of MS, and 250 ng of nonanoic acid dissolved in a few microliters of methanol was placed at several locations on the host skin to induce rapid attachment. Benzaldehyde and 2-methylpropanoic acid replaced MS for *A. hebraeum* (cf. Apps et al., 1988). Ticks attached generally within the first 30–45 min. Unattached ticks were removed after 1 hr to ensure good synchronization.

*Chemicals.* All chemicals for pheromone extractions and analysis were of analytical or HPLC (high-performance liquid chromatography) grade.

*Pheromone Extractions.* Five to 10 ticks were removed from the host with forceps at selected times during feeding, placed in methanol, cut into small pieces with fine scissors, and the volume made up to 1 ml/5 ticks. The extract was sonicated for about 2 min in an ultrasonic bath and then stored at –18°C. Recovery rates were estimated to be about 95% as judged from an experiment where known amounts of ONP and MS were injected into unfed males before extraction.

*Hydrolysis.* A 0.1-ml aliquot of the methanolic extracts from unfed males or mature males, which had fed for 14 days, corresponding to about one-half tick equivalent, was mixed with 0.4 ml H<sub>2</sub>O and 0.5 ml KOH (5 M) and then boiled under reflux for 20 min. After cooling, an aliquot of 0.1 ml was acidified to about pH 2 with 6 M hydrochloric acid. Methanol was added to 30% before separation by HPLC.

*Dissection.* Dissection of ticks was performed in cell-culture medium TC199 (Seromed) under a binocular microscope with very fine scissors and forceps. The various organs were rinsed several times in fresh dissecting medium before extraction and sonication in methanol.

*Histology.* Pieces of dorsal and ventrolateral cuticle were fixed in 2.5% glutaraldehyde in a phosphate buffer (Sørensen, 0.1 M at pH 7.2, with 1.5% saccharose for 2 hr at 4°C) followed by postfixation in a mixture containing 1% OsO<sub>4</sub>, 1.25% K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, and 11% saccharose for 24 hr at 4°C. After three rinses in distilled water containing 11% saccharose for 30 min, the pieces were dehydrated in acetone and embedded in Spurr's medium. Semithin sections were cut with a diamond knife and stained with toluidine blue.

Some of the dehydrated cuticle pieces were critical-point-dried in CO<sub>2</sub> in a Balzers CPD device for scanning electron microscopy. The mounted specimens were coated with carbon and then gold sputtered in a Balzers sputtering apparatus. The dermal glands were observed in a Philips 500 PSEM scanning electron microscope.

*Sampling of Volatiles.* Sampling of volatile pheromones was performed by absorption to reverse-phase Sep-Pak Light cartridges (C<sub>18</sub>, Millipore) coated with NaOH (Kuwata and Tanaka, 1988). To collect volatiles from ticks on the host, a simple apparatus was constructed from a polypropylene funnel, diam. 3.2 cm. A small polypropylene Y piece as an air inlet was glued into the funnel wall; the stem of this Y piece, acting as inlet, was connected by a short piece of plastic tubing (PVC) to an air-purifying system consisting of (1) an activated charcoal filter (20 cm × 2 cm), (2) a Sep-Pak cartridge, and (3) a small pipet containing Porapak (50–80 mesh, Millipore), in series. A short tubing led from the funnel outlet to another Sep-Pak cartridge followed by a Porapak-filled pipet connected to a water pump. The modified funnel was lightly pressed against the skin of the rabbit with the attached ticks, and the air sucked through the device at a rate of about 30 ml/min for 15 min.

In the case of sampling from ticks removed from the host, the funnel was replaced by a 500-ml gas-wash bottle containing the ticks.

The absorbed volatiles were eluted by gently pushing about 100 μl of methanol through the Sep-Pak cartridge with a small syringe. Generally, the first two drops were yellow, indicating the presence of ONP. We observed no breakthrough into the back-up Porapak cartridge.

