



Identification of a New Blend of Host Fruit Volatiles from Red Downy Hawthorn, *Crataegus mollis*, Attractive to *Rhagoletis pomonella* Flies from the Northeastern United States

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Abstract

A new blend of volatiles was identified for the fruit of downy red hawthorn, *Crataegus mollis*, that is attractive to *Rhagoletis pomonella* flies infesting this host in the northeastern USA. The new blend was as attractive as the previously identified mixture but is more complex in the number of odorants (six in the old versus ten in the new) and differs significantly in the ratio of three volatiles, 3-methylbutan-1-ol, butyl hexanoate, and dihydro- β -ionone, that are common to both blends and exerted agonist or antagonist effects on behavior in a flight tunnel assay. However, behavioral results with the old and new northern hawthorn blends, as well as modified blends with substituted ratios of 3-methylbutan-1-ol, butyl hexanoate, dihydro- β -ionone, indicated that the ‘agonist’ or ‘antagonist’ effects of these volatiles depended on the ratio, or balance of compounds within the blend. In addition, the new blend contains a number of esters identified from the headspace of domesticated apple, *Malus domestica*, that are attractive to apple-origin *R. pomonella*, and present in the five other blends from southern hawthorns, including the southern *C. mollis* var. *texana* blend, but are not part of the previously identified blend from northern *C. mollis* fruit. This finding supports the hypothesis that in addition to providing specificity to the odor blends of the northern and southern hawthorn populations, the presence of the significant amounts of ester compounds in the new northern hawthorn blend might have provided a source of standing variation that could help explain the shift in host preference by *C. mollis*-infesting flies to introduced apple in the mid-1800’s.

Keywords Apple maggot fly · Olfaction · Fruit odor discrimination · Flight tunnel · Host races · Sympatry · Host shifts

Introduction

The apple maggot fly, *Rhagoletis pomonella* (Diptera: Tephritidae) is a model system for ecological divergence in sympatry in the face of gene flow (Berlocher and Feder 2002; Coyne and Orr 2004; Egan et al. 2015; Funk et al. 2002). In

the mid 1800’s, *R. pomonella* shifted from infesting the fruit of its primary native host in the northeastern USA, red downy hawthorn, *Crataegus mollis*, to introduced, domesticated apple, *Malus domestica* (Bush 1966, 1969). Laboratory flight tunnel and field trapping studies have shown that apple- and hawthorn-origin flies preferentially respond to the volatile blends of their natal host fruit and tend to avoid the odor of the alternative host fruit (Forbes et al. 2005; Forbes and Feder 2006; Linn et al. 2003, 2005a, b; Sim et al. 2012). This olfactory preference for fruit volatiles together with differences in diapause/life history traits generates ecological reproductive isolation between apple and hawthorn flies (Feder et al. 1994; Powell et al. 2014).

Northern *C. mollis* is not the only hawthorn species attacked by *R. pomonella* across its range in the eastern USA and Mexico (Berlocher and Enquist 1993; Bush 1966; Rull et al. 2006). In the southern USA several different hawthorn species are infested by *R. pomonella*, including eastern and western mayhaw (*C. aestivales* and *C. opaca*, respectively), blueberry hawthorn

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(*C. brachyacantha*), sand hawthorn (*C. flava*), green hawthorn (*C. viridis*), and southern red hawthorn, *C. mollis* var. *texana* (Buckl.) (Berlocher and Enquist 1993). In Mexico, *C. mexicana*, *C. gracilor*, and *C. rosei* are infested by *R. pomonella* (Rull et al. 2006). Studies have shown that these hawthorn species differ from one another not only in their fruiting phenology (Lyons-Sobaski and Berlocher 2009; Powell et al. 2014), but also in their fruit volatile profiles (Cha et al. 2011a, b; Powell et al. 2012).

Two important findings emerged from the gas chromatography with electroantennographic detection (GC-EAD) profiles obtained from the examination of fruit odors in southern hawthorn species (Cha et al. 2011a, b, 2012a) with potentially important implications for understanding the shift from downy hawthorn to apple in the northeastern USA. First, southern hawthorn fruit had more complex odor profiles than reported previously for northern *C. mollis* fruit (Nojima et al. 2003). Second, southern hawthorn fruit contained several ester compounds found in the northern apple blend (Zhang et al. 1999) but not in the previously identified northern *C. mollis* blend.

Our original identifications of volatiles from northern *C. mollis* hawthorn (Nojima et al. 2003) and apple (Zhang et al. 1999) involved the use of both SPME and adsorbent extracts for GC-EAD analysis. However, the synthetic blends reported were based mainly on the SPME analysis using relatively short collection duration (< 10 min) and comprised volatiles displaying consistent and significant GC-EAD peaks. At the time of original identifications, SPME was still a relatively new technique and our original working hypothesis was that SPME could provide for a relatively rapid collection of headspace volatiles reflecting the natural release of compounds from fruit. The SPME technique offers the advantage of rapid collection and less sample preparation but is a static volatile collection method and issues related to misrepresentation of volatile profile have been raised that can lead to loss of potential biological activity due to the lower collection time, as well as differences in differential adsorption of volatile organic compounds by different types of SPME fiber (Agelopoulos and Pickett 1998; Bartelt 1997; Matich et al. 1996, Matich 1999). In contrast to SPME, dynamic volatile collection methods using adsorbent traps, such as activated charcoal or Super-Q, have been suggested to be able to depict more complete volatile profiles (Tholl et al. 2006). These results raised the possibility that the northern *C. mollis* hawthorn fruit might possess a more complex volatile profile, with greater similarity to the southern hawthorn blends, especially the southern *C. mollis* blend, than was previously found, while retaining the functional differences in the agonist/antagonist behavioral effects of key volatiles that provide for the odor preference observed in the flight tunnel and the field.

