

Identification of Host Fruit Volatiles from Snowberry (*Symphoricarpos albus*), Attractive to *Rhagoletis zephyria* Flies from the Western United States

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Abstract A mixture of behaviorally active volatiles was identified from the fruit of snowberry, *Symphoricarpos albus laevigatus*, for *Rhagoletis zephyria* flies reared from snowberry fruit. A nine-component blend containing 3-methylbutan-1-ol (3%), dimethyl trisulfide (1%), 1-octen-3-ol (40%), myrcene (8%), nonanal (9%), linalool (13%), (3*E*)-4,8-dimethyl-1,3,7nonatriene (DMNT, 6%), decanal (15%), and β-caryophyllene (5%) was identified that gave consistent electroantennogram activity and was behaviorally active in flight tunnel tests. In other flight tunnel assays, snowberry flies from two sites in Washington state, USA, displayed significantly greater levels of upwind oriented flight to sources with the snowberry volatile blend compared with previously identified volatile blends from domestic apple (*Malus domestica*) and downy hawthorn (*Crataegus mollis*) fruit from the eastern USA, and domestic

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apple, black hawthorn (*C. douglasii*) and ornamental hawthorn (*C. monogyna*) from Washington state. Selected subtraction assays showed that whereas removal of DMNT or 1-octen-3-ol significantly reduced the level of upwind flight, removal of myrcene and β -caryophyllene, or dimethyl trisulfide alone did not significantly affect the proportion of upwind flights. Our findings add to previous studies showing that populations of *Rhagoletis* flies infesting different host fruit are attracted to unique mixtures of volatile compounds specific to their respective host plants. Taken together, the results support the hypothesis that differences among flies in their behavioral responses to host fruit odors represent key adaptations involved in sympatric host plant shifts, contributing to host specific mating and generating prezygotic reproductive isolation among members of the *R. pomonella* sibling species complex.

Keywords Gas chromatography · Electroantennogram detection · Flight tunnel · Sympatric speciation · Habitat choice · Reproductive isolation

Introduction

The apple maggot, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae), is a model for understanding ecologically based speciation via sympatric host plant shifting (Berlocher and Feder 2002; Dres and Mallet 2002; Feder 1998; Funk et al. 2002). The shift of the fly from native host downy hawthorn, *Crataegus mollis* Scheele (Rosales: Rosaceae), to introduced Eurasian apple, *Malus domestica* Borkhausen (Rosales: Rosaceae) in the mid-1800s is often cited as an example of host race formation in the absence of geographic isolation and in the presence of gene flow (Bush 1966, 1969; Berlocher et al. 1993; Berlocher and Feder 2002; Coyne and Orr 2004;

Feder et al. 1988; McPheron et al. 1988). The shift of *R. pomonella* to apple also resulted in the fly becoming a major pest of commercial apple production in the northeastern and midwestern regions of the USA.

One of the key ecological adaptations involved in the host shift of R. pomonella to apple involves host choice based on fruit odor discrimination (Averill et al. 1988; Linn et al. 2003; Nojima et al. 2003a, b; Zhang et al. 1999). Rhagoletis pomonella use volatiles emitted from ripe fruit as the first long to intermediate range cue to find and distinguish between different host plants (Fein et al. 1982; Prokopy et al. 1973, 1987; Prokopy and Roitberg 1984; Reissig et al. 1982). Fruit odor discrimination is important because Rhagoletis flies mate exclusively on or near the fruit of their respective host plants (Feder et al. 1994; Prokopy et al. 1971, 1972). Apple-origin flies in the eastern USA have rapidly evolved to prefer the odor of apple fruits and avoid that of hawthorns, while the opposite is true for the ancestral downy hawthorn race (Feder and Forbes 2007, 2008; Forbes et al. 2005; Forbes and Feder 2006; Linn et al. 2003, 2004, 2005a, b).

Additional studies have shown a similar pattern of host fruit volatile preference for populations of *R. pomonella* that infest different native species of hawthorns in the southern USA (Berlocher 2000; Berlocher and Enquist 1993; Cha et al. 2011a, b, 2012a; Lyons-Sobaski and Berlocher 2009; Powell et al. 2012), as well as flies infesting flowering dogwood, *Cornus florida*, found throughout the eastern USA, and the immediate sister species to *R. pomonella* (Berlocher 1999, 2000; Nojima et al. 2003b).