*HPLC.* HPLC separations were performed with a Kontron liquid chromatograph consisting of two 420 pumps, a Rheodyne 7125 injector with a 1-ml sample loop, and a 250 mm × 4 mm or a 125 mm × 4 mm reverse-phase column RP-18 (Nucleosil 120-5 C<sub>18</sub>, Macherey-Nagel). For separation, a gradient of methanol–aqueous buffer (KH<sub>2</sub>PO<sub>4</sub>, 0.02 M at pH 4) was employed at a flow rate of 1.2 ml/min at room temperature: 30 sec at 30% methanol, then step to 50% followed by a gradient to 100% methanol in 5 min and a final purge at 100% for 10 min. Before injection, sample aliquots of up to 400 μl were prepared by adjusting with buffer to contain 30% methanol and then centrifuged, if particles were present. In general, we injected aliquots of between 0.1 and 0.5 tick equivalents for whole tick extracts, and half or the entire extract in the case of the sampled volatiles.

UV-VIS detection of the eluting compounds was achieved with Hewlett-Packard HP 1040 photodiode array detector controlled by a HP ChemStation with chromatograms registered simultaneously at 210, 254, and 280 nm. For identification, the retention times and spectra of unknown compounds were compared with spectra from various reference compounds injected at the same conditions. Quantification of ONP and MS was done with the external standard method at 210 nm. The minimum quantities of ONP or MS for unambiguous identification were about 30–50 ng.

The reproducibility of retention times and area measurements was tested by repetitive injections of standards. For ONP, the retention time and the area showed coefficients of variation of 0.3 and 2.2%, respectively. In view of the good reproducibility, we injected only one aliquot of the different extracts. Between injections, both syringe and injector were carefully rinsed with methanol. Blank runs revealed no cross contamination.

*GC Electrophysiology.* The presence of ONP, MS, and nonanoic acid in a few extracts was tested by injection of 1- $\mu$ l extract aliquots onto a capillary GC (Carlo-Erba HRGC 5160 Mega) fitted with an on-column injector and a FID and ECD detector in tandem. Separation was achieved on a G&W DB-wax fused silica capillary column (30 m, 0.32 mm ID) with H<sub>2</sub> (linear flow at 0.5 m/sec) using a temperature program of 80–230°C at 8°C/min. The column was simultaneously interfaced with the ONP-sensitive dII wp/sw/A sensilla of the Haller's organ on the tarsus of the first pair of legs (Schöni, 1987) via a glass Y-piece outlet splitter. The branch of the splitter leading to the biological detector passes through a heated (250°C) transfer line in the wall of the chromatograph. Upon exit to the exterior, the effluent is swept over the tick sensillum by a temperature- (22°C) and humidity- (100% relative humidity) controlled airstream in a water-jacketed glass tube, at a flow rate of 35 cm/sec. Responses of the olfactory cells were recorded with a glass electrode in contact with the severed tip of the sensillum. The reference electrode, also of glass, was placed at the base of the leg. Action and receptor potentials were recorded using an amplifier (Syntech, Hilversum, Netherlands) linked to a pulse-code modulator and video-cassette recorder (Gödde, 1985). Identification was based on the selective response of the biological detector to ONP and matching of retention times of unknowns with authentic standards.

## RESULTS

*Biosynthesis of Attachment-Aggregation Pheromone during Feeding.* In whole extracts of *Amblyomma variegatum* males that fed on rabbits, traces of ONP were detectable by HPLC for the first time on day 4 after onset of feeding (150 ng/tick). The concentration increased during the following two days to 1.3–2  $\mu$ g/tick at day 6. After this and up to day 19, variable amounts of about

1.3–7.3  $\mu\text{g}/\text{tick}$  were measured (mean  $3.4 \pm 0.7 \mu\text{g SEM}$  er tick;  $N = 9$ ). Methyl salicylate was detected only at day 8 (40 ng/tick), and thereafter reached about 0.6  $\mu\text{g}/\text{tick}$  after day 10. Males removed at day 1, 2, or 3 after onset of feeding and kept alive at 30°C and 90% relative humidity did not contain detectable amounts of ONP or MS if assayed later at day 8. After hydrolysis of extracts of unfed males, no ONP or salicylic acid (from MS) was present. Hydrolysis of extracts from mature males also did not increase the pheromone content cited above.

Extracts of *A. hebraeum* males by day 14 of feeding contained about 1.9  $\mu\text{g}$  ONP per tick; benzaldehyde was not detectable under our conditions.