Here, we report results obtained on the composition and GC-EAD profile of the volatile blend produced by northern *C. mollis* using the same volatile collection techniques

and analytical procedures as performed for southern hawthorns (Cha et al. 2011a, b, 2012a). Flight tunnel bioassays were then carried out to compare flight behaviors of *R. pomonella* flies to the old and newly identified blends from northern *C. mollis*, the blend from southern *C. mollis*, the blend from northern apple fruit, and various modified blends. Flies from northern and southern *C. mollis* hawthorn and from northern apple were tested with the aim of understanding the odor preferences of the flies to their natal and non-natal blends.

Methods and Materials

Insects *Rhagoletis pomonella* flies used in the study were collected as larvae in infested host fruit and reared to adulthood in the laboratory at the University of Notre Dame, Notre Dame, Indiana, USA, following the methods described in Neilson and McCallen (1965) and Dambroski and Feder (2007). Southern *C. mollis* var. *texana* flies came from Palmetto State Park, Gonzales Co., Texas (TX), USA (Lat: 29.22, Long: 96.05), and Brazos Bend State Park, Fort Bend Co., TX (Lat: 29.22, Long: 95.34). Northern *C. mollis* flies came from Grant and Fennville, Michigan (MI), USA (Lat: 43.21, Long: 85.54 and 42.36, 86.09 respectively). Apple-origin flies came from Grant, MI. Following eclosion, adult flies were kept in a walk-in environmental chamber at 23–24 °C, 16 L: 8D photoperiod, and 65% relative humidity, on an artificial diet (Fein et al. 1982) prior to flight tunnel testing. Odor-naïve adult flies at 0–7 and 10–21 days old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively.

Collection of Volatiles Northern *C. mollis* fruit were collected from trees at the New York State Agricultural Experiment Station in Geneva, New York (NY) in 2009 and 2010, the same trees used, along with fruit from Grant, MI, in the original hawthorn blend identification (Nojima et al. 2003). Fruit headspace volatiles used for the GC-EAD analysis were collected from whole fruit using 2.4 L closed volatile collection chambers (ARS, Inc., Gainesville, Florida, USA; Glass Shop, Cornell University, Ithaca, NY, USA). Field-collected fruits (500–800 ml in volume) were gently cleaned with distilled water, thoroughly dried and then immediately put into a collection chamber. Clean air was pushed into the chamber at 0.7 L/min 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 samplings were made over a 5-d period. Volatiles were eluted with 500 µl methylene chloride every 24 h and combined across the 5 collection days. The combined extract was kept at –20 °C and subjected to GC-EAD, GC-MS, and flight tunnel analysis.

Gas Chromatography with Electroantennographic Detection (GC-EAD) Analyses were performed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a non-polar EC-1 capillary column (30 m × 0.25 mm ID, 0.25 µm 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) with splitless injection. The oven temperature was programmed at 40 °C for 5 min, then increased by 15 °C/min to 250 °C and held for 5 min. Injector and detector temperatures were set at 280 °C and 270 °C, respectively. Nitrogen gas was the carrier at a flow of 2 ml/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 were made of individual flies for GC-EAD analysis (Nojima et al. 2003). The head was separated from the body and its antennae positioned between two gold wire electrodes immersed in saline filled (*Drosophila* Ringer’s 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 amplified by a customized high input impedance DC amplifier and recorded on an HP 3390A integrator.

Gas Chromatography Coupled to Mass Spectrometry (GC-MS) Volatile identification, based on 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 or a polar EC-Wax Econo-Cap capillary column. Helium was the carrier gas (54 kPa at 1.1 ml/min). The GC conditions and temperature program were as for the GC-EAD analyses. The interface temperature was set at 260 °C. Volatile compounds were identified by comparison of chromatographic retention times and mass spectra with those of authentic standards analyzed on the same instrument. For further verification, we tested the authentic standards (at 5 ng each) on the fly antennae using GC-EAD. Quantification of the relative ratio of the compounds that elicited EAD responses was made from the adsorbent collection based on total ion abundances from GC/MS analyses. As we did not add an internal standard to the adsorbent sample for behavioral testing purposes, standard solutions containing 0.1, 1, 10, and 100 ng of each compound that elicited EAD responses in 1 µl of methylene chloride were prepared and analyzed to obtain calibration curves.

Chemicals Pentyl hexanoate, butyl acetate, ethyl acetate, hexyl butanoate, butyl hexanoate, propyl hexanoate, pentyl butanoate, pentyl hexanoate, butyl butanoate, 3-

methylbutan-1-ol, isoamyl acetate, isoamyl butanoate, isoamyl hexanoate, butyl propionate, hexyl propionate (all >98%), and dihydro-β-ionone (> 90%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The 4,8-dimethyl-1,3(*E*),7-nonatriene was synthesized according to Greenwald et al. (1963), and further purified (with >97% (*E*)-isomer by GC/MS) using flash chromatography on silica gel eluting with hexanes followed by Kugelrohr distillation.

