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IDENTIFICATION OF HOST FRUIT VOLATILES FROM HAWTHORN (*Crataegus* spp.) ATTRACTIVE TO HAWTHORN-ORIGIN *Rhagoletis pomonella* FLIES

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Abstract—Solid-phase microextraction (SPME) and gas chromatography coupled with electroantennographic detection (GC-EAD) were used to identify volatile compounds from hawthorn fruit (*Crataegus* spp.) acting as behavioral attractants for hawthorn-infesting *Rhagoletis pomonella* flies. Consistent EAD activity was obtained for six chemicals: ethyl acetate (94.3%), 3-methylbutan-1-ol (4.0%), isoamyl acetate (1.5%), 4,8-dimethyl-1,3(*E*),7-nonatriene (0.07%), butyl hexanoate (0.01%), and dihydro-β-ionone (0.10%). In a flight-tunnel bioassay, there was a dose-related increase in the percentage of flies flying upwind to the six-component mixture. Hawthorn-origin flies also made equivalent levels of upwind flight with the synthetic blend and an adsorbent extract of volatiles collected from whole fruit, each containing the same amount of the 3-methylbutan-1-ol compound. Significantly lower levels of upwind flight occurred to a previously identified volatile blend of ester compounds that attracts *R. pomonella* flies infesting domestic apples, compared with the hawthorn volatile mix. Selected subtraction assays showed further that the four-component mixture of 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, and dihydro-β-ionone also elicited levels of upwind flight equivalent to the six-component mix. Removal of 3-methylbutan-1-ol from the four-component blend resulted in complete loss of upwind flight behavior. Removal of dihydro-β-ionone, 4,8-dimethyl-1,3(*E*),7-nonatriene, or butyl hexanoate from the

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four-component mixture resulted in significant decreases in the mean number of upwind flights compared to the four- or six-component mixtures.

Key Words—Solid-phase microextraction, gas chromatographic electroantennographic detection, flight tunnel, *Crataegus* spp., fruit volatiles.

INTRODUCTION

The apple maggot fly, *Rhagoletis pomonella* (Walsh), occurs in North America as two races, one on native hawthorn (*Crataegus*), and one on introduced apple (Bush, 1966; Feder, 1998). Electrophoretic analysis has confirmed the taxonomic status of apple- and hawthorn-infesting populations of *R. pomonella* as partially reproductively isolated "host races," the hypothesized first stage in sympatric speciation (Feder et al., 1988, 1990, 1994, 1998; Feder and Bush, 1989; Berlocher and Feder, 2002). Behavioral studies have shown that differences in host plant preference play an important role in reproductively isolating the apple and hawthorn host races, and volatile host fruit odors have been proposed as one of several important cues in locating host plants (Prokopy et al., 1988; Feder et al., 1993, 1994). In earlier work, apple-origin flies were shown to be attracted to odors from apples (Prokopy et al., 1973) and to synthetic compounds isolated from apple fruit (Fein et al., 1982; Reissig et al., 1982; Zhang et al., 1999).

Here we present details of a protocol, using solid-phase microextraction [SPME, Supelco (see Bartelt, 1997; Agelopoulos and Pickett, 1998)], in conjunction with GC-EAD analysis that allowed for effective screening of key volatiles from hawthorn fruit to which adult flies are most sensitive. We also present evidence that hawthorn-origin flies from a well-studied field site at Grant, Michigan, USA (Feder et al., 1998), respond behaviorally to, and display a preference for, a blend of volatiles identified from hawthorn fruit that is different from the volatile blend identified from domestic apple.

METHODS AND MATERIALS

Insects. Hawthorn-origin *R. pomonella* pupae were collected from fruit at Grant, Michigan, USA. Postdiapause pupae (Feder et al., 1994) were shipped to the laboratory in Geneva, New York, USA, and kept in an environmental chamber at 23–24°C temperature, 16L:8D photoperiod, and 55–60% relative humidity. Adults were maintained on an artificial diet (Fein et al., 1982). Adult flies at 0–7 and 10–21 days old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively. The difference in age was because younger flies were more stable in the EAD apparatus, whereas flies do not respond behaviorally to volatiles until reproductive maturity at 7–10 days old.

Adult *R. pomonella* from a laboratory colony maintained on Red Delicious apples (Neilson and McAllen, 1965) also were used for some of the EAD

measurements (see GC-EAD section below). Adults were kept in an environmental chamber at 23°C temperature, 50% relative humidity, and 16L:8D photoperiod and maintained on artificial diet.

Fruit. Hawthorn fruit was collected at Grant, Michigan, and shipped to the New York laboratory. Hawthorn fruit from trees at the Experiment Station in Geneva, New York, also was analyzed for volatiles. This allowed us to compare the profiles from fruit that had been freshly picked versus shipments of fruit sent via overnight express. Fruit was collected at the two sites during August and September to include fruit that was in preripe (green fruit), ripe (red), and overripe (fruit that had fallen from the tree and might be infested) stages. Fruit from the Michigan site was held in cold storage (4°C; never more than 2 weeks) until sampled. Fruit was taken from cold storage and placed in the sampling containers (see below) at room temperature at least 1 hr before SPME sampling began. Analysis of fruit taken from cold storage continued for up to 1 week, whereas analysis of freshly picked fruit in New York began immediately after picking and continued for 1–2 days.