*Emission Rates of Attached Ticks.* On one rabbit, traces of ONP emitted by *A. variegatum* males and the surrounding host skin covered by the sampling device were first detected at day 3 after onset of the blood meal (7 ng/hr per tick, see Figure 1A). Thereafter emission rose rapidly and reached a plateau at day 6 (620 ng/hr per male tick).

When four female ticks were added to these males at day 12, the ONP emission rate of 975 ng/hr per male tick just before contact, dropped after the encounter between the sexes over the next 4 hr to a level of 110 ng/hr per tick, but thereafter increased again to variable levels of 310–1650 ng/hr per male tick (Figure 1A). At day 12 we began to observe another control group of four males in parallel on the same rabbit (Figure 1A). The emission was comparable to that of the paired females–males, with variable rates between 350 and 1780 ng/hr per male tick.

Traces of volatile MS was detectable for the first time in *A. variegatum* males at day 4 of feeding (5 ng/hr per tick; Figure 1B). Increase in emission over the next days followed the pattern described for ONP. A drop occurred after pairing of the males with the females. The variable rates were lower than for ONP (20–600 ng/hour per tick). The ratio between emitted ONP and MS after 12 days of feeding varied between 2:1 and 7:1 with a mean value of about 4:1.

Host skin adjacent to 12-day feeding ticks also emitted pheromone products: ONP at 40 ng/cm<sup>2</sup>/hr and MS at 10 ng/cm<sup>2</sup>/hr. An area beginning about 5 cm away from the nearest tick gave off ONP at about 10 ng/cm<sup>2</sup>/hr. No emission was detected from areas further away. In addition, feces collected near a tick contained about 140 ng ONP and 35 ng MS per milligram dry weight.

*A. hebraeum* males began to emit ONP one day later than *A. variegatum* males at a rate of 8 ng/hr per tick (Figure 2). The release appeared to reach a plateau after seven days of feeding with rates of 225–280 ng/hr per tick.

*Emission Rates of Forcibly Removed Ticks.* Mature ticks of both *Amblyomma* species, forcibly removed after 14 days of feeding, released ONP during the first few minutes at rates comparable to attached ticks (Figure 3; 950 and 250 ng/hr per male tick for *A. variegatum* and *A. hebraeum*, respectively).

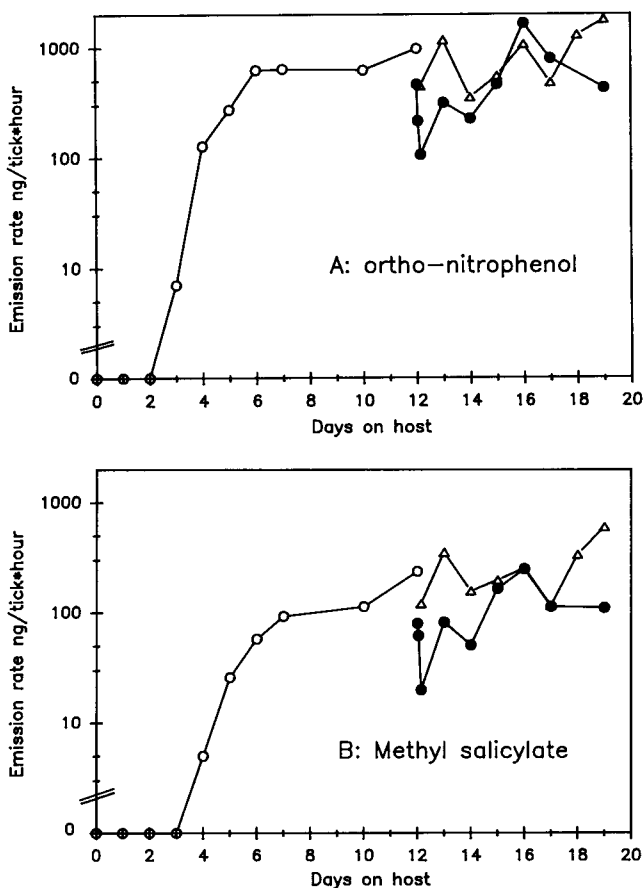


FIG. 1. Emission rate of *o*-nitrophenol (A) and methyl salicylate (B) and four males of *Amblyomma variegatum* after attachment on the rabbit (open circles). At day 12, four females were added; after an initial drop, the males continued to emit at high rates (filled circles). Open triangles: another group of four males without females on the same rabbit.

