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ORIGINAL PAPER

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The chemosensory basis for behavioral divergence involved in sympatric host shifts. I. Characterizing olfactory receptor neuron classes responding to key host volatiles

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Abstract The recent shift of *Rhagoletis pomonella* from its native host hawthorn to introduced, domestic apple has been implicated as an example of sympatric speciation. Recent studies suggest that host volatile preference might play a fundamental role in host shifts and subsequent speciation in this group. Single sensillum electrophysiology was used to test a proposed hypothesis that differences in *R. pomonella* olfactory preference are due to changes in the number or odor specificity of olfactory receptor neurons. Individuals were analyzed from apple, hawthorn, and flowering dogwood-origin populations, as well as from the blueberry maggot, *Rhagoletis mendax* Curran (an outgroup). Eleven compounds were selected as biologically relevant stimuli from previous electroantennographic/behavioral studies of the three *R. pomonella* populations to host fruit volatiles. Cluster analysis of 99 neuron responses showed that cells from all tested populations could be grouped into the same five classes, ranging from those responding to one or two volatiles to those responding to several host volatiles. Topographical mapping also indicated that antennal neuron locations did not differ by class or fly taxa. Our results do not support the hypothesis that differences in host preference among *Rhagoletis* populations are a result of alterations in the number or class of receptor neurons responding to host volatiles.

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Abbreviations GC–EAD: Gas chromatography coupled with electroantennographic detection · ORN: Olfactory receptor neuron

Introduction

In the 17th century, domestic apple trees were virtually nonexistent in the United States. By 1875, settlers had planted roughly eighteen million trees in New York State alone (Chapman 1971). It was there that Benjamin Walsh (1867) first cited the shift of *Rhagoletis pomonella* (Walsh) (a true fruit fly) from its native host hawthorn (*Crataegus* spp.) to the new viable host, apple (*Malus pumila* P. Mill) as the formation of a new host race. Bush (1969) further proposed that members of the *R. pomonella* group were established by sympatric speciation via host plant shifts. The *R. pomonella* species complex contains several host-specific populations lacking fixed allozyme differences, including hawthorn, apple, and flowering dogwood-origin flies (hereafter referred to simply as dogwood) (Berlocher 2000). In fact, phylogenetic data suggest that multiple host shifts have occurred within the *pomonella* species group (Berlocher 2000).

Monophagous *Rhagoletis* flies mate exclusively on or near the fruit of their host (Prokopy et al. 1971), and identify each host through visual, tactile and olfactory cues (Bush 1969; Fletcher and Prokopy 1991, and references therein). Mark-recapture studies indicate that flies can travel hundreds of meters from their point of origin (Feder et al. 1998), so host-specific cues are of primary importance for host location. Variation in host preference therefore creates positive assortative mating that serves as a premating barrier to gene flow among flies infesting different plant species (Feder et al. 1994). Speciation can thus ensue without the necessity of a geographic barrier. Behavioral analyses show distinct

preferences of *Rhagoletis* populations for unique volatile blends identified from each host fruit (see Table 1; Zhang et al. 1999; Nojima et al. 2003a, b). Such discrimination persists even when hawthorn-origin flies have been reared on apples for two generations (Linn et al. 2003). In fact, recent studies show that *Rhagoletis* flies not only preferentially orient to their own host volatile blends in the field (Linn et al. 2003), but they also avoid the blends of non-hosts (Linn et al. 2005a).

One of the foremost issues in understanding the proposed shifts in host volatile preference is how such levels of olfactory discrimination could be established within a mere 150 generations (as is the case for apple and hawthorn-origin flies), or indeed, exist in sympatry at all. Behavioral studies indicate that maximal levels of upwind flight occur to unique blends of chemicals, but it is not known whether this is because flies possess unique sets of olfactory receptor neurons (ORNs), or whether flies have altered the behavioral functionality of compounds associated with a specific host type. In *Rhagoletis*, it has been proposed that "minor genetic changes affecting the number or odor specificity of a specific receptor cell type may thus be an important mechanism promoting host shifts and speciation" (Frey and Bush 1990). Although plant-odor ORNs have often been considered to be broadly tuned in relation to pheromone receptor neurons, recent studies show they can possess highly sensitive and specific receptor neurons (e.g., Dickens 1990; Anderson et al. 1995; Wibe and Mustaparta 1996; Hansson et al. 1999; Stensmyr et al. 2001). Therefore, it is possible that host-shifting populations have generated unique ORN specificities for certain host volatiles, or have dramatically altered the number of ORNs responding to those volatiles. A recent study by Stensmyr et al. (2003) found a shift in specificity among three different *Drosophila* species in a single ORN type as well as a complete loss of a sensillum type in *Drosophila sechellia*. Additionally, other studies in *Drosophila* have found that ORNs with similar specificities reside in morphologically localized areas on the antennal surface (de Bruyne et al. 2001). If such localization also occurs in *Rhagoletis*, could host-shifting populations also differ as to the position of specific ORNs?