Flight Tunnel Tests

Flight Tunnel The responses of flies to host fruit volatiles were measured in a sustained-flight tunnel (Cha et al. 2011b; Nojima et al. 2003). Flight-tunnel conditions were 23–24 °C, 50–70% relative humidity, 35 cm/s wind speed, 1500 lx light intensity. Male and female sexually mature adult flies, 10–21-d-old post-eclosion that had never been previously exposed to the fruit volatile blends (i.e., fruit odor-naïve flies) were tested during hours 3–11 of the 16 h photophase period. Flies 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 then allowed to acclimatize for at least 30 min before testing. Individual flies were transferred to a screen holding cage which was then placed on a release stand such that the open end of the cage faced upwind at a distance of 1 m from the odor source. Flies were observed for 1 min and scored for two behaviors: (1) taking flight, i.e. fly took flight from the release cage but did not exhibit oriented upwind flight toward the source, but rather flew to the side, top or floor of the tunnel; (2) upwind flight, i.e. fly faced upwind, walked to the edge of the release cage, took flight and exhibited upwind oriented flight over the 1 m distance to the source. With one exception, all flies exhibiting upwind flight contacted the target red sphere. For each of the three flight tunnel tests (below) all of the flies for each geographic location/host fruit type were tested sequentially to all of the blend sources in each test. On each test day a cohort of at least 5 flies was tested to the sequence of blends, with results grouped over days for analysis. Each fly was tested only once to the sequence of blends in each test. Fresh rubber septa sources and red spheres were used for each replicate.

Synthetic Blends and Sources Synthetic blends used for flight tunnel testing (see Fig. 2) were prepared as described in Zhang et al. (1999), Nojima et al. (2003), Linn et al. (2003), and Cha et al. (2011a, b). A total volume of 200 µl of a synthetic blend at a concentration of 1 µg/µl was applied to a red rubber septum (hexane-washed, cat. no. 1780 J07; Thomas Scientific, Swedesboro, New Jersey, USA) attached to a red

plastic sphere 7.5 cm in diameter (Gempler's, Mt. Horeb, Wisconsin, USA). We have previously shown that 200 µg of synthetic volatile blend induces maximal levels of behavioral response by *R. pomonella* flies in the flight tunnel (Linn et al. 2003).

A first experiment compared upwind flights of *R. pomonella* flies from three origins to a whole-fruit adsorbent collection and four synthetic blends, as shown with results in Fig. 2. Flies were from Grant and Fennville, MI, northern *C. mollis* fruit, Palmetto and Brazos Bend, TX, southern *C. mollis* var. *texana* fruit, and Grant MI, apple fruit. Synthetic blends were the original northern *C. mollis* blend (NR old), southern red hawthorn (SR), the new northern red hawthorn (NR new), and apple (northern apple).

For NR old, the 1 µg/µl value refers to the amount of 3-methylbutan-1-ol, with the remaining volatiles added in the proportions shown in Fig. 2, and thus, the total quantity of volatiles for the NR old lure was actually greater than 200 µg. This approach of standardizing the amount of 3-methylbutan-1-ol across blends was used in the original hawthorn blend identification (Nojima et al. 2003). The other synthetic blends used here were prepared so that the total concentration of all the blend components was 1 µg/µl, as was done in the studies with southern hawthorn plants (Cha et al. 2011a, b). The whole-fruit adsorbent collections from northern *C. mollis* were diluted with hexane or concentrated under a gentle stream of nitrogen to approximate the concentrations of GC-EAD active volatiles in the extracts to those of the corresponding synthetic blend prepared at 1 µg/µl total concentration.

In two subsequent experiments, hawthorn-origin and apple-origin flies from the two northern locations (Grant and Fennville, MI) were tested against the NR new synthetic blend and five different modifications of the NR new blend and one modified blend of the NR old blend (details with Results in Figs. 3 and 4). The composition of the five modified blends differed from the NR new blend by either (1) changing amounts of selected volatiles to ratios present in the NR old blend (butyl hexanoate, hexyl butanoate, dihydro-β-ionone, 3-methylbutan-1-ol); (2) adding a volatile from the SR blend (butyl acetate); or 3) removing a component completely from the NR new blend (3-methylbutan-1-ol). The modified NR old blend contained the amount of dihydro-β-ionone found in the NR new blend. The remaining volatiles in each modified blend were maintained at levels found in the complete NR new blend.

Statistical Analysis The total number of flies in each treatment that exhibited upwind flight was converted to a percentage value for graphical display. Differences in the frequencies of upwind flight to fruit volatile blends were compared for statistical significance using Fisher's exact tests ($P < 0.05$).

Results

Identification of EAD-Active Components of Northern Red Hawthorn Fruit Volatiles In GC-EAD analyses the northern *C. mollis* hawthorn fruit volatiles EAD responses to 10 compounds were recorded from the antenna of a northern *C. mollis*-origin fly from Grant, MI (Fig. 1). These were identified as 3-methylbutan-1-ol (I), butyl butanoate (II), isoamyl butanoate (III), propyl hexanoate (IV), (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT; V), butyl hexanoate (VI), hexyl butanoate (VII), isoamyl hexanoate (VIII), pentyl hexanoate (IX), and dihydro-β-ionone (X). The relative amounts of the EAD-active compounds in the northern *C. mollis* extract estimated by GC-FID and GC-MS are listed in Fig. 2 (NR new), along with the compositions of the previously identified northern *C. mollis* blend (NR old), the southern *C. mollis* blend (SR), and northern apple blends.

Behavioral Responses of Hawthorn- and Apple-Origin Flies to the Northern *C. mollis* Extract and Synthetic Blends Northern *C. mollis* flies collected from Grant and Fennville, MI ($N = 46$), displayed maximal levels of upwind flight to the collections of volatiles from northern *C. mollis* fruit (NR extract), the previously identified northern blend (NR old) of synthetic compounds, and the new blend of synthetic volatiles identified from northern *C. mollis* (NR new) (65%, 63%, and 70% respectively; Fig. 2). Significantly lower levels of upwind flight were observed for northern *C. mollis* flies with the southern *C. mollis* (SR) and northern apple synthetic blends (17 and 13% respectively; $P < 0.001$ in all cases compared to the northern hawthorn extract and blends, as determined by Fisher's exact tests; Fig. 2).