Adsorbent Sampling. A glass desiccator (20 liters) with a charcoal filter tube in the inlet was used for collection of volatiles from whole fruit. Volatiles from the head space were drawn (ca. 0.5 liters/min) by a vacuum pump onto an ORBO solvent desorption tube (150 mg active coconut charcoal, 20–40 mesh, 4 mm ID, 70 mm length; Supelco Inc., Bellefonte, Pennsylvania, USA). Adsorbent samplings were made for 7 days at room temperature from 3 kg of ripe fruit collected at the Grant, Michigan, USA site. The ORBO tubes were changed every day. The volatiles were eluted with 1 ml methylene chloride (Nanograde, Mallinckrodt Baker, Inc., Phillipsburg, New Jersey, USA), dried over anhydrous sodium sulfate, and then combined. The extract was kept in a freezer (−20°C) and subjected to GC and GC-MS analysis and flight tunnel bioassay.

SPME Sampling. A glass jar (500 ml) with Teflon liner screwcap (Wheaton, Milliville, New Jersey, USA) was used for a sampling container. The inner wall of the container was deactivated with 5% dimethylchlorosilane in toluene (Glass Prep; Alltech Associates, Inc., Deerfield, Illinois), and then cleaned and oven baked (ca. 250°C). Fruit (ca. 150 g) was placed in the container for SPME sampling. A carboxen–polydimethylsiloxane-coated SPME fiber (film thickness 85 μm; Supelco) was conditioned in the GC injector (280°C) for 20 min, and then passed through the small hole on the cap into the headspace of the jar. After a 10 to 20-min exposure, collected volatiles on the fiber were immediately subjected to GC-EAD analysis. For the chemical analysis of the volatiles with GC-MS, SPME samplings were made for a longer time (a few hours to overnight) if necessary.

Coupled Gas Chromatographic–Electroantennographic Detection (GC-EAD) Analysis. A Hewlett Packard 5890 series II gas chromatograph equipped with a nonpolar SPB-1 capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness; Supelco) or a polar EC-Wax Econo-Cap capillary column (30 m × 0.25 mm

ID, 0.25 mm film thickness; Alltech Associates, Inc., Deerfield, Illinois, USA) in the splitless mode was used for GC-EAD analysis. For SPME sample injection, a 0.75-mm-ID glass inlet liner (Supelco) was used, but for liquid sample injection, a 4-mm-ID liner was used. Nitrogen was used as the carrier gas at a head pressure of 138 kPa (flow rate, 2.0 ml/min). The time for splitless injection was 0.25 and 1.0 min for SPME samples and liquid samples, respectively. Oven temperature was set initially at 40°C for 2 min, increased at 15°C/min to 250°C, and held for 10 min. Injector and detector temperatures were set at 280°C. Septum purge flow rate was set at 3 ml/min and total flow rate at 60 ml/min.

The column effluent was combined with nitrogen makeup gas (30 ml/min) and then split 1:1 to the flame ionization detector (FID) and EAD with a TCD capillary column adapter (Agilent Technology, Wilmington, Delaware, USA), two deactivated capillary columns (nonpolar, 0.25 mm ID, Alltech Associates) of equal length as transfer lines, and a universal capillary Y connector (Alltech Associates). The TCD adapter and the Y connector were connected with a short length of deactivated wide-bore capillary column (0.53 mm ID) and a stainless reducing union (3.2 mm OD to 1.6 mm OD). The end of the column was fixed to the TCD adapter with a regular column nut so that the column was extended to the inside of the wide-bore column to facilitate easy column changes.

An extra FID port of the GC was modified and used for the EAD. The transfer line in the EAD heating block was protected and insulated with glass-lined stainless steel tube (1.6 mm OD $\times$ 0.3 mm ID, Alltech Associates), and a wider regular grade stainless steel tube. The EAD port also was insulated with glass wool and capped with a stainless steel cap. The EAD outlet was through a small hole on the top of the cap and was secured in a charcoal-filtered and humidified air stream, which was refrigerated by a modified condenser flushed with 0°C water (Zhang et al., 1999) flowing at ca. 500 ml/min over a *Rhagoletis* fly antennal preparation.