However, the emission dropped within a few hours to very low levels of less than 10 ng/hr per male tick in both species. Emission of MS in *A. variegatum* (not shown) diminished in a similar way. After one day, ticks still contained ONP in amounts comparable to attached males (one to several micrograms of ONP).

**Localization of Pheromone Production.** Analysis of a small piece of dorsal cuticle bearing the two foveal glands of mature *A. variegatum* or *A. hebraeum* males revealed only traces of ONP; by contrast, nearly all of the total extract-

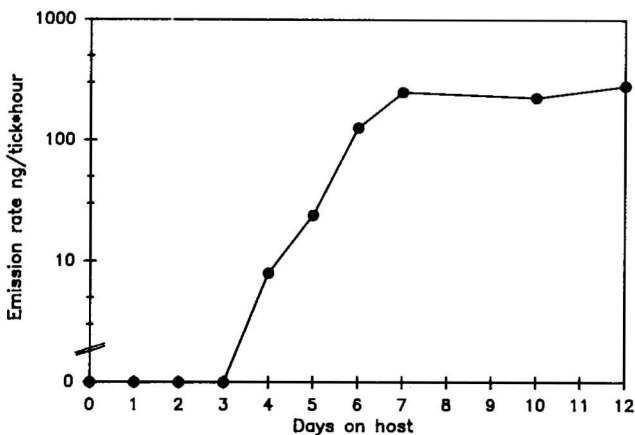


FIG. 2. Emission rate of *o*-nitrophenol from 25 males of *Amblyomma hebraeum* during the first 12 days following attachment on the rabbit.

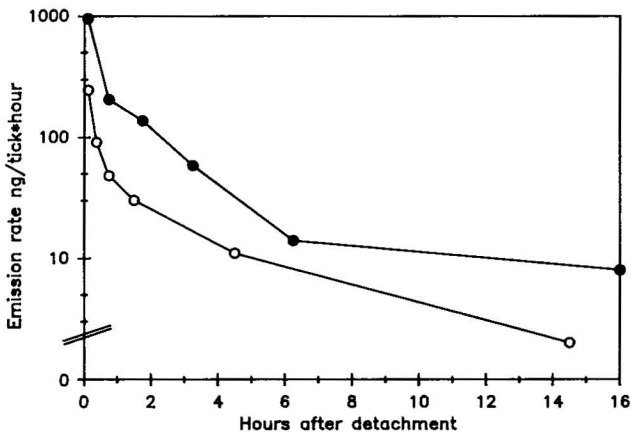


FIG. 3. Emission rates of *o*-nitrophenol in forcibly detached males of *Amblyomma variegatum* (filled circles,  $N = 20$ ) and *Amblyomma hebraeum* (open circles,  $N = 25$ ). Each point represents the release rate, calculated as the mean pheromone amount collected during the sampling period per hour and per tick, at the midpoint of the sampling period.

able ONP and MS (in *A. variegatum*) were found in the rest of the tick. Systematic analysis of different organs from *A. variegatum* males showed that the pheromone was nearly absent from (1) dorsal cuticle without the lateral border, (2) legs, and (3) internal organs such as gut, salivary glands, and excretory,

nervous, and genital systems. Nearly all of the ONP and MS was associated with the ventral cuticle behind the genital opening and with the lateral border.

Dissection and histology of the cuticle revealed the presence of very large dermal glands type 2 (variable, from 200 up to 500  $\mu\text{m}$  diam.) on the ventro lateral side, but not on the dorsal cuticle (Figure 4). In unfed males, these dermal glands were much smaller (60–80  $\mu\text{m}$  diam.).

HPLC analysis of several extracts from carefully dissected and rinsed dermal glands type 2 allowed unambiguous detection of ONP and MS by comparison of retention times and UV spectra of the natural products with that of authentic standards (Figure 5). Presence of ONP was confirmed by combined capillary GC linked electrophysiology, using the ONP-sensitive dII wp/sw/A sensillum on the first pair of legs as a selective biological detector (Figure 6). We conclude that ONP and MS accumulate in these type 2 dermal glands and not in the dorsally located foveal glands.