Southern *C. mollis* flies collected from Palmetto and Brazos Bend, TX ($N = 66$) displayed maximal levels of upwind flight to the southern *C. mollis* synthetic blend (74%), and significantly lower levels of upwind flight to the with the northern collection of volatiles (17%), the NR old synthetic blend (20%), the NR new blend (17%), and the northern apple blend (10%; $P < 0.0001$ in all cases; Fig. 2). Apple-origin flies collected from Grant, MI ($N = 32$) displayed maximal level of upwind flight to the northern apple synthetic blend (72%), and significantly lower levels of upwind flight to the northern *C. mollis* collection of volatiles (9%), NR old synthetic blend (12%), NR new synthetic blend (19%), and SR synthetic blend (6%; $P < 0.0001$ in all cases; Fig. 2).

Behavioral Responses of Northern Hawthorn- and Apple-Origin Flies to Modified Northern *C. mollis* Blends Northern *C. mollis* flies collected from hawthorn fruit at Grant and Fennville, MI ($N = 51$) displayed maximal levels of upwind flight to the NR new blend (designated NR1 in Fig. 3, 75%). When the amounts of butyl hexanoate and hexyl butanoate in the NR1 blend were changed to the levels in the NR old blend,

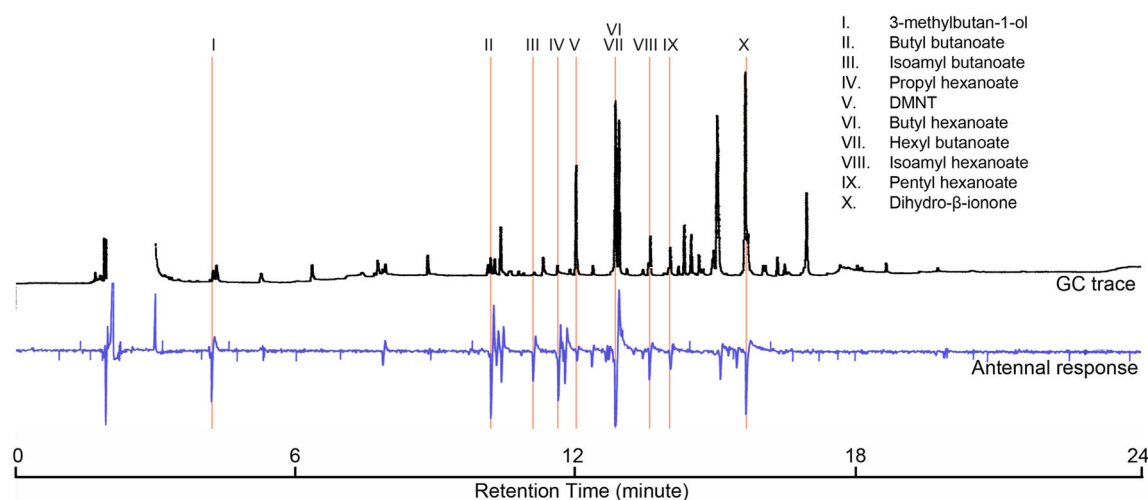


Fig. 1 Representative GC-EAD analysis of volatiles collected from northern *Crataegus mollis* with the antenna of a northern *C. mollis*-origin fly from Grant, MI and identification of EAD-active compounds (DMNT = (3*E*)-4,8-dimethyl-1,3,7-nonatriene)

the level of upwind flight of hawthorn flies decreased significantly to 53% (NR2; $P = 0.039$; Fig. 3). Reducing the amount of dihydro- β -ionone in the NR1 blend (blend NR3, Fig. 3) to the level found in the NR old blend also resulted in a significant decrease in upwind flight (blend NR3, Fig. 3; 13%) of

hawthorn flies compared with the new red hawthorn blend NR1 and blend NR2 (Fig. 3, 75 and 53%, respectively; $P < 0.0001$ for both comparisons). When the amount of dihydro- β -ionone in the NR old blend was changed to that found in the NR1 (new) blend a similar and significant

Fig. 2 Upwind flight response (% of total tested) of *Rhagoletis pomonella* flies from Grant and Fennville, MI, northern *Crataegus mollis* fruit, Palmetto and Brazos Bend, TX, southern *C. mollis* var. *texana* fruit, and Grant MI, apple fruit, to the adsorbent extract of northern red hawthorn fruit collected at the Agricultural Experiment Station in Geneva, NY, the previously identified northern *C. mollis* synthetic blend (northern red haw old), the new synthetic blend identified from northern *C. mollis* fruit (northern red haw new), the synthetic blend identified from southern *C. mollis* var. *texana* fruit (southern red haw), and the synthetic blend for northern apple fruit (Northern apple). *N* for each fly location indicates total number of flies tested to all of the blends, and numbers in columns indicate proportion and quantity (μ g) of each volatile in the blend. DMNT = (3*E*)-4,8-dimethyl-1,3,7-nonatriene