A fly head, separated at the prothorax, was mounted on the tip of a plastic pipet chip (1 ml), which was filled with *Drosophila* ringer solution (46 mmol NaCl, 182 mmol KCl, 3 mmol $CaCl_2$ and 10 mmol Tris HCl at pH 7.2), and equipped with a pure gold wire indifferent electrode. The chip was mounted with adhesive glue on a custom acrylic holder (Figure 1). A pure gold wire recording electrode was extended into the cavity (2 mm ID) in the acrylic holder, and a capillary tube (3 μl micropipet, Drumond Scientific Company, Broomall, Pennsylvania, USA) was fixed in a hole (2 mm ID) at the end of the acrylic holder with a tiny rubber stopper (made with a GC septa). The cavity and the capillary tube were filled with saline. The plastic pipet chip with a mounted fly head was slid back and forth on the acrylic holder so that the tips of both antennae were maneuvered to make electrical contact with the saline of the capillary tube. This device allowed easy mounting of variable size fly heads. The holder was placed inside a cooling condenser and was maintained at 5°C. The distance between the antennae and the EAD outlet

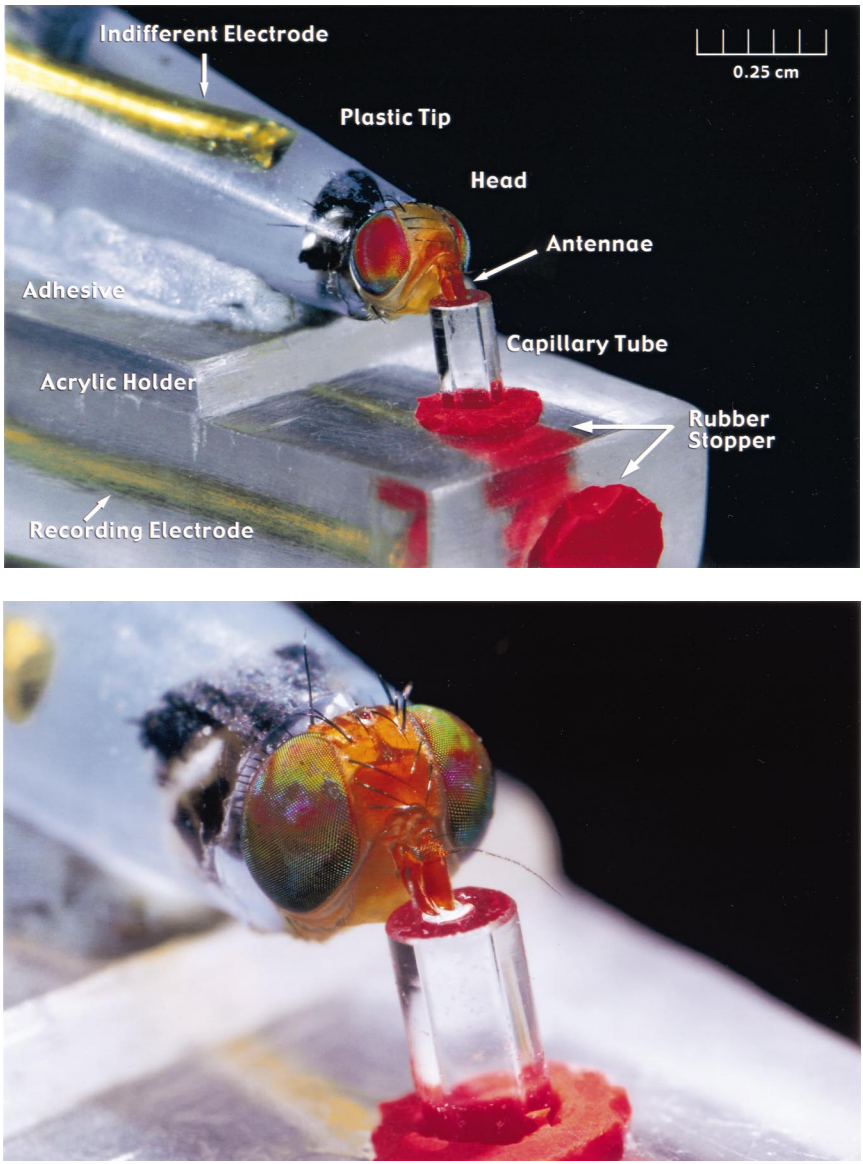


FIG. 1. (A) Acrylic holder for antennal EAD preparation; (B) close-up view.

was 1 cm. With this apparatus, excellent EAD recordings could be made for a few hours on an antennal preparation without cutting off the tip of the antenna.

The output signal from the antennae was amplified by a customized single-step high-input impedance DC amplifier. An OPA 121 precision monolithic dielectrically isolated FET operational amplifier chip (Burr-Brown, Tucson, Arizona, USA) was used at 100 $\times$ amplification. The output signal was filtered by a simple resistance/capacitance (*RC*) high-pass filter (Horowitz and Hill, 1989) with a cutoff frequency of about 0.5 Hz, experimentally determined by examining the signal-to-noise level. A metallized polyester film capacitor (3.3 μF, Matsushita Electric Industrial Co., Ltd., Tokyo, Japan) and a metal film 1/4 W resistor (100 kΩ, Yageo, Taipei, Taiwan) were used for the *RC* filter. All electrical parts were obtained through Digi-Key Corporation, Thief River Falls, Minnesota, USA (www.Digi-key.com) or RadioShack, Fort Worth, Texas, USA (www.radioshack.com). A dual-tracking variable constant voltage power supply (Akidzuki Co. Ltd, Tokyo, Japan) was used at $\pm$ 15 V and connected to the amplifier with a shielded three-conductor cable. The signal was recorded on an HP 3390 A integrator and synchronized with a GC integrator. The amplifier and the integrator were connected with a shielded coaxial cable with a BNC connector. All devices were grounded to a clean source, so that the signal could be recorded without a Faraday cage.