The geographic range of the apple maggot fly is not limited to the eastern and southern USA, however. In the late 1970s, *R. pomonella* was detected in the Pacific Northwest, believed to be the result of human mediated transport of larval infested apples from the eastern USA (AliNiazee and Penrose 1981; AliNiazee and Brunner 1986; AliNiazee and Westcott 1986). Populations of *R. pomonella* in the western USA also infest native black hawthorn, *C. douglasii*, and the introduced ornamental hawthorn, *C. monogyna* (Hood et al. 2013; Yee 2008; Yee and Goughnour 2008; Yee et al. 2012, 2015). As in the eastern and southern USA, volatile blends have been identified for each of these western fruit types (Cha et al. 2012b; Linn et al. 2012; Sim et al. 2012).

Here, we expand our studies on the chemical ecology of *Rhagoletis* flies in the western USA by investigating a sibling species of *R. pomonella*, the snowberry fly, *R. zephyria*. The geographic range of *R. zephyria* extends from British Columbia to central California and eastward across the northern USA and southern Canada (Bush 1966; Foote et al. 1993; Gavrilovic et al. 2007). The fly infests the fruit of common snowberry, *Symphoricarpos albus* variety *laevigatus*, a plant native to the western USA, ranging from San Luis Obispo in California north to Alaska. The fly also infests western snowberry, *S. occidentalis*, in the northern Great Plains states in the

USA (Smith and Bush 2000; Gavrilovic et al. 2007), and *S. albus* variety *albus*, which is native to eastern North America. We report the identification of a behaviorally active blend of volatiles from *S. albus* variety *laevigatus* fruit and flight tunnel assays comparing the responses of *R. zephyria* and *R. pomonella* to snowberry and several other identified blends from apple and different hawthorn hosts infested by flies.

Methods and Materials

Insects Snowberry-origin fly larvae were collected in 2009 from fruit at two sites, Burnt Bridge Creek, Vancouver, WA and St. Cloud Park, Stevenson, WA and reared in the laboratory using standard R. pomonella husbandry techniques (Dambroski and Feder 2007; Linn et al. 2003; Neilson and McCallen 1965). Black hawthorn and ornamental hawthorn fly larvae were similarly reared from fruit collected from the Burnt Bridge Creek site from 2008 to 2010, and black hawthorn and apple-origin fly larvae from fruit collected at the St. Cloud Park site from 2008 to 2010. In addition, eastern downy hawthorn larvae collected in 2010 from fruit at Fennville, Michigan (MI) were reared to adulthood in the laboratory. Eclosing adults from all locations were sent to the Geneva NY lab and kept in an environmental chamber at 23-24 °C, 16 L: 8D photoperiod, and 65-70% relative humidity, and maintained on an artificial diet containing water, sugar, vitamins, casein hydrolysate, and salt (Fein et al. 1982). Adult flies 0-7 and 10-21 days old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively. Eastern appleorigin R. pomonella tested in the flight tunnel in 2010 came from a laboratory colony maintained on Red Delicious apples at the New York State Agricultural Experiment Station, Barton Laboratory in Geneva, NY (Linn et al. 2003; Neilson and McCallen 1965).

Fruit Snowberry fruit were collected at the Geneva Experiment Station from an ornamental planting of the species *Symphoricarpos albus laevigatus*, as identified in Gavrilovic et al. (2007). Collected snowberry fruit were placed in sampling containers (see below) at room temperature for preparation of headspace volatile collections.

Headspace Volatile Sampling Adsorbent samples of fruit headspace volatiles were made from whole fruit using 2.4 L closed volatile collection chambers (ARS, Inc., Gainesville, Florida, USA). Field collected snowberry fruit (500–800 ml in volume) were gently cleaned with distilled water, dried, and then put into a collection chamber. Clean air was pushed into the chamber at 0.5 L min⁻¹, and volatiles were pushed out through volatile traps (activated charcoal filters, ORBO32-small, Supelco Inc., Bellefonte, Pennsylvania, USA) on the

bottom of the chamber. For each sample of fruit, adsorbent collections were made over a 5-d period. Volatiles were eluted with 1 ml methylene chloride every 24 hr and combined across the 5 collection days. The combined extract was kept at -20 °C and subjected to GC-EAD, GC-MS (mass spectrometry), and flight tunnel analyses.