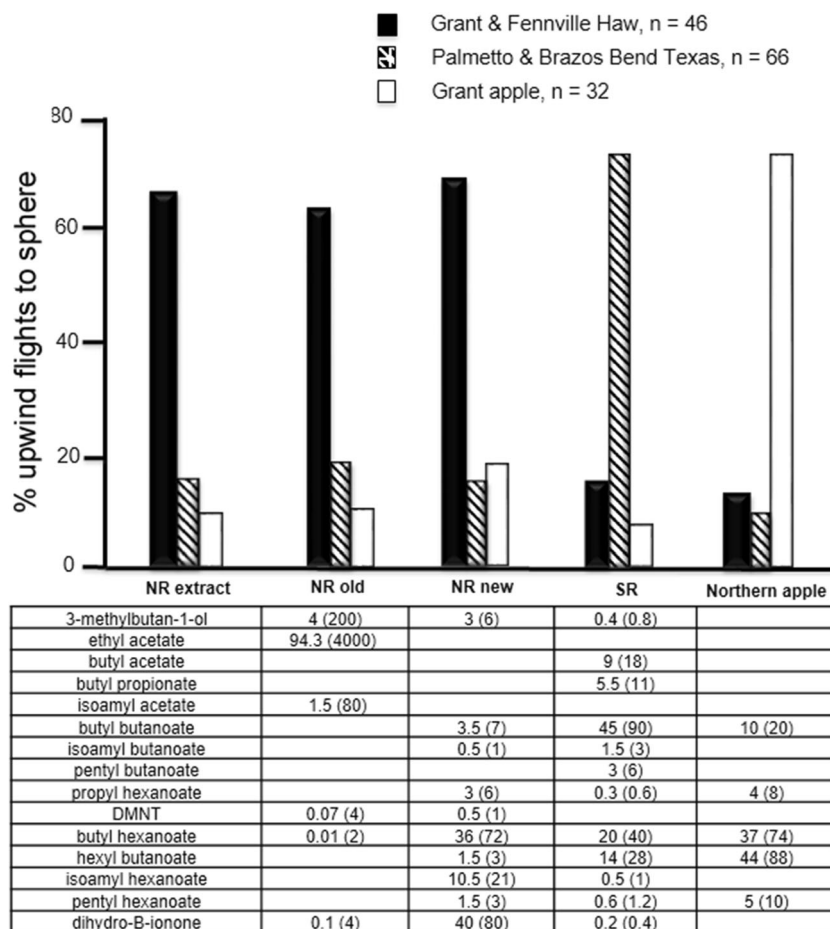
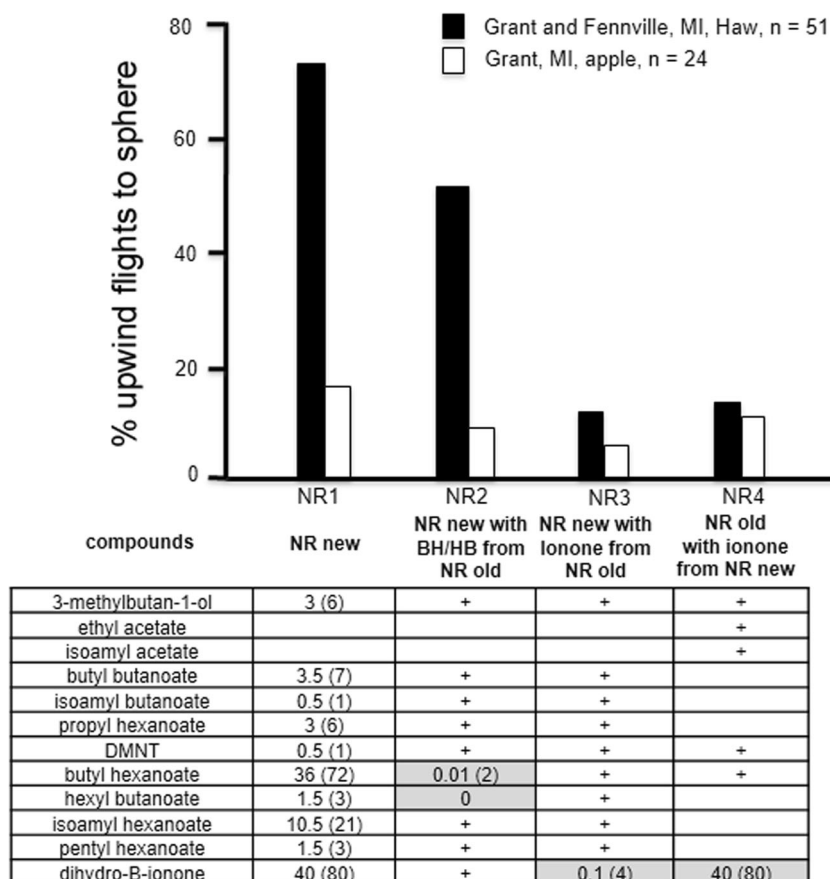


Fig. 3 Upwind flight response (% of total tested) of *Rhagoletis pomonella* flies from Grant and Fennville, MI, northern *Crataegus mollis* fruit, and Grant MI, apple fruit, to the new synthetic blend identified from northern *C. mollis* fruit (NR1), and three modified blends: NR2, the new NR1 blend with the proportion of butyl hexanoate/hexyl butanoate from the northern red haw old blend; NR3, the NR1 new blend with the proportion of dihydro- β -ionone from the northern red haw old blend; and NR4, the northern red haw old blend with the proportion of dihydro- β -ionone from the NR1 new blend. *N* for each fly location indicates total number of flies tested to all of the blends, and numbers in column for NR1 new blend indicates the proportion and quantity (μg) of each volatile in the blend. The + symbol indicates that the compound was in the blend at the proportion and amount indicated for the northern red haw new blend. DMNT = (3*E*)-4,8-dimethyl-1,3,7-nonatriene



decrease in upwind flights occurred for northern *C. mollis* flies (blend NR4 20%; $P < 0.001$ in both cases; Fig. 3).

Apple-origin flies from Grant, MI ($N = 24$) were also tested to the NR1 blend and the set of three modified blends (Fig. 3). Apple-origin flies exhibited low levels of upwind flight to the NR1 blend (17%) as well as the three modified NR2 (8%), NR3 (4%), and NR4 (13%) blends.