Table 1 Key host plant volatiles determined through GC–EAD and behavioral assays of host fruit (from Zhang et al. 1999; Nojima et al. 2003a, b)

Host fruit	Compounds
Apple (*Malus pumila*)	Butyl hexanoate
	Hexyl butanoate
	Butyl butanoate
	Pentyl hexanoate
	Propyl hexanoate
Hawthorn (*Crataegus* spp.)	Butyl hexanoate
	3-methylbutan-1-ol
	Isoamyl acetate
	4,8-dimethyl-1,3*(E)*, 7-nonatriene
	Dihydro-β-ionone
	Ethyl acetate
Dogwood (*Cornus florida*)	1-octen-3-ol
	Isoamyl acetate
	3-methylbutan-1-ol
	Ethyl acetate

To test these hypotheses, we have carried out a comparative study using four closely related *R. pomonella* taxa: two host races (apple and hawthorn origin), a sibling species (dogwood origin), and the most closely related confirmed species as an outgroup comparison (*Rhagoletis mendax* Curran, the blueberry maggot) (Berlocher 2000), using biologically relevant olfactory stimuli. The relevant host volatiles for the *R. pomonella* populations were identified by gas chromatography/electroantennographic detection (GC–EAD) and flight-tunnel behavioral studies with host fruit (Zhang et al. 1999; Nojima et al. 2003a, b). These two methods allowed hundreds of host fruit compounds to be screened for activity in order to identify unique blends of key host compounds specific for each host taxon (see Table 1). Therefore, instead of examining hundreds of potential ligands for contacted ORNs, we chose only those compounds that had been identified as relevant to *Rhagoletis* host preference and a potential role in host shifts. In this study, we show that all populations tested possess similar numbers of single and multiple compound-responding ORNs and that there are no qualitative differences in ORN classes among the *Rhagoletis* taxa when tested at a high (10 μg) stimulus load. In the companion paper (Olsson et al. 2005), we present a detailed quantitative analysis of population differences in ORN response characteristics (e.g., sensitivity and temporal firing pattern) to host stimuli throughout a range of odor concentrations.

Materials and methods

Rhagoletis *origins and rearing conditions*

Female *Rhagoletis* flies were selected from lab-reared or field-collected populations as follows (see Linn et al. 2005b for detailed description of *R. pomonella* origins): *R. pomonella* [apple (lab colony) origin]—Lab colony, Geneva, NY; *R. pomonella* [apple (wild) origin]—Grant, MI; *R. pomonella* (hawthorn origin)—Grant, MI; *R. pomonella* (dogwood origin)—Granger, IN; and *R. mendax* (blueberry origin)—Burlington County, NJ.

Field-collected flies were gathered as larvae from fruit at the site of origin, and post-diapause pupae (Feder et al. 1994) shipped to Geneva, New York. They were 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). Lab-reared flies were maintained on Red Delicious apples (Neilson and McAllen 1965). Adults were kept in an environmental chamber at 23°C temperature, 50% relative humidity, 16L:8D photoperiod, and maintained on the same artificial diet.

All flies used for neurophysiological analyses were between 0 and 20 days of age. Flies generally survive

up to 4 weeks and can be used for behavioral analyses at any point after 8–10 days of age, the age of reproductive maturity (Zhang et al. 1999; Nojima et al. 2003a, b). Thus, senescence was not suspected during the first 20 days of life, and in most cases recordings were made from flies between 0 and 7 days old because of the increased vigor of younger flies. Flies 0–7 days old were also used in previous GC–EAD identification of the host volatiles (Zhang et al. 1999; Nojima et al. 2003a, b).