In preliminary experiments, no significant qualitative differences in EAD responses were found between apple-origin flies from our laboratory colony and hawthorn-origin flies from nature, or between sexes within each race in the various GC-EAD runs. Because of limited availability of hawthorn-origin flies needed for behavioral testing, key host volatiles from hawthorn fruit were first estimated by using the laboratory colony flies reared on apple, and then the results were confirmed with hawthorn-origin flies from Grant, Michigan.

Chemical Analysis. GC-MS analyses were performed on a Shimadzu GC-17A equipped with a nonpolar DB-1ms capillary column (30 m $\times$ 0.25 mm ID, 0.25 mm film thickness; J&W Scientific, Folsom, California, USA) or a polar EC-WAX Econo-Cap capillary column (30 m $\times$ 0.25 mm ID, 0.25 mm film thickness; Alltech Associates) and coupled to a Shimadzu QP-5000 quadrupole mass spectrometer running in the EI (at 70 eV) scan mode. For SPME sample injection, a 0.75-mm-ID glass inlet liner (Supelco) was used, but for liquid injection, a 3-mm-ID liner was used. Helium was used as the carrier gas at an initial head pressure of 54 kPa at constant flow rate (1.0 ml/min). The time for splitless injection was 0.3 and 1.0 min for SPME and liquid samples, respectively. Oven temperature was programmed at 40°C for 2 min, increased at 15°C/min to 250°C, and held for 10 min. The injector and interface temperatures were set at 280°C and 260°C, respectively. Volatiles were identified by mass spectral matches to library spectra as well as by retention time matches to available known standards. The EAD activity of compounds were verified by the GC-EAD analysis with authentic standards. Quantification of the relative ratio of the EAD active compounds was made from

the adsorbent collection. Compounds were quantified from their ion abundances from GC-MS analyses, or FID responses, according to the standard curves made from each authentic standard.

Chemicals. Butyl hexanoate, 3-methylbutan-1-ol, isoamyl acetate, and ethyl acetate were purchased from Aldrich, Milwaukee, Wisconsin, USA (purities $> 98\%$). Dihydro-β-ionone was purchased from Scientific Exchange, Inc., Center Ossipee, New Hampshire, USA, and purity was $>89\%$ based on GC-MS analysis. The 4,8-dimethyl-1,3(*E*),7-nonatriene compound was synthesized according to Greenwald et al. (1963), producing an 87:13 ratio of *E* and *Z* isomers, based on GC-MS analysis. The net proportion of isomers was $>96\%$.

Flight Tunnel. The response of flies to host fruit volatiles was measured in a sustained-flight tunnel (Zhang et al., 1999). Outside dimensions of the glass and metal frame tunnel were 183 cm long and 61 $\times$ 61 cm square. Flight-tunnel conditions were 23–24°C, 50–70% relative humidity, 35–40 cm/sec wind speed, and 800 lux light intensity. Light was provided by fluorescent bulbs hung from the ceiling. Adult flies were tested during the third to eleventh hours of the 16-hr photophase period (6 AM to 10 PM EST). All flight tunnel tests involved hawthorn-origin flies from the Grant, Michigan, site.

The floor of the tunnel was covered with white paper and 15 green paper circles of 10 cm diameter placed in a random pattern. The sides of the tunnel also were covered with white paper, since this was found to decrease the flies' distraction by visual stimuli in the testing environment, significantly increasing the number of flies exhibiting upwind flight in the odor plume (Cossé and Baker, 1996). Adult flies (mixed-sex, 10–21 days old) were selected from holding cages located in a separate, environmentally controlled, room, placed singly in glass vials, taken to the room housing the flight tunnel, and allowed to acclimate in the room for 15 min.

Individual flies then were transferred to a screen holding cage, which was placed inside the tunnel on a screen stand 1 m downwind of the odor source, a red rubber septum (Thomas Scientific, Swedesboro, New Jersey, USA; Cat. No. 1780J07) containing solutions of volatile extracts or synthetic compounds prepared in methylene chloride. Rubber septa were prepared 60 min prior to a test and were clipped onto the bottom of a 7.5-cm red plastic sphere (Gempler's Inc., Mt. Horeb, Wisconsin, USA), which was hung from the ceiling at the upwind end of the tunnel. Blank spheres contained rubber septa treated with methylene chloride. Fresh sources and red spheres were used for each test period. Flies were recovered after testing and used on consecutive days, but never more than four times in a weekly period. Each fly was given 1 min to respond, and a positive response was scored if the fly flew upwind in a continuous flight and landed on the red sphere containing the rubber septum source.