Analysis by Gas Chromatography with Electroantennographic Detection (GC-EAD) Coupled GC-EAD analysis was performed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a non-polar EC-1 capillary column (30 m \times 0.25 mm ID, 0.25 μ m film thickness; Alltech Associates, Inc., Deerfield, Illinois, USA) or a Shimadzu GC-17A gas chromatograph equipped with a polar EC-Wax Econo-Cap capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; Alltech) in the splitless mode. Injector and detector temperatures were set at 250 °C and 280 °C respectively. For both GC columns the oven temperature was programmed for 5 min at 40 °C, and 5 °C/min increase to 250 °C and then held for 5 min. Nitrogen was the carrier gas at a flow rate of 2.0 ml/min). The time for splitless injection was 1.0 min. The column effluent was split 1:1 in the oven via a "Y" glass splitter (Supelco). One arm of the splitter led to the flame ionization detector (FID) (270 °C) and the other to the heated EAD port (270 °C). Whole head preparations (Nojima et al. 2003a) were made of individual flies for GC-EAD analysis. The head was separated from the body and antennae positioned between two gold wire electrodes immersed in saline-filled (Drosophila ringer solution; 46 mmol NaCl, 182 mmol KCl, 3 mmol CaCl₂ and 10 mmol Tris HCl at pH 7.2) micropipettes in an acrylic holder. The antennal holder was placed inside a humidified condenser and maintained at 5 °C. The output signal from the antenna was recorded on an HP 3390A integrator. For the GC-EAD analysis of snowberry fruit volatiles, a total of 11 different antennal pairs of R. zephyria flies (2-9 replicated runs/pair) were tested to an adsorbent extract. Volatile compounds that were consistently (>80% of the runs) EAD active were then identified as candidate volatiles for behavioral testing.

Chemical Analysis GC-MS was carried out with a Shimadzu GCMS-QP5050A quadrupole mass spectrometer in EI (at 70 eV) scan mode coupled with a Shimadzu GC-17A equipped with a nonpolar DB-1 ms capillary column (30 m \times 0.25 mm i.d., 0.25-mm film thickness; J&W Scientific, Folsom, CA, USA) or a polar EC-Wax Econo-Cap capillary column (30 m \times 0.25 mm i.d., 0.25-mm film thickness (Alltech Assoc.). Helium was the carrier gas (54 kPa at 1.1 ml/min). The GC conditions and temperature program were as for the GC-EAD analysis. Mass spectra and retention indices of fruit volatiles of interest were used to assign tentative structures, which were confirmed by comparisons with

mass spectra and retention indices of authentic standards. Identities of EAD active compounds were verified by comparing antennal responses of individual fruit volatiles to antennal responses of standards. EAD active compounds were verified by antennal responses in the GC-EAD analyses compared with standards. Quantification of the relative ratio of the EAD active compounds was made from the adsorbent collection based on total ion abundances from GC/MS analyses according to the standard curves made from each authentic sample.

Chemicals Pentyl hexanoate, hexyl butanoate, butyl hexanoate, propyl hexanoate, pentyl butanoate, butyl butanoate, 3methylbutan-1-ol, L-limonene, ethyl acetate, isoamyl acetate, hexyl acetate, ethyl heptanoate, isoamyl butanoate, isoamyl isobutanoate, isoamyl propionate, (Z)-3-hexenyl acetate, hexyl isobutanoate, hexyl propionate, 1-octen-3-ol, nonanal, decanal, β -caryophyllene, myrcene (purities $\geq 98\%$), and linalool (purity \geq 97%), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dihydro-β-ionone was purchased from Scientific Exchange, Inc. (Center Ossipee, NH, USA) with purity >89%. Dimethyl trisulfide was purchased from Acros Organics (Pittsburgh, Pennsylvania, USA), with purity >98%. The compound (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was synthesized by oxidation of geraniol followed by Wittig reaction with methylenetriphenylphosphorane (Greenwald et al. 1963) and purified (> 97% with >97% E-isomer by GC/MS) using flash chromatography on silica gel. The synthesis product was eluted with hexane and then subjected to Kugelrohr distillation (b.p. ~60-70 °C/3.0 mmHg) to remove non-volatile impurities, such as traces of silica.