Olfactory stimuli

Propyl hexanoate, hexyl butanoate, 3-methylbutan-1-ol, isoamyl acetate, 1-octen-3-ol, and butyl hexanoate were purchased from Aldrich, Milwaukee, WI, USA (purity >98%); butyl butanoate and pentyl hexanoate from Pfaltz and Bauer, Stamford, CT, USA; ethyl acetate from Fisher Scientific, Fair Lawn, NJ, USA (purity >99%); and dihydro-β-ionone from Scientific Exchange, Inc., Center Ossipee, NH, USA (purity was >89% based on GC-MS analysis). 4,8-Dimethyl-1,3*(E)*,7-nonatriene was synthesized according to Greenwald et al. (1963) (87:13 ratio of E and Z isomers with >96% purity based on GC-MS analysis). The individual chemicals were dissolved in hexane at a concentration of 1 μg/μl.

Stock solutions (1 μg/μl) of individual key fruit volatiles and specific fruit blends in hexane were prepared as follows: apple blend (propyl hexanoate, hexyl butanoate, butyl butanoate, pentyl hexanoate, and butyl hexanoate), hawthorn blend (butyl hexanoate, 3-methylbutan-1-ol, isoamyl acetate, 4,8-dimethyl-1,3*(E)*,7-nonatriene, dihydro-β-ionone, and ethyl acetate), and dogwood blend (1-octen-3-ol, 3-methylbutan-1-ol, isoamyl acetate, and ethyl acetate).

Ten microliters of each diluted compound or blend were pipetted onto small pieces of filter paper (ca. 5 mm×15 mm) placed in disposable Pasteur pipettes to establish a 10 μg stimulus load. Blank cartridges, containing only filter paper plus solvent, were also prepared. In order to prevent evaporation and contamination, cartridges were not used after 2.5 h.

Electrophysiological recording

Female *Rhagoletis* were confined in the tapered, cut end of a 100 μl pipette tip with only their heads protruding. Heads were immobilized at the base with dental wax to restrict movement. A sharpened tungsten wire was inserted into the right eye, serving as a ground electrode. Tungsten microelectrodes, electrolytically sharpened in KNO_2 solution, were used to establish contact with the ORNs. The recording electrode was positioned at, or near, the base of sensilla using a preparation microscope with up to 200× magnification and an electrophysiological recording unit with combined joystick micromanipulators and amplifier (Syntech INR-5, Hilversum). In most cases, the ventral portion of the left antenna was used for recording.

A constant flow of charcoal-filtered and humidified air passed over the antenna from a stimulus air controller at approximately 2.6 l/min (Syntech, CS-5, Hilversum). The constant flow included both a continuous (~1.5 l/min) and a complimentary (~1.3 l/min) air stream. Air passed through a metal tube attached to the recording unit whose outlet protruded approximately 10 mm from the antenna. Stimulation was performed by inserting the tip of the test pipette into a hole in the metal tube, approximately 10 cm before the outlet. The test pipette was connected to the stimulus air controller, which generated air puffs (~1.3 l/min during 0.5 s) through the pipette and replaced the complimentary air stream during that time period.

The analog signal originating from the ORNs was amplified (10×) (Syntech INR-5, Hilversum), sampled (31746.0 samples/s), and filtered (200–3000 Hz with 50/60 Hz suppression) via USB-IDAC connection to a computer (Syntech, Hilversum). Action potentials were extracted as digital spikes from the analog signal according to top–top amplitudes using Syntech Auto Spike 32 v. 1.1b and 2.2 software. When different spike heights occurred in the same recording, individual neurons were sorted manually for each recorded trace based on differences in the amplitude of their action potentials (spikes). Consideration was taken for changes in amplitude due to excessive firing (i.e., "pinching"). Differences in amplitude between co-located spikes were measured in μV for each recording based upon signal output (see Table 2).

In the event of a contact, ORNs were first screened with the three fruit blends at a 10 μg stimulus load (apple, hawthorn, and dogwood), and the blank (hexane). These stimuli were tested at least once at the beginning and, in nearly all cases, end of each recording period to observe if cell responsiveness was preserved throughout the recording session. All stimuli were presented in 0.5 s air puffs at approximately 1-min intervals to allow the ORNs to return to baseline firing rate. If the neuron(s) responded to one or more of the blends (see below for definition of a response), then all 11 components of the three blends were tested individually at a stimulus load of 10 μg, as in Stensmyr et al. (2003) and analogous to the 10^{-2} dilutions used in de Bruyne et al. (1999, 2001). To establish possible topographic relationships, the location of the electrode tip was noted on a template drawing of the antenna after the recording had been performed.