## DISCUSSION

*Biosynthesis and Emission in A. variegatum.* Our studies on the time course of biosynthesis and emission of ONP and MS in *A. variegatum* males demonstrated that unfed and feeding male ticks up to day 2 did not contain measurable amounts of ONP or MS or hydrolyzable conjugates. This contrasts with the case of the sex attractant pheromone 2,6-DCP, which is already present in unfed females of other metastriate species (Sonenshine, 1984a, 1986; Sonenshine et al., 1982). Trace of ONP emission was detected for the first time by HPLC from ticks at day 3, and within the male at the fourth day. Thus, release of ONP begins apparently as soon as synthesis starts. The mean ONP content of whole mature male ticks was 3.4  $\mu\text{g}$  (range 1.3–7.3  $\mu\text{g}$ ). A similar amount of ONP (2  $\mu\text{g}$ /tick) in males fed for six days was described (Schöni, 1987; Schöni et al., 1984). These substantial pheromone quantities represent up to about 0.25% of the weight of a fed male (average weight of 32 mg) or, in comparison, about 20 g for an average adult human. This contrasts sharply again with the sex pheromone 2,6-DCP, which occurs only in nanogram quantities, e.g., only 16 ng/tick were detected in hexane extracts of sexually active female *A. variegatum* that had been feeding for six days (Wood et al., 1975).

The timing of MS emission followed a similar pattern to that of ONP, but was only detectable one day later. Unlike ONP however, we detected MS in whole-body extracts not before day 8. This very low initial body content is very different from the reported value of about 1  $\mu\text{g}$ /tick found previously in males feeding for six days on cows (Schöni, 1987; Schöni et al., 1984).

The emission from males and their surroundings on the host as measured with the Sep-Pak cartridge proved to be variable. Clearly, an impregnation of