Flight Tunnel Assays The responses of snowberry-, black hawthorn-, ornamental hawthorn-, and apple-origin flies collected from WA, and hawthorn- and apple-origin flies from MI and NY respectively, to host fruit volatiles were measured in a sustained-flight tunnel (Nojima et al. 2003a). Flighttunnel conditions were 23-24 °C temperature, 50-70% relative humidity, 35 cm/s wind speed, and 1000 lx light intensity. Flies were tested during the 3rd to 11th hours of the 16 hr photophase period (6 am to 10 pm EST). Adult flies (mixedsex, 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 for 15 min. Individual flies then were transferred to a screen release cage, which was placed on a screen stand inside the flight tunnel. The stand was positioned 1 m downwind of a clear plastic sphere (7.5 cm dia.) used as a release point for volatile fruit blends (see below). A clear sphere was used for snowberry flies instead of the typical red commercial sphere used for R. pomonella because snowberry fruit are white in color. The red sphere was used for black hawthorn-, ornamental hawthorn-, eastern downy

hawthorn, and apple-origin flies. The release cage was open at one end, with the open end facing in the upwind direction. Flies were given 1 min to respond, and scored for the following behaviors: 1) taking flight = flight from the release cage (100 cm from the source, may or may not be odor stimulated); and 2) upwind flight = upwind-directed flight to a point 20 cm from the source. From our previous studies, we have determined that an accurate measure of discrimination of blend quality from a distance is whether or not a fly exhibits 'upwind directed flight' and we have yet to observe a fly displaying this behavior in control experiments in the absence of a synthetic fruit blend (Linn et al. 2003).

Solutions of volatile extracts in methylene chloride, or synthetic compounds prepared in hexane, were applied to red rubber septa (Thomas Scientific, Swedesboro, New Jersey, No. 1780 J07) that had been hexane-washed. The rubber septum was attached to a 7.5 cm clear or red plastic sphere and hung from the ceiling at the upwind end of the tunnel. The proportion of chemicals in the complete synthetic snowberry blend, and volatile blends for Washington state apple (WA), eastern apple (EA), eastern downy hawthorn (EDH), black hawthorn (BH), and ornamental hawthorn (OH) are shown in Table 1 and are the same blend proportions as reported and used in Linn et al. (2012). The reported dose of each tested mixture (200 µg) reflects the total amount of the compounds. Sources were prepared 60 min prior to a test, and were clipped onto the bottom of the sphere. Blank spheres used for control experiments contained rubber septa treated with hexane. Fresh volatile sources and spheres were used for each test period. In all experiments 5-10 flies were tested each day to each treatment, until a minimum of 20 different flies had been tested per treatment. Over the course of the studies a total of 35 snowberry flies was also tested to a control sphere containing a solvent treated rubber septum.

Statistical Analysis The total number of flies in each treatment that exhibited upwind flight at least 20 cm from the source was converted to a percent value for graphical display. Differences in the frequencies of upwind flight to fruit volatile blends were compared for statistical significance using G-heterogeneity tests, or for selected comparisons using Fisher's exact in R (R development core team, Vienna, Austria).

Results

Identification of Key Snowberry Fruit Host Volatiles Adsorbent extracts of headspace volatiles from snowberry fruit samples from the Geneva Experiment Station campus were analyzed using GC-EAD. A total of 11 different antennal pairs of *R. zephyria* flies were tested for the study (2–9 runs/pair). The flies tested were collected and reared from

 Table 1
 Relative percentages of chemical compounds comprising fruit volatile blends used in the study

Volatile Compound	SN	BH	OH	EDH	WA	EA
Dimethyl trisulfide	1					
1-Octen-3-ol	40					
Myrcene	8					
Nonanal	9					
Linalool	13					
Decanal	15					
β-Caryophyllene	5					
3-Methylbutan-1-ol	3	20	10	5		
DMNT	6	20	4	0.1		
Ethyl acetate				92.75		
Isoamyl acetate				2		
Dihydro β ionone				0.1		
Isoamyl propionate			1			
Isoamyl isobutanoate			1			
Isoamyl butanoate			1			
Pentyl butanoate			2			
Hexyl isobutanoate			40			
(Z)-3-Hexenyl acetate		5				
L-Limonene ^a		5				
Ethyl heptanoate		5				
Hexyl propionate		5	3		9	
Hexyl butanoate		20	38		25	44
Butyl hexanoate		20		0.05	25	37
Butyl butanoate					12	10
Hexyl acetate					14	
Propyl hexanoate					8	4
Pentyl hexanoate					7	5