For comparative purposes, the dosage of the hawthorn synthetic blends and the adsorbent extract (reported below) reflects the amount of 3-methylbutan-1-ol,

TABLE 1. ACTIVE CHEMICALS FROM HAWTHORN FRUIT DETERMINED BY INTEGRATION OF GC-FID AND GC-MS TOTAL ION CURRENT USING ADSORBENT SAMPLE FROM RIPE FRUIT COLLECTED AT GRANT, MICHIGAN[a]

Chemicals	GC/MS; GC/FID (%)	FT (%)
I. Ethyl acetate	94.3	2000
II. 3-methylbutan-1-ol	4.0	100
III. Isoamyl acetate	1.5	40
IV. 4,8-dimethyl-1,3(E),7-nonatriene	0.07	2
V. Butyl hexanoate	0.01	1
VI. Dihydro-β-ionone	0.10	2

[a] FT indicates the percentage of each compound in the synthetic blend used in the flight tunnel.

with the other components added in the proportions shown in Table 1. Thus, 100 μg doses of the adsorbent extracts of head space volatile collections from whole fruit and the synthetic blend both contained approximately 100 μg of 3-methylbutan-1-ol. Hawthorn-origin flies also were tested to the synthetic blend of ester compounds identified from domestic apple (Zhang et al., 1999). The synthetic apple volatile mixture, in methylene chloride, comprised the following compounds and proportions: butyl butanoate (10%), propyl hexanoate (4%), butyl hexanoate (37%), hexyl butanoate (44%), and pentyl hexanoate (5%). The reported dose of this mixture reflects the total amount of the compounds.

Four different experiments were conducted. In the first, a 100-μg dose of the adsorbent volatile extract from hawthorn fruit was compared with a 100-μg dose of the complete six-component hawthorn fruit blend identified from SPME-EAD analysis. The 100-μg dose was selected based on analysis of the adsorbent extract showing that it contained approximately 0.5 μg/μl 3-methylbutan-1-ol, resulting in 100 μg of the compound when 200 μl of the solution was applied to the rubber septum. In the second experiment, 50- and 200-μg doses of the synthetic blend were compared. In the third experiment, the 200-μg dose of the six-component blend was compared with a 200-μg dose (total quantity) of the 5-component apple volatile blend. In the fourth experiment, the 200 μg dose of the 6-component blend was compared with mixtures in which single or multiple compounds were removed from the complete blend. In all experiments, 25 flies were tested individually each day with each treatment until there were $N = 100$ flies for experiments 1 and 2, and $N = 75$ for experiments 3 and 4. On each day when testing occurred, 10–15 males were tested to a red sphere with a solvent-treated rubber septum.

Statistical Analysis. The number of flies in each treatment that flew upwind to the source was converted to a percent value for graphical display. Treatment

comparisons within each experiment were made using χ^2 analysis, according to the JMP statistical analysis program for Macintosh ($P < 0.05$).

RESULTS

Identification of Key Host Volatiles from Hawthorn Fruit. A total of 9 fruit samples collected during August and September, 2000 (6 samples) and 2001 (3) at the Michigan (5) and New York (4) sites, were analyzed using SPME with GC-EAD. A total of 98 different antennal pairs (1–6 runs/pair; 28 female and 24 male colony apple-origin flies; 31 female and 15 male hawthorn-origin flies) were used for the analysis. Comparison of EAD samples from Michigan and New York hawthorn fruit with both Michigan hawthorn-origin flies and New York apple-origin flies allowed us to determine the key group of volatiles that consistently gave EAD responses. Figure 2 shows a GC-EAD recording from the antennae of an apple-origin female *R. pomonella* exposed to SPME samples of host hawthorn fruit. This sample was from fruit collected at the Geneva site in 2000, at a stage when the majority of the fruit had turned red and were in the ripe stage. The corresponding active compounds were identified as (I) ethyl acetate, (II) 3-methylbutan-1-ol, (III) isoamyl acetate, (IV) 4,8-dimethyl-1,3(*E*),7-nonatriene, (V) butyl hexanoate, and (VI) dihydro-β-ionone.

All of the responses in Figure 2, except for the one earlier than compound I, were consistently found in the majority of fruit samples taken from the two