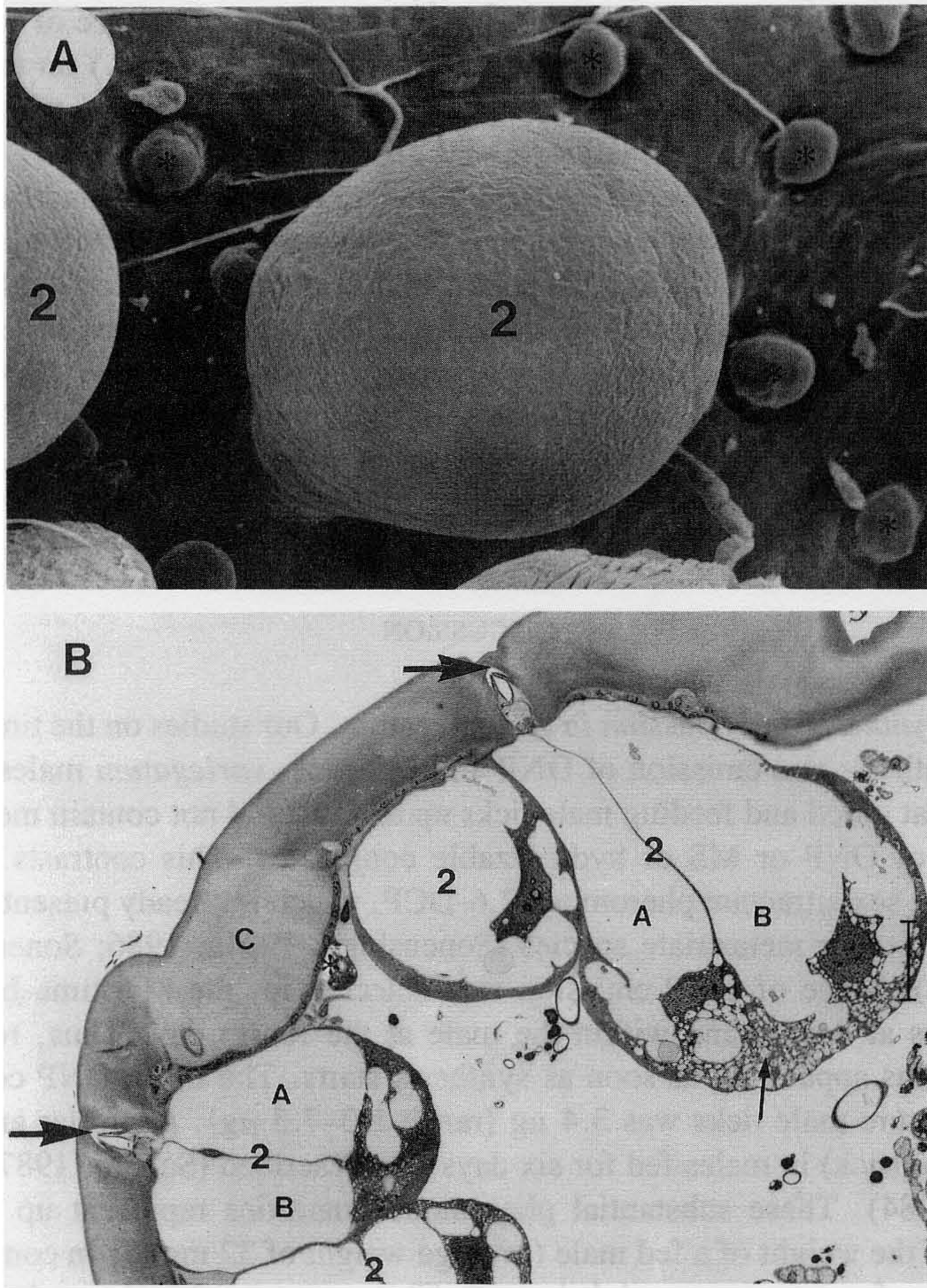


FIG. 4. Mature male of *Amblyomma variegatum* after 12 days of feeding. (A) Scanning electron micrograph of an exposed piece of ventral cuticle. Numerous small dermal glands type 1 (\*) are visible; they are found all over the dorsal and ventral cuticle. Two very large dermal glands type 2 (2) are present; they are only associated with the ventrolateral cuticle. Magnification:  $340\times$  (B) Semithin section from the ventral cuticle. One small dermal gland type 1 (\*) and four very large dermal glands type 2 (2) are present. The huge gland lumens (A,B) and the cytoplasm with many small secretory vesicles (  $\longrightarrow$  ) are visible. (C) cuticle.  $\longrightarrow$  : two canals with valvelike regions. Magnification:  $190\times$ .

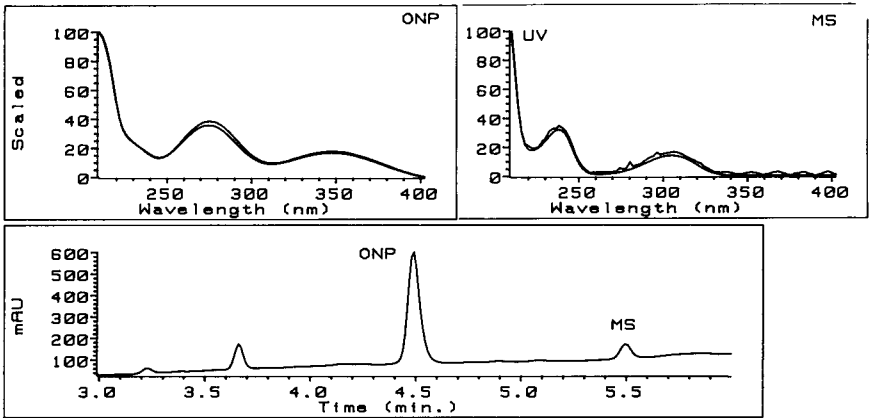


FIG. 5. Presence of *o*-nitrophenol (ONP; 890 ng) and methyl salicylate (MS; 60 ng) in a methanolic extract corresponding to about 75 dissected dermal glands type 2 of *Amblyomma variegatum* males at day 12 of feeding. Lower trace: HPLC separation on a reverse-phase column RP<sub>18</sub> (125 × 4 mm) at 210 nm. The retention times and the UV-VIS spectra (upper traces) of the unknowns and of authentic standards are nearly identical.

the skin and feces occurred in the immediate vicinity, presumably through direct contact with the male, and up to a few centimeters away through adsorption from the vapor phase. We therefore collected not only from males per se, but also from the surroundings covered by the funnel. The true emission rates from attached males remains unknown; nevertheless, most of the collected pheromones should stem from the ticks because forcibly detached animals showed comparable release rates over the first few minutes in the absence of a contaminated substrate. Interestingly, release seemed to be temporarily lower during the first few hours after pairing with females. Females are apparently not disturbed by subsequent exposure to large quantities of pheromone in the immediate vicinity of the males.