SN snowberry, *BH* black hawthorn, *OH* ornamental hawthorn, *EDH* eastern downy hawthorn, *WA* western apple, *EA* eastern apple. *DMNT* (*3E*)-4,8-dimethyl-1,3,7-nonatriene

^a L-limonene was designated in error as D-limonene in Cha et al. (2012b) and Sim et al. (2012)

St. Cloud Park, WA. Figure 1 shows a GC-EAD trace from the antenna of a St. Cloud Park snowberry-origin fly to the Geneva NY snowberry extract. The corresponding nine consistently (at least 75% of the GC-EAD traces) active EAD compounds were identified as (1) 3-methylbutan-1-ol, (2) dimethyl trisulfide, (3) 1-octen-3-ol, (4) myrcene, (5) nonanal, (6) linalool, (7) DMNT, (8) decanal, and (9) β -caryophyllene. The relative ratio of the EAD active compounds in the snowberry fruit extract estimated with GC-FID and GC-MS, are listed in Table 1.

Behavioral Responses of Snowberry Flies to Control Spheres Only 2 of the 35 snowberry-origin flies tested individually to a blank control treatment (clear sphere with solvent



Fig. 1 A GC-EAD trace from the antenna of a St. Cloud Park site snowberry-origin fly to the Geneva NY whole fruit snowberry extract. The corresponding nine consistent (at least 75% of the GC-EAD traces)

EAD-active compounds were identified as (1) 3-methylbutan-1-ol, (2) dimethyl trisulfide, (3) 1-octen-3-ol, (4) myrcene, (5) nonanal, (6) linalo-ol, (7) DMNT, (8) decanal, and (9) β -caryophyllene

treated septum) took flight from the release cage, and neither of these flies initiated upwind flight toward the sphere. Thus, upwind flight was taken as representing positive behavioral orientation of *R. zephyria* to fruit volatiles in subsequent flight tunnel tests, as no fly displayed this behavior to blank control spheres.

Behavioral Responses of Snowberry Flies to Synthetic Blends of Snowberry Volatiles Snowberry-origin flies (n = 21-35) were tested to the adsorbent snowberry fruit extract, the nine-component snowberry synthetic mix, and six other blends with selected volatiles removed from the complete blend (Fig. 2). Because of limited numbers of flies only a few subtraction tests could be completed. Volatiles in the snowberry synthetic mix were selected for subtraction based on their relative high impact in our previous studies with R. pomonella host populations (blends V, VI, and VII) but also to ensure that subtractions to some degree involved all of the volatiles. Maximal levels of upwind flight occurred with the adsorbent extract (56.7%, n = 30), the complete blend (62.9%, n = 35, blend I) and blends with either dimethyl trisulfide (60.0%, n = 30, blend II) or myrcene and nonanal (48.0%, n = 25, blend III) removed (P = 0.298, df = 1). The level of upwind flight to the blend with nonanal, linalool, decanal, and β -caryophyllene removed (32.1%, n = 28, blend IV), was significantly lower than the level to the complete blend (P = 0.023, df = 1) but not significantly different from the blend with myrcene, linalool, and β-caryophyllene removed (P = 0.2725, df = 1). There was no upwind flight observed with the blend in which 3-methylbutan-1-ol was removed (n = 25, blend VII, P = 0.0020, df = 1).

Responses of Snowberry Flies to *R. pomonella* **Host Race Blends** The upwind flight responses of snowberry-origin flies from the Burnt Bridge Creek and St. Cloud Park sites were also measured to five identified volatile blends from fruit infested by *R. pomonella* flies (Fig. 3). There was significant variation in upwind flight across the snowberry and five *R. pomonella* blends tested (G = 66.4, P < 0.0001, df = 5). Maximal response of *R. zephyria* flies was observed to the nine-component snowberry blend (66.7%, n = 24) and was significantly lower to black hawthorn (11.1%, n = 27), ornamental hawthorn (7.7%, n = 26), eastern downy hawthorn (4.5%, n = 22), and the western (0%, n = 23) and eastern apple blends (4.3%, n = 23) (P < 0.0001, df = 1 in all cases).