Data analysis

For each ORN testing period, spike frequencies of the blank (hexane) were calculated every 600 ms (equal to the 500-ms stimulus period plus an additional 100 ms) for the entire 10.8 s recording period (including 1 s pre- and 9.8 s post-stimulus onset). In the majority of cases, more than one blank trial was presented. Spike counts

Table 2 Description of all contacted ORNs for each *Rhagoletis* population with ORN classes and response profiles as in Fig. 1

Class	Fly population	ORN response profile	Recording description
A	Apple (lab)	O3OL	1 ORN
A	Apple (lab)	O3OL	1 ORN
A	Apple (lab)	O3OL	1 ORN
A	Apple (wild)	O3OL	1 ORN
A	Blueberry	O3OL	1 ORN
A	Dogwood	O3OL	1 ORN
A	Hawthorn	O3OL	1 ORN
A	Hawthorn	O3OL	1 ORN
A	Hawthorn	O3OL	1 ORN
A	Hawthorn	O3OL	1 ORN
A	Hawthorn	O3OL	1 ORN
A	Hawthorn	O3OL	1 ORN
A	Apple (wild)	O3OL	1 ORN of 2 Close
A	Dogwood	O3OL	1 ORN of 2 Close
A	Apple (lab)	O3OL	1 ORN of 2, +100
A	Apple (lab)	O3OL	1 ORN of 2, −200
B	Apple (lab)	HB	1 ORN
B	Apple (lab)	HB	1 ORN
B	Apple (wild)	HB	1 ORN
B	Apple (wild)	DBI	1 ORN
B	Blueberry	DBI	1 ORN
B	Dogwood	HB	1 ORN
B	Dogwood	HB	1 ORN
B	Dogwood	HB	1 ORN
B	Hawthorn	DBI	1 ORN
B	Apple (wild)	DBI	1 ORN of 2 Close
B	Apple (lab)	DBI	1 ORN of 2, +100
B	Apple (wild)	HB, PrH	1 ORN of 2, +100
B	Blueberry	HB	1 ORN of 2, +100
B	Apple (lab)	DBI	1 ORN of 2, +200
B	Apple (lab)	HB	1 ORN of 2, −200
C	Apple (lab)	3 MB, PrH	1 ORN
C	Apple (lab)	DNT, PrH, PeH	1 ORN
C	Apple (lab)	3 MB, HB, PeH, BH	1 ORN
C	Apple (wild)	DNT	1 ORN
C	Apple (wild)	DNT, 3 MB, DBI, EA, IAA, O3OL	1 ORN
C	Blueberry	DNT, PrH	1 ORN
C	Blueberry	IAA	1 ORN
C	Blueberry	DNT, 3 MB, PeH	1 ORN
C	Hawthorn	DNT	1 ORN
C	Hawthorn	DNT	1 ORN
C	Dogwood	DNT, 3 MB, IAA	1 ORN of 2 Close
C	Apple (lab)	DNT	1 ORN of 2, +100
C	Blueberry	DNT	1 ORN of 2, +100
C	Blueberry	DNT, BB, O3OL	1 ORN of 2, +100
C	Apple (wild)	DNT	1 ORN of 2, +200
C	Blueberry	DNT	1 ORN of 2, +200
C	Blueberry	DNT, PrH	1 ORN of 2, +200
C	Blueberry	DNT, PrH	1 ORN of 2, +200
C	Blueberry	DNT, PrH	1 ORN of 2, +200
C	Blueberry	DNT, 3 MB, IAA, O3OL	1 ORN of 2, +200
C	Hawthorn	DNT	1 ORN of 2, +200
C	Hawthorn	DNT, PrH	1 ORN of 2, +200
C	Blueberry	DNT, PrH	1 ORN of 2, +300
C	Blueberry	DNT, O3OL	1 ORN of 2, +300
C	Apple (wild)	DNT, 3MB	1 ORN of 2, +400
C	Apple (wild)	DNT	1 ORN of 2, −200
C	Apple (lab)	3 MB, IAA	2 ORNs of 2, −100
C	Dogwood	PrH, PeH	2 ORNs of 2, −100
C	Hawthorn	DNT, 3 MB, HB	2 ORNs of 2, −100
D	Apple (lab)	PrH, BH	1 ORN
D	Apple (lab)	PrH, HB, BB, PeH, BH, O3OL	1 ORN
D	Apple (lab)	PH, HB, PeH, BH	1 ORN
D	Apple (lab)	PrH, HB, BB, PeH, BH	1 ORN
D	Blueberry	PrH, BB, PeH, BH	1 ORN
D	Hawthorn	PrH, HB, PeH, BH, DNT, EA	1 ORN
D	Dogwood	PrH, HB, BB, DNT	1 ORN of 2 Close