**Biosynthesis and Emission in *A. hebraeum*.** Whole-body content of ONP in *A. hebraeum* was similar to *A. variegatum*, but emission rates appeared to be somewhat lower at about 250 ng/tick/hr. This value agreed with the maximum release of 400 ng/tick/hr as estimated by capillary GC-MS after tapping with the dynamic solvent effect (Apps, personal communication).

Apps et al. (1988) found that no MS, but benzaldehyde instead, was detectable in volatiles from *A. hebraeum*. Thus, the attachment-aggregation pheromone of both species contains ONP as a nonspecific main component, but differs with respect to the secondary components. *A. variegatum* probably uses MS and nonanoic acid in addition, whereas *A. hebraeum* uses benzaldehyde and 2-

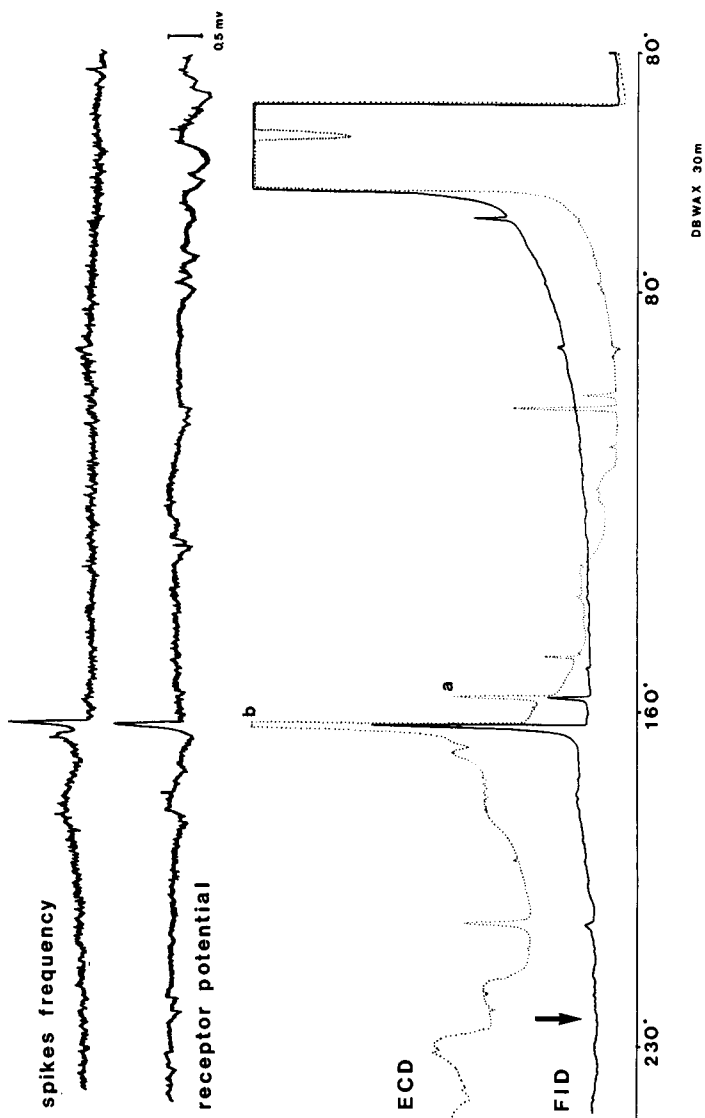


FIG. 6. Capillary gas chromatography-linked single sensillum analysis of a dermal gland type 2 methanolic extract of *Amblyomma variegatum* males at day 12 of feeding. FID, flame ionization detector; ECD, electron capture detector of the chromatograph. Recordings were made of receptor and action potentials of the *o*-nitrophenol-sensitive cell of the DII wp/sw/A sensillum on the Haller's organ of an *Amblyomma variegatum* male, placed opposite the effluent conduit of the gas chromatograph. Frequency/voltage conversion was applied to the AC trace to provide an on-line readout of the spike frequency. Note the selectivity of the olfactory unit for *o*-nitrophenol (b), with no response to methyl salicylate (a), the second major aromatic component of the extract. The elution temperature of nonanoic acid is indicated by the arrow. Detection limit for this product was about 1 ng.