In a second set of tests using modified blends, *C. mollis*-origin flies collected from Grant and Fennville, MI ($N = 42$) displayed maximal levels of upwind flight to the new NR blend (NR1 in Fig. 4; 57%). When the amount of butyl acetate, an agonist compound for southern *C. mollis* flies but not found in the NR new or old blends, was added to NR1 (blend NR5 in Fig. 4), the proportion of upwind flights by northern *C. mollis* flies was significantly decreased to 10% ($P < 0.0001$). Two additional tests were performed involving the key volatile 3-methylbutan-1-ol. When 3-methylbutan-1-ol was removed entirely from the NR1 blend (blend NR6; Fig. 4), no upwind flights were recorded, a result that was also found in the original identification of the northern *C. mollis* blend (Nojima et al. 2003). Changing the amount of 3-methylbutan-1-ol in NR1 (6 μg ; blend NR7; Fig. 4) to the amount in the NR old blend (200 μg) resulted in significantly fewer hawthorn flies flying upwind compared with NR1 (14% vs. 57%; $P < 0.0001$).

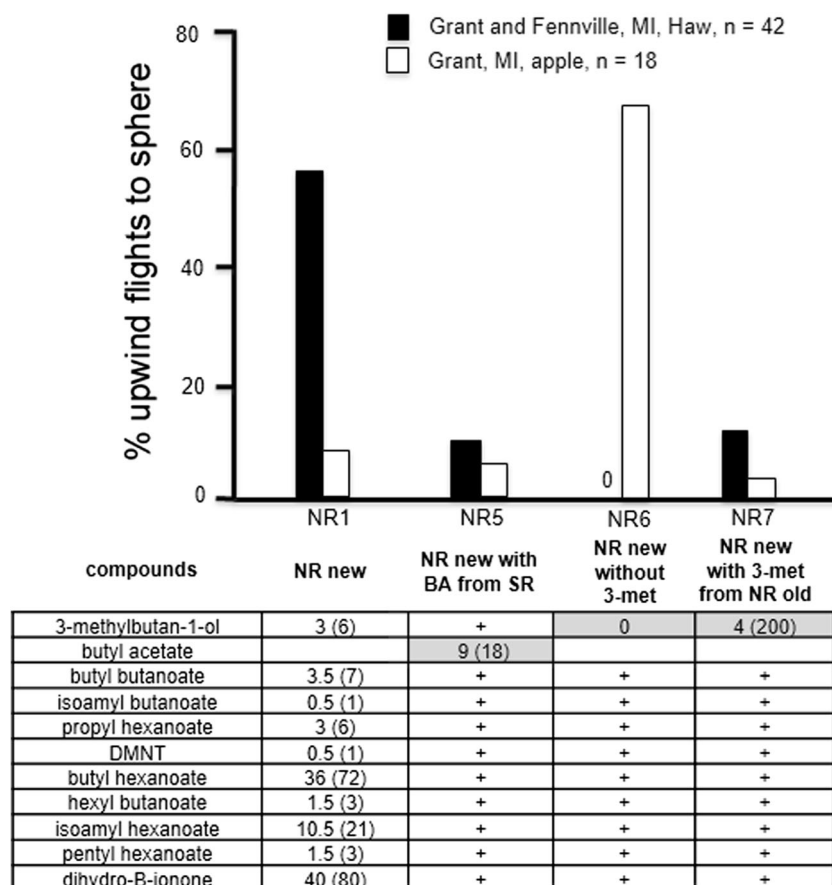
Apple-origin flies from Grant, MI ($N = 18$) were also tested to the second set of modified blends (Fig. 4). These flies exhibited low levels of upwind flight with the NR1 blend (11%), the NR1 blend with butyl acetate from the southern *C. mollis* blend added (blend NR5; 6%), and the NR1 blend with the amount of 3-methylbutan-1-ol equal to the amount in the NR old blend (NR7; 200 vs 6 μg ; 6%). Interestingly, with blend NR1 lacking the volatile 3-methylbutan-1-ol (blend NR6), apple-origin flies exhibited high levels of upwind flight (69%), a value not significantly different from the response to the apple blend (Fig. 2, 72%; $P = 0.72$). Northern *C. mollis* flies exhibited low levels of upwind flight to the apple blend (16%; Fig. 2).

For all of the different populations tested, all of the flies that responded to non-natal blends also responded to their natal blends. Thus, none of the flies displayed a preference solely for a blend that was not from their natal host.

Discussion

We have identified a new blend of volatiles from the fruit of downy red hawthorn, *C. mollis*, attractive to *R. pomonella* flies infesting this host in the northeastern

Fig. 4 Upwind flight response (% of total tested) of *Rhagoletis pomonella* flies from Grant and Fennville, MI, northern *Crataegus mollis* fruit, and Grant MI, apple fruit, to the new synthetic blend (NR1) identified from northern *C. mollis* fruit (NR1), and three modified NR1 new blends: NR5, the new NR1 blend with butyl acetate added (a key volatile in the southern *C. mollis* var. *texana* blend); NR6, the NR1 new blend with 3-methylbutan-1-ol removed from the blend; and NR7, the NR1 new blend containing the proportion of 3-methylbutan-1-ol found in the northern red haw old blend. *N* for each fly location indicates total number of flies tested to all of the blends, and numbers in column for NR1 new blend indicates the proportion and quantity (μg) of each volatile in the blend. The + symbol indicates that the compound was in the blend at the proportion and amount indicated for the northern red haw new blend. DMNT = (3*E*)-4,8-dimethyl-1,3,7-nonatriene