Table 2 (Contd.)

Class	Fly population	ORN response profile	Recording description
D	Hawthorn	PrH, PeH, BH	1 ORN of 2 Close
D	Apple (wild)	PrH, PeH, BH	1 ORN of 2, +100
D	Dogwood	PrH, BB, PeH, BH	1 ORN of 2, +100
D	Blueberry	PrH, BH	1 ORN of 2, +150
D	Apple (wild)	PrH, HB, BH, DNT, O3OL	1 ORN of 2, +200
D	Apple (wild)	PrH, PeH, BH	1 ORN of 2, +200
D	Blueberry	PrH, PeH, BH	1 ORN of 2, +200
D	Blueberry	PrH, HB, BB, BH	1 ORN of 2, +300
D	Blueberry	PrH, HB, BB, PeH, BH, O3OL	1 ORN of 2, +400
D	Dogwood	PrH, BH	1 ORN of 2, +50
D	Dogwood	PrH, BB, PeH, BH	1 ORN of 2, +50
D	Apple (wild)	PrH, HB, BB, BH, DNT	1 ORN of 2, +70
D	Apple (lab)	PrH, BH, EA, IAA	1 ORN of 2, −100
D	Dogwood	PrH, BB, PeH, BH, DNT	2 ORNs of 2, +100
D	Hawthorn	PrH, BH	2 ORNs of 2, +100
E	Apple (lab)	PrH, BB, PeH, BH, 3 MB, IAA	1 ORN
E	Apple (lab)	PrH, HB, BB, PeH, BH, DNT, EA, 3 MB, IAA, O3OL	1 ORN
E	Apple (wild)	PrH, HB, PeH, BH, DNT, IAA, O3OL	1 ORN
E	Apple (wild)	PrH, HB, BB, PeH, DNT, DBI, 3 MB, IAA	1 ORN
E	Apple (wild)	PrH, HB, BB, PeH, BH, DNT, DBI, EA, 3 MB, IAA, O3OL	1 ORN
E	Blueberry	PrH, HB, PeH, BH, DNT, DBI, IAA, O3OL	1 ORN
E	Blueberry	PrH, HB, BB, PeH, BH, DNT, 3 MB, IAA, O3OL	1 ORN
E	Dogwood	PrH, PeH, DNT, 3 MB, IAA, O3OL	1 ORN
E	Dogwood	PrH, BB, BH, DNT, 3 MB, IAA	1 ORN of 2 Close
E	Apple (lab)	PrH, PeH, BH, DNT, IAA	1 ORN of 2, +100
E	Hawthorn	PrH, HB, PeH, BH. DNT, 3 MB, IAA, O3OL	1 ORN of 2, +100
E	Hawthorn	PrH, HB, BB, PeH, BH, DNT, EA, IAA, O3OL	1 ORN of 2, +150
E	Hawthorn	PrH, HB, PeH, BH, DNT, 3 MB, IAA	1 ORN of 2, −100
E	Apple (wild)	PrH, HB, PeH, BH, DBI, EA, 3 MB, IAA, O3OL	1 ORN of 2, −120
E	Dogwood	PrH, HB, PeH, BH, DBI, 3 MB, IAA	1 ORN of 2, −200
E	Dogwood	PrH, HB, PeH, BH, DNT, 3 MB, IAA, O3OL	1 ORN of 3 Close
E	Apple (lab)	PrH, BB, PeH, BH, 3 MB, IAA	2 ORNs of 2, +100