Responses of R. pomonella Flies from WA to the Snowberry Blend There was significant variation in upwind flight of western apple-origin flies from the St. Cloud Park site (G = 242.9, P < 0.0001, df = 5), ornamental hawthorn flies from the Burnt Bridge Creek site in Vancouver (G = 298.0, P < 0.0001, df = 5), and black hawthorn flies from either the Burnt Bridge Creek or St. Cloud Park site (G = 336.6, P < 0.0001, df = 5) across the six blends [snowberry (SN), black hawthorn (BH), ornamental hawthorn (OH), eastern downy hawthorn (EDH), western apple (WA) and eastern apple (EA)]. No black hawthorn-origin (n = 229, Fig. 4a), ornamental hawthorn-origin (n = 120, Fig. 4b), or appleorigin (n = 60, Fig. 4c), fly displayed upwind flight to the nine-component snowberry blend. In contrast, all three *R. pomonella* populations showed >60% upwind flight to their natal fruit blends (Fig. 4a-c), comparable to the level of response observed for snowberry flies to the snowberry blend. For black hawthorn flies (Fig. 4a) significantly more

Fig. 2 Responses of snowberryorigin R. zephyria flies (percentage of upwind-directed flight in laboratory flight tunnel assays) collected from the Burnt Bridge Creek and St. Cloud Park sites in WA to whole snowberry fruit extracts, the nine-component volatile blend (designated I), and six other blends in which different volatiles were removed from the complete blend (designated II - VII). The volatiles and their percentages in the complete blend are shown, along with those present in blends with missing compounds (indicated by gray cells). All the blends were prepared so that the same compound was always in the same amount as in corresponding complete blend, thus eliminating the potential effect of variation in concentration. Sample sizes (n = number of snowberry fliestested) are also given. Responses that do not share a letter in common indicate a significant difference between blends, as determined by Fisher's exact tests





Fig. 3 Responses of snowberry-origin *R. zephyria* flies (percentage of upwind-directed flight in laboratory flight tunnel assays) collected from the Burnt Bridge Creek and St. Cloud Park sites in WA to the nine-component snowberry volatile fruit blend, as well as the volatile blends for black hawthorn, ornamental hawthorn, eastern downy hawthorn, western apple, and eastern apple fruit (Table 1). Sample sizes (n = number of snowberry flies tested) are given. Responses that do not share a letter in common indicate a significant difference between blends, as determined by Fisher's exact tests

upwind flight occurred with the black hawthorn blend than with the western apple blend (P < 0.0001, df = 1); for ornamental hawthorn flies (Fig. 4b) significantly more upwind flight occurred with the black hawthorn blend than with the western apple blend (P < 0.0001, df = 1), and for western apple flies (Fig. 4c) significantly more upwind flight occurred with the western apple blend than with the eastern apple blend (P = 0.0010, df = 1).

Responses of *R. pomonella* Flies from MI and NY to the Snowberry Blend Hawthorn- and apple-origin *R. pomonella* flies from sites in the eastern USA showed >65% upwind flight to their natal fruit blends (65.6%, n = 32 for MI downy hawthorn, Fig. 5a; 69.6%, n = 56 for NY apple, Fig. 5b), comparable to the level of response observed for snowberry flies to the nine component snowberry blend (see Fig. 4). Hawthorn- and appleorigin *R. pomonella* flies also displayed levels of upwind flight that were significantly lower to their respective non-natal apple (hawthorn-origin flies, 16.7%, n = 30) or hawthorn (apple-origin flies, 18.2%, n = 56) than to their natal blends (P < 0.001, df = 1 for both comparisons). No *R. pomonella* fly tested from the Fennville, MI site (n = 33), or apple-origin fly from the Geneva NY colony (n = 65) displayed upwind flight to the nine