USA. The new blend, NR new, is as attractive as a previously identified mixture, NR old, but is more complex in the number of odorants and differs in the ratio and concentration of three volatiles that are common to both blends. In addition, the NR new blend contains a number of volatiles found in the apple blend that are attractive to apple-origin *R. pomonella* and found in other southern hawthorn blends (Cha et al. 2011a, b), including the southern *C. mollis* var. *texana* blend (Cha et al. 2012a), but were not part of the previously identified NR old blend (Nojima et al. 2003). Our results also show that red hawthorn-infesting flies from Texas in the southern USA that respond preferentially to a blend of volatiles from *C. mollis* var. *texana* (Buckl.) fruit (Cha et al. 2012a) are not attracted to the NR new blend, even though NR new resembles the southern *C. mollis* blend, SR, more than the NR old blend does. The SR blend shares nine volatiles in common with the NR new blend (3-methylbutan-1-ol, butyl hexanoate, butyl butanoate, isoamyl butanoate, dihydro- β -ionone, and four esters), but also contains at least one compound, butyl acetate, that is necessary for high levels of upwind flight for southern hawthorn flies that is antagonistic to northern flies (Cha et al. 2012a, blend NR5 in Fig. 4).

Variation in Behaviorally Active Mixtures and the Importance of Balanced Sensory Inputs for Understanding Agonist and Antagonist Effects on Behavior Our finding that northern *C. mollis* flies made equivalent levels of upwind flight to two GC-EAD active blends differing in the number and concentration of volatiles provides evidence that, although these flies can distinguish natal and non-natal hosts based on agonist/antagonist inputs from host/non-host volatiles, there is still a high degree of variation in blend quality that can stimulate oriented flight to host fruit odor. Some differences were due to the different methods used for collection and analysis of volatiles. Ethyl acetate and isoamyl acetate were detected in the previous analyses using SPME, but these compounds eluted with the solvent in the new analyses using adsorption on a solid adsorbent and solvent desorption. We do not know the extent to which the observed variability reflects natural variation in the headspace volatile profile from fruit, which could, for example, be related to many different factors such as fruit ripeness and different biotic or abiotic environmental conditions, but it might also be expected given the fact that specific fruit volatile mixtures do not function as signals to attract phytophagous insects. Rather, insects are using mixtures of volatiles from the many released from plants as cues for locating a particular host. Thus, it might be expected that there

would be greater plasticity and redundancy in the range of mixtures an insect will behaviorally respond to, than for example a sex pheromone that is required for mate location. Even within the same set of GC-EAD active components there can be different subsets that provide for same maximal attraction, as has been found in certain plant-insect systems, such as the grape berry moth (Cha et al. 2008, 2011c).

Our results not only provide evidence for a high degree of variation in blend quality/quantity, but also indicate that whether a volatile elicits an ‘agonist’ or ‘antagonist’ effect on behavior depends on the balance of sensory inputs that is a function of the ratio of odorants in the blend (see Baker 2008). First, 3-methylbutan-1-ol differed significantly in amount in the NR new versus NR old blend (6 μg vs 200 μg) but this difference did not affect maximal upwind flight levels. However, when the amount of 3-methylbutan-1-ol in the NR new blend (6 μg) was replaced by the high amount in the NR old blend (200 μg), upwind flight was significantly reduced, suggesting that the agonist activity of 3-methylbutan-1-ol was dependent on its ratio to other odorants in the mixture.

Second, the NR new blend contained a significantly higher proportion of butyl hexanoate than the NR old blend (36 vs 0.01%; 72 μg vs 1 μg), a proportion that at first suggests a conflict with previous flight tunnel results (Linn et al. 2005a). In the earlier study, butyl hexanoate, albeit at a low concentration of 0.01% (2 μg), was necessary in the NR old blend for maximal upwind flight of northern hawthorn flies. However, when an elevated level of butyl hexanoate (37%, 74 μg), such as is found in the apple blend, was added to the NR old blend, upwind flight of northern *C. mollis* flies was significantly reduced. Thus, the earlier work implied that an antagonist effect of butyl hexanoate exists for hawthorn flies at high concentrations (Linn et al. 2005a). However, in the current study, the NR new blend contains butyl hexanoate at 36% (72 μg) and is just as attractive to hawthorn flies as the NR old blend. One possible explanation for the difference is that the antagonist action of butyl hexanoate observed when added at a high level to the NR old blend (Linn et al. 2005a) was due to 3-methylbutan-1-ol also being present at an elevated concentration in the mixture. The combination of high levels of butyl hexanoate and 3-methylbutan-1-ol might have created an imbalance in sensory inputs for blend recognition in hawthorn flies, resulting in antagonism. However, when 3-methylbutan-1-ol is present at a lower level, as in the NR new blend, a higher proportion of butyl hexanoate can be present and not affect hawthorn fly behavior. Further, when both 3-methylbutan-1-ol and butyl hexanoate are present at low levels (the NR new blend with the proportion of butyl hexanoate in the old blend; blend NR2 in Fig. 3) response levels were significantly lower, but not drastically reduced, dropping from 75 to 53%. This was probably not due to antagonism but rather less stimulation from the lower quantities

of both volatiles. Overall, the results suggest that, as with 3-methylbutan-1-ol, the agonist/antagonist effect of butyl hexanoate on northern hawthorn fly behavior varies as a function of its concentration/ratio in the blend, in particular, in relation to 3-methylbutan-1-ol.

Third, the conclusions above also depend on the quantity of dihydro- β -ionone in the blend. As with butyl hexanoate, there was significantly more ionone in the NR new blend (40%, 80 μg) compared with the NR old blend (0.1%, 2 μg). Reducing the amount of ionone in the NR new blend to the level of the NR old blend (blend NR3, Fig. 3) significantly reduced the level of upwind flight. This result suggests that the agonist action of low 3-methylbutan-1-ol and high butyl hexanoate is eliminated when an imbalance of a high concentration of ionone is also present.