Recording description presents the number of ORNs responding in the 10.8 s recorded trace as well as the total number of spikes found in that recording. Numbers following the description indicate the relative difference in μV amplitude between co-located cells measured separately for each contact. For each description, (+) values indicate that the responding cell was the larger amplitude cell in the recording, (−) indicate that it was the smaller, and (close) indicates that the cells could be separated by amplitude at values too small to measure accurately. Descriptions are organized by class and recording. ORN response profile abbreviations represent the following compounds:
3 MB 3-methylbutan-1-ol, *BB* butyl butanoate, *BH* butyl hexanoate, *DBI* dihydro-β-ionone, *DNT* 4,8-dimethyl-1,3(*E*),7-nonatriene, *EA* ethyl acetate, *HB* hexyl butanoate, *IAA* isoamyl acetate, *PrH* propyl hexanoate, *PeH* pentyl hexanoate, *O3OL* 1-octen-3-ol

per 600 ms were then averaged across all blank trials. An increase (or decrease) in spike frequency for the 600 ms following stimulus presentation was considered a response if it was >3 SD above or below the blank mean. Changes in ORN spike frequencies below this level were not considered further. In this manner, a response was considered excitatory if it produced a significant increase in spike frequency and inhibitory if it caused a decrease. Although there were clear differences in spike frequency between ORN responses and nonresponses (the latter generally producing no change in frequency at all), the above method was chosen as a way to allow statistical comparisons of responses from several ORNs and fly populations. Comparison to the blank trial was used to confirm a response because time restrictions due to ORN and insect longevity prohibited multiple stimulation comparisons of each compound.

To determine the range of ORN classes (i.e., the number and variety of biologically relevant volatiles to which each ORN responded), cells were classified across fly population [apple (lab colony), apple (wild), hawthorn, dogwood, and blueberry] according to response pattern to the 11 host compounds used in the study. Cluster analysis was performed by SPSS version 11.0 software using Ward's Method with intervals measured by squared Euclidian distance. A χ^2 test was used to compare ORN frequencies for each population among the clusters.

To compare ORN topographical locations, X and Y coordinates of contacted cells were determined from the template drawing location using Adobe Photoshop v. 8.0. Coordinates were then compared for both the effect of fly taxa and response profile by multivariate regression analysis and analysis of variance (MANOVA) including Pillai's Trace, Wilks' Lambda, and post hoc Tukey HSD using SPSS version 12.0 software.

Results

Olfactory receptor neurons (ORNs, $n=99$) from 38 individuals among the four populations were used for

neurophysiological analyses. For *R. pomonella* these included: seven individuals of apple (lab colony) origin (ORN $n = 24$), seven individuals of apple (Grant, MI) origin (ORN $n = 19$), ten individuals of hawthorn origin (ORN $n = 18$), and six individuals of dogwood origin (ORN $n = 16$). For *R. mendax*, eight individuals of blueberry origin (ORN $n = 22$) were used.

ORN excitation/inhibition

ORN responses to host stimuli could be characterized by either an increase in firing frequency during/after stimulus exposure (excitation), or a decrease or temporary termination of firing (inhibition). As all ORNs possessed some degree of spontaneous firing, both inhibition and excitation were potential response characteristics. However, all 99 ORN responses recorded for *Rhagoletis* were characterized by a significant increase in firing frequency following stimulus exposure (excitation). This was also the case when host volatile blends were presented to each ORN.

ORN response classes

To compare ORN responses to the biologically relevant host volatiles, cluster analysis of ORN response pattern was performed for all contacted cells responding to one or more of the tested stimuli at the high 10 μg stimulus load. Results of the cluster analysis are shown as a stylized dendrogram in Fig. 1 and reveal four important findings. First, the figure illustrates that all responding ORNs could be grouped into two major clusters and five subgroups, or classes, representing increasing levels of complexity with respect to the number of compounds eliciting a response, and associations involving, with few exceptions, specific volatiles. Cluster 1 contains ORNs that responded to relatively few key host volatiles. Class A contains ORNs that responded only to 1-octen-3-ol (a dogwood volatile). Class B contains cells that responded to either dihydro-β-ionone (a hawthorn volatile) or hexyl butanoate (an apple volatile), with one ORN also responding to propyl hexanoate (another apple volatile). Class C contains cells that responded to 4,8-dimethyl-1,3*(E)*,7-nonatriene (a hawthorn volatile) and/or 3-methylbutan-1-ol (in both hawthorn and dogwood blends), either exclusively or with one or more other compounds. The exceptions to this categorization are two cells that responded to isoamyl acetate and propyl hexanoate/pentyl hexanoate, respectively.