Fig. 4 Responses of *R. pomonella* flies (percentage of upwind-directed flight in laboratory flight tunnel assays) of (**a**) black hawthorn origin; (**b**) ornamental hawthorn origin; and (**c**) apple origin collected from the Burnt Bridge Creek and/or St. Cloud Park sites in WA to the nine-component snowberry volatile fruit blend (SN), as well as the volatile blends for black hawthorn (BH), ornamental hawthorn (OH), eastern downy hawthorn (EDH), western apple (WA), and eastern apple (EA) fruit. Sample sizes (n = number of snowberry flies tested) are given. Responses that do not share a letter in common indicate a significant difference between blends, as determined by Fisher's exact tests

component snowberry blend (P = 0.0203, df = 1 for snowberry vs. eastern apple, 5A; and P < 0.0001, df = 1 for snowberry vs. eastern hawthorn, 5B).

Discussion

Using a protocol involving GC-EAD analysis previously employed for *R. pomonella* flies infesting several different hosts in the eastern, southern, and western USA (Noiima et al. 2003a, 2003b; Powell et al. 2012; Sim et al. 2012), we have identified a blend of nine volatile compounds emitted from snowberry fruit that gave consistent EAD activity from R. zephyria flies infesting snowberry fruit: 3-methylbutan-1ol, dimethyl trisulfide, 1-octen-3-ol, myrcene, nonanal, linalool, DMNT, decanal, and β-caryophyllene. Flight tunnel tests confirmed the behavioral activity of the synthetic blend, with >60% of the tested snowberry-origin flies exhibiting upwind flight. Subtraction of dimethyl trisulfide did not affect the level of response, but removal of several other components resulted in significantly lower response levels. In particular, removing 3-methylbutan-1-ol resulted in complete loss of upwind flight behavior for snowberry flies. Previous studies have reported a similar finding with respect to the behavior of flowering dogwood- and all of the different hawthorninfesting R. pomonella (Cha et al. 2011a, b, 2012a, b; Forbes et al. 2005; Linn et al. 2003, 2005a, b, Linn et al. 2012; Nojima et al. 2003a, 2003b; Powell et al. 2012; Sim et al. 2012). Thus, 3-methylbutan-1-ol appears to be a key, essential volatile involved in host fruit odor discrimination and host location for all Rhagoletis host races/populations studied to date, with the exception of the apple-infesting host race. 3-methylbutan-1-ol is not a component of the eastern or western apple blends that attract eastern or western appleinfesting populations, respectively, and, in fact, has been shown to antagonize the upwind flight response of eastern flies when added to the apple blend (Cha et al. 2012b, Linn et al. 2005a, 2012; Sim et al. 2012; Zhang et al. 1999).

With respect to volatile identifications we did not further verify the role of enantiomers in this study, but recognize that enantiomeric composition of chiral behaviorally active compounds can play important roles in insect behavior. For example, attraction of males of the solitary bee *Colletes cunicularius* was significantly affected by the ratio of (*S*)-(+)-linalool and (*R*)-(-)-linalool although both enantiomers were EAD active (Borg-Karlson et al. 2003). The importance of the enantiomeric composition of 1-octen-3-ol (found behaviorally active in this study) has also been observed (Thakeow et al. 2008) and needs further attention.

Snowberry-origin flies from WA displayed a significantly higher level of response to their natal snowberry fruit blend compared with alternative apple, black hawthorn, and ornamental hawthorn blends favored by host-specific populations of *R. pomonella* in the Pacific Northwest. Thus, *R. zephyria* appears to conform to the general pattern in the *R. pomonella* complex of being attracted to their natal blend relative to nonnatal fruit volatiles. We cannot currently determine from the flight tunnel tests alone whether snowberry flies tend to avoid the natal fruit volatile blends of *R. pomonella* from the eastern and western USA, as opposed to merely not recognizing them. Field trials are needed comparing side by side capture rates of *R. zephyria* in fruit blend baited versus odorless control traps