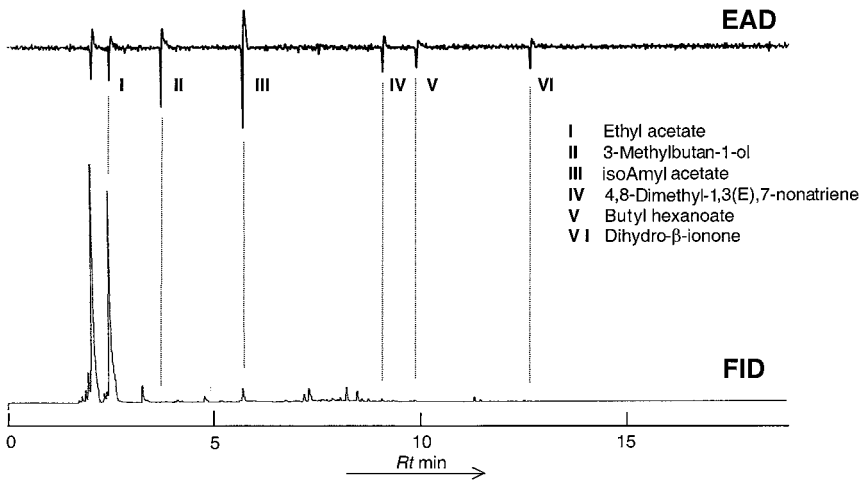


FIG. 2. Simultaneous responses of FID and EAD using the antennae of apple-origin *R. pomonella* female adult to SPME desorbed volatiles from fresh hawthorn fruit collected at the Geneva, New York, site on a nonpolar capillary column.

geographical locations during the period when fruit was red, in the ripe or overripe stage. In green fruit, EAD measurements for compounds III, V, and VI often were low or not evident, and the response to compound IV was higher than in ripe and overripe (infested) fruit. Similarly, in overripe fruit, EAD responses to compounds II, V, and VI increased compared to those from ripe fruit. Samples that were shipped from Michigan often displayed additional EAD peaks, probably due to changes in fruit quality that occurred after collection and shipment. Although these additional peaks were often present, they were not consistent among samples, as were the compounds shown in Figure 2.

A single adsorbent extract of volatiles from whole fruit, for quantitative analysis of key volatiles (GC-MS) and flight tunnel analysis, was made from ripe fruit collected at the Michigan site in September 2000. The relative ratios of the EAD active compounds in this sample, estimated with GC-FID and GC-MS, are listed in Table 1. Flight tunnel tests (see below) confirmed that the proportion of flies responding to this adsorbent collection was equivalent to a sample of the six-component synthetic blend at a comparable dose.

Behavioral Responses of Hawthorn-Origin Flies to Red Sphere with No Volatiles. Over the four experiments, a total of 90 flies were tested individually to a blank treatment, comprised of a red sphere with solvent treated septum. None of these flies initiated upwind flights. The majority presented with a red sphere and no volatiles did not leave the release cage, instead exhibiting bouts of walking and grooming. Flies that did leave the cage flew to the floor or sides of the tunnel. Thus, flies were not attracted by the visual stimulus of the red sphere from a distance of 1 m downwind.

Experiment 1. Behavioral Responses of Hawthorn-Origin Flies to Adsorbent Collection Extract and Synthetic Blend. With the 100-μg dose of extract from volatile collections from whole fruit, 42% of the flies made successful flights to the sphere, and this value was not significantly different from the proportion (44%) of upwind flights to 100 μg of the six-component synthetic blend ($\chi^2 = 0.06$, $P > 0.05$). Flies responding to the volatiles first turned and faced upwind (within 3–5 sec), walked a short distance (0.5–1.5 cm) in the upwind direction, and then initiated upwind flight. Flies that did not reach the sphere typically flew to the floor, side, or top of the tunnel, or remained in the release cage.

Experiment 2. Behavioral Response of Hawthorn-Origin Flies to Doses of the Synthetic Blend. Twenty-two percent of the flies tested with a 50-μg dose of the six-component synthetic blend flew upwind to the sphere (Figure 3). The response level increased significantly to 72% with the 200-μg source ($\chi^2 = 44.4$, $P < 0.05$). Although not tested at the same time, the data from experiment 1 show that the 42% response value for the 100-μg dose falls between those for the lower 50-μg and the higher 200-μg doses.

Experiment 3. Behavioral Response of Hawthorn-Origin Flies to Hawthorn and Apple Volatile Blends. Significantly fewer hawthorn-origin flies flew upwind

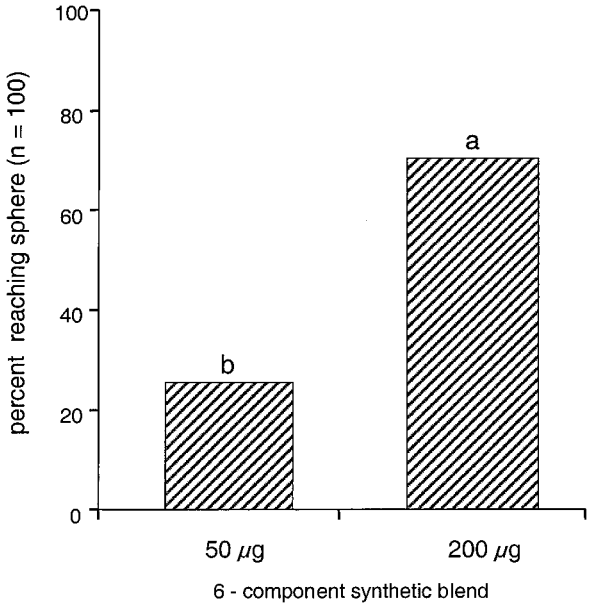


FIG. 3. Percentage of hawthorn-origin *R. pomonella* flying upwind in a sustained-flight tunnel to a red sphere + rubber septum containing 50 or 200 μg of the six-component synthetic mix of hawthorn fruit volatiles (Table 1). The doses are amounts of 3-methylbutan-1-ol applied to the septum, with the remaining compounds in the proportions given in Table 1. $N = 100$ adult flies, mixed-sex, 10–21 day old per treatment. Different letters indicate significant differences in response according to χ^2 analysis ($P < 0.05$).