Cluster 2 contains multiple compound-responding ORNs, all including at least two longer chain esters (i.e., not acetates; henceforth referred to simply as "esters"). Esters are found almost exclusively in the apple host blend, with butyl hexanoate as a minor component in the hawthorn blend (Zhang et al. 1999; Nojima et al. 2003a). Class D contains ORNs that responded to esters and occasionally one or two other compounds. Although class D contains ORNs that responded to 4,8-dimethyl-1,3*(E)*,7-nonatriene as in class C, 4,8-dimethyl-1,3*(E)*,7-nonatriene responders in class D were only found in conjunction with response to at least three esters, and only five such cells were found. Furthermore, no class D cells responded to 3-methylbutan-1-ol as in class C. Finally, class E contains cells that responded to at least two esters along with several other compounds, including 3-methylbutan-1-ol and/or 4,8-dimethyl-1,3*(E)*,7-nonatriene.

A second finding is that many of the contacted cells, if responding to any fruit blends, only responded to select compounds in those blends at the 10 μg stimulus load used. Some ORNs, such as those responding to 1-octen-3-ol (Class A cells), were highly selective to a single compound of the 11 tested. Other cells responded only to a specific chemical group, such as esters (most Class D cells).

Figure 1 also illustrates that *Rhagoletis* ORNs, though selective to the tested volatiles, were not specific to any population. The four *Rhagoletis* taxa were spread across the different ORN classes and no clustered group was exclusive to any specific population. Thus, the populations did not differ as a function of presence or absence of response to a particular volatile.

A fourth finding is that there did not appear to be a large bias in number of contacted ORNs among the clusters for any fly taxa. A χ^2 test did not reveal any significant differences among or between clusters $\chi^2(16, n = 99) = 18.103$, NS.

Sensillum organization

All electrophysiological recordings performed on *Rhagoletis* contained between 1 and 3 spontaneously active ORNs according to manual separation of their spikes by amplitude. However, in only three cases (Fig. 1 lettered symbols) did multiple cells respond to host volatiles concurrently within the same recording [two cells each from an apple (lab colony) (a), hawthorn (b), and dogwood (c) fly recording]. In all other recordings, only one cell responded to the tested volatiles, and in many cases only one cell was found within the trace. Table 2 provides a description of the recording from which each ORN in Fig. 1 was found. The table illustrates that each cluster, or class, in Fig. 1 contains ORNs that were found both co-localized as well as unaccompanied within each recorded trace. Although the exact location of each sensillum could not be confirmed at the magnification used in these methods, the variety in recorded traces indicates the presence of multiple sensillum types, and possibly multiple ORN types within each class. However, further analysis of Table 2 reveals that several ORNs from each *Rhagoletis* population not only responded similarly to the host volatiles, but likely resided in similar sensillum types. For example, all populations contained an ORN recorded singly that responded to 1-octen-3-ol (class A). Each population also contained