The concentrations and ratios of other volatiles in addition to 3-methylbutan-1-ol, butyl hexanoate, and dihydro- β -ionone might also affect *R. pomonella* behavior. Due to limitations in the number of available flies we could test in the flight tunnel, we were not able to conduct exhaustive tests with modified blends adding and subtracting the other volatiles present in the NR new blend. Rather, we focused on 3-methylbutan-1-ol and butyl hexanoate, because of their previously known major effects on fly behavior. The third volatile, dihydro- β -ionone, was selected because of the great variability in the amount of this compound found in the NR new versus NR old blend (40%, 80 μg versus 0.1%, 4 μg). The impact of DMNT, butyl butanoate, and other volatiles found in the new hawthorn blend could therefore provide further insight into the nature of how particular compounds, alone and in combination, affect host preference. In this regard, it is of interest that apple-origin flies exhibited high levels of upwind flight to the NR new blend when 3-methylbutan-1-ol was removed (Fig. 4, blend NR6), implying that they are not antagonized by DMNT, dihydro- β -ionone, or any other volatile in the NR new blend. This result also emphasizes the importance of 3-methylbutan-1-ol in the shift that occurred from red downy hawthorn to apple.

Esters in the Northern Hawthorn Blend and the Origin of the Apple Host Race As noted above, one of the important findings in the current study was that there were a number of ester compounds in the NR new blend that are found in the northern apple blend, a property that is mirrored in the series of southern hawthorn blends (Cha et al. 2011a, b; see Powell et al. 2012, 2014). One of the motivations for the southern hawthorn studies was to address a key question concerning the origin of the formation of the apple host race in the northern USA. Was the introduction of a geographically differentiated, hawthorn-infesting form of *R. pomonella* from the South the source of the apple-infesting race in the North (Carson 1989; see Bush et al. 1989)? The studies with southern hawthorns did show the presence of apple-like compounds in the GC-

EAD and behaviorally active profiles, but the hypothesis was not supported as evidenced by the fact that 1) flies collected from all of the southern hawthorn varieties exhibited very low response levels with the apple blend in flight tunnel assays; and 2) all of the apple-responding southern hawthorn flies also responded to their natal fruit extract or synthetic blend. Rather than a pre-existing apple population in the southern hawthorns, our results support the hypothesis that the presence of esters in the southern and northern hawthorn fruits provided a level of standing variation in the odor space of *Rhagoletis* flies. This pre-existing ability to detect the ester compounds, also evidenced by the results of single-sensillum recordings showing that flies in both of the host races are able to detect all of the volatiles in the respective blends (Olsson et al. 2006, 2009), could facilitate the shift to apples, especially given the fact that esters dominate the volatile profile for mature apple fruit (Bengtsson et al. 2001; Brown et al. 1966; Carle et al. 1987; Mattheis et al. 1991).

A recent report by Tait et al. (2016) supports the hypothesis that the shift from downy hawthorn to apple involved a change in chemosensory processing in which the balance between 3-methylbutan-1-ol and butyl hexanoate (at minimum), two key volatiles that exert agonist and antagonist effects on the response of hawthorn- and apple-origin flies to their respective blends, was altered. Electrophysiological recordings were made from antennal olfactory sensory neurons of flies infesting northern apple and downy red hawthorn fruit (see Olsson et al. 2006, 2009). A total of 28 different classes of neurons were identified, but butyl hexanoate and 3-methylbutan-1-ol were shown to be recognized by only four olfactory neurons that were co-localized as responding pairs in two sensilla. The authors hypothesized that a subset of the downy hawthorn fly population exhibited a reverse in valence between the co-localized neurons. A switch between the neuronal pairs in these specific sensilla could produce a fly phenotype that showed avoidance of the key 3-methylbutan-1-ol volatile rather than attraction, and attraction to the esters present in the apple blend rather than avoidance. The importance of the antagonist effect of 3-methylbutan-1-ol on the preference of apple-origin flies is evidenced by the fact that removal of this volatile from the new northern red hawthorn blend removed all inhibition on upwind flight (Fig. 4).

A final important question is whether our results call for a re-examination of the apple blend: Would a more detailed analysis reveal the presence of, for example, 3-methylbutan-1-ol, butyl acetate, or other hawthorn-specific volatiles that can exert antagonistic effects on the response of apple-origin flies to their natal blend? We contend that the answer to this question is no. In the study of *Rhagoletis* flies infesting different hosts in the western USA (Cha et al. 2012b, Linn et al. 2012), GC-EAD profiles for apple-origin flies collected from an introduced population in Washington state (Fig. 1a in Cha et al. 2012b) and for a laboratory colony established from Geneva,

New York, for the adsorbent extract from red delicious apples at the Agricultural Experiment Station in Geneva, contained all of the identified behaviorally active apple ester volatiles. Moreover, there was no evidence for the presence of 3-methylbutan-1-ol or butyl acetate, a result that also applies to two earlier studies characterizing behaviorally active apple volatile blends for apple-origin flies (Fein et al. 1982; Zhang et al. 1999).

Conclusions Our identification of a new northern *C. mollis* blend provides insight into the nature of how chemosensory cues in *R. pomonella* affect host location behavior and how specific compounds present in the headspace of host fruit may have affected the shift to apple. Foremost among these findings is the presence of previously unidentified, behaviorally active apple esters in the fruit of northern downy hawthorns. Our results will aid in future investigations into the neurophysiological and genetic differences between hawthorn and apple flies responsible for their alternate preferences for apple versus hawthorn fruits.

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