Fig. 5 Responses of R. pomonella flies (percentage of upwind-directed flight in laboratory flight tunnel assays) of (a) eastern downy hawthornorigin from Grant, MI, and; (b) eastern apple-origin flies from the Geneva NY colony to the nine-component snowberry volatile fruit blend, as well as the volatile blends for eastern downy hawthorn and eastern apple fruit. Sample sizes (n = number of flies)of each fruit origin tested) are given. Responses that do not share a letter in common indicate a significant difference between blends, as determined by Fisher's exact tests



to confirm avoidance behavior for snowberry flies. Such studies have shown that eastern and western populations of *R. pomonella* tend to avoid non-natal fruit volatiles (Forbes et al. 2005; Forbes and Feder 2006; Sim et al. 2012). However, a portion of *R. pomonella* flies, albeit a relatively low proportion, still positively orient to non-natal fruit blends of alternate fly populations in flight tunnel assays (Linn et al. 2003, Linn et al. 2005a, b, 2012; Powell et al. 2012), as also seen here (Fig. 4a-c). This variation likely reflects a degree of ongoing gene flow and indicates standing behavioral potential for inter-host migration among host-associated *R. pomonella* populations.

A similar pattern was observed for snowberry flies in the current study, with a low proportion of *R. zephyria* from WA orienting to the black hawthorn and ornamental hawthorn blends in flight tunnel assays (Fig. 3). No snowberry fly responded to the western apple blend, however. Due to the relatively low sample size (n = 23) of *R. zephryia* tested to non-natal blends in the flight tunnel, the lack of response to the western apple blend could be a statistical artifact and may not represent a complete lack of behavioral attraction to apple volatiles in snowberry fly populations, as one of 23 *R. zephyria* flies tested did respond to the eastern apple blend. Thus, it would appear that there is at least some potential for inter-host migration of *R. zephyria* into *R. pomonella* populations in the Pacific Northwest and low level hybridization and introgression.

In contrast, no WA *R. pomonella* fly originating from apple, black hawthorn, or ornamental hawthorn oriented to the nine-component snowberry blend in flight tunnel assays (Fig. 4a-c). In the case of *R. pomonella*, the lack of response

cannot be explained due to a small sample size, as a total of 409 flies were tested to the snowberry blend. The same was true for the total of 98 eastern *R. pomonella* flies from downy hawthorn and apple origin tested to the snowberry blend. Thus, if variation exists within *R. pomonella* to recognize and orient to snowberry fruit volatiles, then it is present at comparatively very low frequency within populations.

The difference in the potential for inter-host migration between R. zephyria and R. pomonella is consistent with a genetic study of the flies in the Pacific Northwest implying that hybridization is primarily occurring, perhaps at a rate as high as $\sim 1\%$ per generation, in the direction of snowberry into black hawthorn populations (Arcella et al. 2015). It has been argued that hybridization has potentially resulted in the introgression of alleles conferring higher resistance to desiccation from R. zephyria into R. pomonella populations, facilitating the spread of R. pomonella from its original source of introduction in the Portland, Oregon area eastward into the more arid and hotter apple-growing regions of central WA, where it is a quarantine pest (Arcella et al. 2015; Green et al. 2013). Our results do not provide direct evidence supporting or refuting the introgression hypothesis. Nevertheless, they are consistent with hybridization and introgression, if it is ongoing between the two species in the Pacific Northwest, principally occurring from R. zephyria into R. pomonella populations. However, the effective introgression of genes would appear to be limited to only certain phenotypes like desiccation resistance, as there was no evidence in the current study for any R. pomonella fly behaviorally orienting to snowberry fruit. Thus, it would appear that alleles affecting snowberry volatile choice might

be selected against and quickly eliminated from *R. pomonella* populations, unless they are recessive and/or epistatic to those for apple, black hawthorn, and ornamental hawthorn.

In conclusion, the development of a nine-component blend for snowberry fruit and flight tunnel testing of R. zephvria and R. pomonella flies from WA extends previous work on fruit odor discrimination for apple and hawthorn-infesting host races of R. pomonella and flowering dogwood flies phylogenetically deeper into the R. pomonella complex. Like other members of the R. pomonella species complex, R. zephyria show strong attraction to its natal snowberry fruit blend and only low-level responses to non-natal blends. It would therefore appear that most snowberry flies avoid the fruit volatiles of R. pomonella host plants, but that a few individuals in the population may still be attracted to these compounds, potentially enabling low level hybridization between the two species. However, unlike earlier studies, no R. pomonella fly responded to the snowberry blend. Thus, our results imply that host fruit odor discrimination is also an important ecological adaptation and barrier to gene flow between R. pomonella complex species, as well as host races, and may be even stronger between different species than among races.

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