methylpropanoic acid with maximum release rates of about 17 and 950 ng/tick/hr, respectively; in addition, variable amounts of medium-chain fatty acids such as nonanoic acid might be present (Apps et al., 1988).

*A. hebraeum* males matured at a slightly slower pace than *A. variegatum* on rabbits. This is in good agreement with earlier observations (Norval and Rechav, 1979; Rechav, 1977; Schöni, 1987; Schöni et al., 1984). In both species, the development of the clasping behavior was concomitant with the increase in pheromone release. In *A. variegatum* fed on cows, approximately 40% of the males showed clasping for the first time at day 3 (Schöni, 1987). This correlates with the time when we detected the first traces of emitted ONP in our rabbit-fed ticks. We observed that the remaining immature males developed the clasping behavior during the following two days, at a time when we noted a clear increase in ONP content and emission rates from the feeding ticks. It is tempting to speculate that the two phenomena may be controlled by the same hormones. Males prematurely detached during the first three days of feeding did not synthesize the pheromone; permanent contact with the host is a prerequisite for normal development.

*Localization of Pheromone Glands.* We found that ONP and MS are not associated with the foveal glands, as we initially suspected, but rather with the enormous dermal glands type 2 from the ventrolateral cuticle. Other tissues such as hypodermal cells or dermal glands type 1 from the same localization could also be implicated. However, this appears unlikely, because the dorsal cuticle, which contains these same cell types, is pheromone-free.

Dermal glands type 2 have not been reported previously to produce pheromones. Like the dermal glands type 1, in metastriate ticks, they are suspected to participate in the production of such secretions as molting fluid, the waterproofing wax layer or lipidic material on the cuticle of repleted ticks (Balashov, 1972; Lees, 1947). Our preliminary studies showed a secretory structure quite comparable to the foveal glands. Each dermal gland type 2 consists of two very large secretory cells, which contain many secretory vesicles. The glandular products are discharged into a huge lumen and gain the exterior through a cuticular duct. An innervated valvelike region in the duct might control the passage of secretion. This could represent the basis for a controlled release mechanism and explain the rapid decline in pheromone emission after forced detachment of males, and the temporary decrease in release rate after first contact with a female.

*Nonanoic Acid.* Schöni et al. (1984) found approximately 8  $\mu\text{g}$  of nonanoic acid per tick in an ether extract of males at day 6 of feeding. Our HPLC technique was not sensitive enough to explore its biosynthesis and possible release, but neither did capillary GC did not this substrate in dermal gland extracts or in collected volatiles. Offered separately, nonanoic acid showed no influence on attraction, but together with MS in the blend it contributed to complete pheromone activity (Schöni et al., 1984; Schöni, 1987). It may function rather as a

contact chemostimulant. Male *A. hebraeum* intermittently released small amounts of nonanoic acid, among other medium-chain acids, but its behavioral role has not been elucidated (Apps et al., 1988).

*Attachment-Aggregation Pheromones as Part of Host Location* In both *Amblyomma* species, very high pheromone emission rates were observed. We estimate a maximum emission from a cow, infested with 152 male *A. variegatum* as reported by Hoogstraal (1956), to be in the order of about 7 mg/day! This complements various observations that indicate male infested steers or pheromone sources prove to be very attractive over a distance of several meters in the field; CO<sub>2</sub> further increased efficiency by rendering ticks active (Hess and De Castro, 1986; Norval et al., 1989a,b). These aggregation-attachment pheromones are thus efficient in signaling suitable hosts and must play an important part in the host-location strategy of the two *Amblyomma* species studied.

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