and made contact with a red sphere containing a 200-μg dose of the apple volatile blend compared with a 200-μg dose of the hawthorn blend (Figure 4, 16% vs. 67%, $\chi^2 = 54$; $P < 0.05$).

Experiment 4. Behavioral Responses of Hawthorn-Origin Flies to Partial Mixtures of Synthetic Blend. There was no significant difference in the proportion of upwind flights to a four-component mixture of 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, and dihydro-β-ionone (66%; Figure 5, treatment 2), and the response to the full six-component blend (62%, Figure 5, treatment 1; $\chi^2 = 0.06$; $P > 0.05$). Removing 3-methylbutan-1-ol from the four-component mix resulted in complete loss of upwind flight behavior, most likely because 3-methylbutan-1-ol comprised 95% of the amount applied to the rubber septum (Figure 5, treatment 3; see Table 1). Removing 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, or dihydro-β-ionone from the four-component mix (treatments 4–6, Figure 5) resulted in levels of upwind flight that were not different from each other (21, 32, and 24%, respectively; $\chi^2 = 2.36$; $P > 0.05$). However,

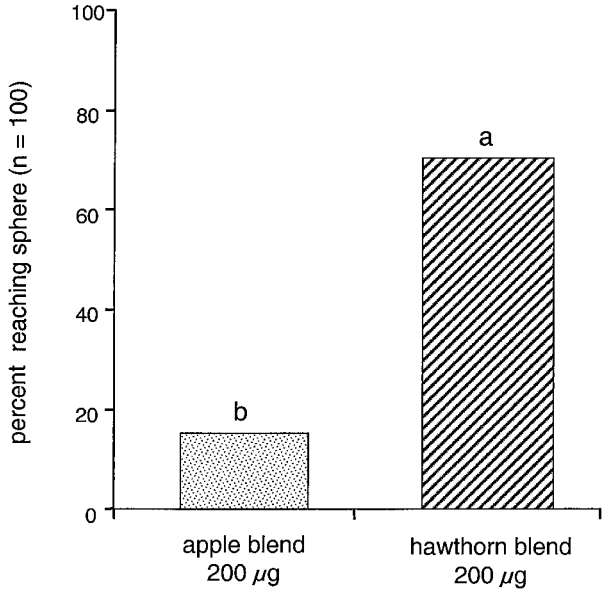
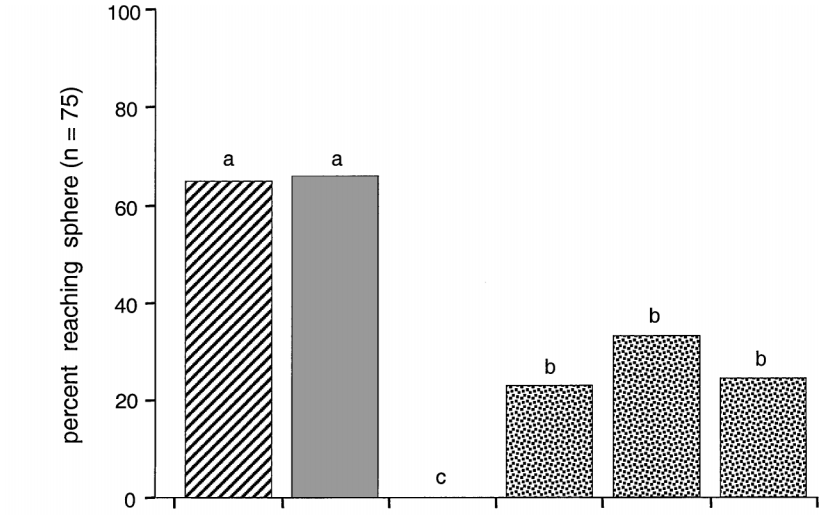


FIG. 4. Percentage of hawthorn-origin *R. pomonella* flying upwind in a sustained-flight tunnel to a red sphere + rubber septum containing the six-component synthetic blend (200 μg 3-methylbutan-1-ol with the remaining compounds in the proportions given in Table 1), compared with response of a 200-μg dose of the apple volatile blend. $N = 75$ adult flies, mixed-sex, 10–21 day old per treatment. Different letters indicate significant differences in response according to χ^2 analysis ($P < 0.05$).

the response to the four-component blend lacking butyl hexanoate (32%; treatment 5, Figure 5) was lower than the response with the six-component mix (64%; treatment 1; $\chi^2 = 15.5$; $P < 0.05$).

DISCUSSION

Identification of Key Host Volatiles from Hawthorn Fruit. We have identified a blend of volatiles from hawthorn fruit, each chemical giving consistent EAD activity from *R. pomonella* flies originating from hawthorn fruit: ethyl acetate, isoamyl acetate, 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, and dihydro-β-ionone. Adult hawthorn-origin flies responded in a flight tunnel assay at equivalent levels to an adsorbent collection of volatiles from whole fruit and a synthetic blend of the six chemicals that contained the same amount of the 3-methylbutan-1-ol compound. However, based on results of subtraction assays, the four-component mix of 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, and dihydro-β-ionone also provided full activity. The



treatment	1	2	3	4	5	6
Ethyl acetate	x					
3-Methylbutan-1-ol	x	x		x	x	x
isoAmy acetate	x					
Nonatriene	x	x	x		x	x
Butyl hexanoate	x	x	x	x		x
Ionone	x	x	x	x	x	

FIG. 5. Percentage of hawthorn-origin *R. pomonella* flying upwind in a sustained-flight tunnel to a red sphere + rubber septum containing the six-component synthetic blend (200 μg 3-methylbutan-1-ol with the remaining compounds in the proportions given in Table 1); a four-component blend containing 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*), 7-nonatriene, butyl hexanoate, and dihydro-β-ionone (200 μg 3-methylbutan-1-ol); or sources of the latter four-component blend with one of the components removed. $N = 75$ adult flies, mixed sex, 10–21 days old per treatment. Different letters indicate significant differences in response according to χ^2 analysis ($P < 0.05$).

results also show that hawthorn-origin flies from Grant, Michigan, responded in greater proportions to the hawthorn volatile blend than to a previously identified blend of ester compounds from domestic apple, a blend known to attract *R. pomonella* in apple orchards (Zhang et al., 1999).

The use of SPME with GC-EAD allowed very small quantities of headspace to be analyzed. Our hypothesis was that if the flies are sensitive to a specific set of volatiles, the SPME analysis at very low concentrations of chemicals should reveal those few compounds among the many volatiles being released from the fruit. The procedures we used obtained clear EAD responses from small quantities of headspace volatiles. In particular, the use of *Drosophila* ringer solution and

an acrylic holder and amplifier custom-designed for the *Rhagoletis* head/antenna complex allowed stable EAD recordings from an antennal preparation to be made for a few hours (see Bjostad, 1998), and the analysis of key volatiles from minimal headspace volumes. Furthermore, whereas there are qualitative and quantitative differences in headspace volatiles between hawthorn fruit picked fresh versus late in the season (green fruit just beginning to express a red blush versus red, maggot-infested fruit), we believe the results reflect the set of key volatiles that hawthorn-origin flies use in host selection. In almost all samples, the same group of key volatiles elicited EAD responses, and there was no difference between the behavioral response of flies to an adsorbent extract of volatiles collected from ripe fruit and the synthetic mixture.

Our flight tunnel assay was modified (after Cossé and Baker, 1996) from that in Zhang et al. (1999), resulting in individual flies making upwind oriented flights to the odor source. This is the first flight tunnel demonstration that *R. pomonella* flies can exhibit odor-modulated anemotaxis and supports the hypothesis that flies can recognize specific odor mixtures from a distance and make directed flights to a particular tree. Other studies demonstrating that adult flies can execute oriented flights to an odor source include tsetse flies, *Glossina* spp. (Gibson and Brady, 1985; Brady et al., 1989; Colvin et al., 1989), the Queensland fruit fly, *Bactrocera tryoni* (Meats and Hartland, 1999); a biting midge, *Culicoides impunctatus* (Bhasin et al., 2000); and the house fly, *Musca domestica* (Cossé and Baker, 1996).

Host Fruit Volatiles and Host Fidelity in Rhagoletis flies. Because *Rhagoletis* adults court and mate exclusively on or near the fruit of their host plants (Prokopy et al., 1971, 1972), differences in host preference behaviors translate directly into mate choice decisions and premating isolation (Prokopy et al., 1988; Feder et al., 1994; Berlocher and Feder, 2002). Both *R. pomonella* host races coexist in sympatry at the Grant, Michigan, site and have been the focus of study for 20 years (Feder, 1998). Here, we show that hawthorn-origin flies from the Grant site responded to, and displayed a preference for, a blend of volatiles from hawthorn fruit that is different from that previously characterized for domestic apple, supporting the hypothesis that discrimination of host fruit odors is an important trait in premating isolation. The fact that 16% of the hawthorn-origin flies made upwind flights to the apple blend is in agreement with allozyme analysis indicating that there is gene flow between the two sympatric host races at the Grant site (Feder, 1998). Additional behavioral tests, which include apple-origin flies from Grant, Michigan, as well as field trapping tests for both the apple and hawthorn populations, will be reported elswhere.

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