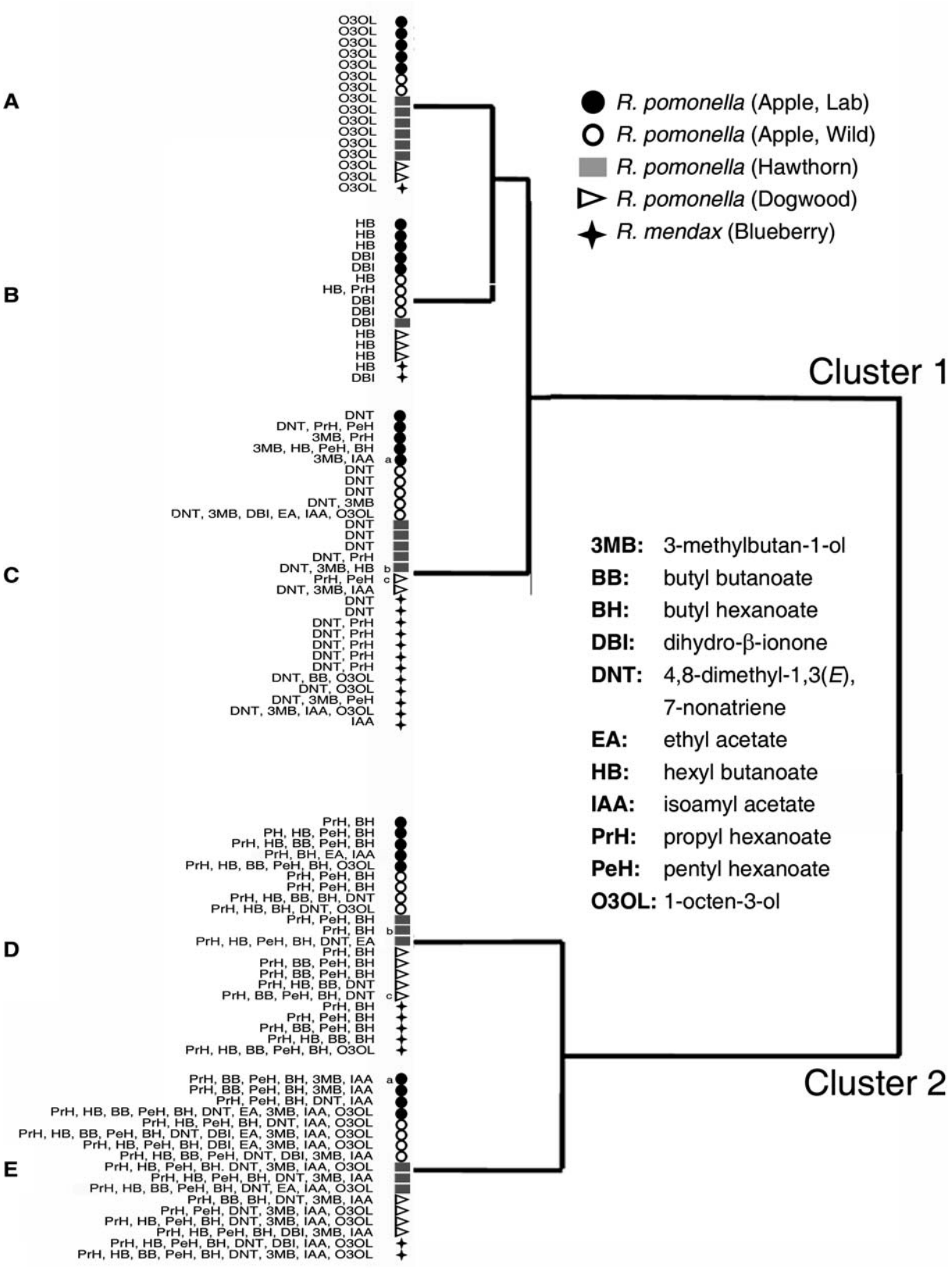

Fig. 1 Dendrogram depicting clusters (classes) based on ORN responses to 10 μg stimulus loads of the compounds listed on the *right side* of the figure. *Symbols* represent specific *Rhagoletis* ORNs for each population. The response of each ORN is listed to the *left* of its corresponding symbol. *Letters* to the *left* of ORN *symbols* indicate multiple ORNs responding to stimuli within the same recorded trace [2 cells each from an apple (**a**), hawthorn (**b**), and dogwood (**c**) recording]

ORNs responding to class C and D compounds recorded with another, non-responding ORN, although it cannot be confirmed that this ORN was located in the same sensillum.

Nevertheless, there are several notable differences in the recorded traces that may indicate variation in sensillum organization among the populations. First, only apple and dogwood populations contained ORNs responding to 1-octen-3-ol that were recorded with a non-responding ORN (class A). Second, only apple and blueberry populations contained cells responding to class B compounds that were co-localized with a non-responder. Third, the dogwood population lacked any class C- or D-responding ORNs recorded singly. This is also true for the hawthorn population with class E and the wild apple population for class D. Finally, although in the majority of cases, the larger amplitude ORN responded to host volatiles, a few cases (6 ORNs among classes B–E) possessed ORNs responding with a smaller amplitude co-localized with non-responders.

ORN topographical mapping

In addition to classifying ORN responses according to the range of volatiles eliciting a response, cells were also mapped to determine if ORN classes were morphologically localized among fly varieties. During contact, the antennae were observed under 200× magnification. Although specific sensilla could not be identified, their location on the antenna could be determined with some accuracy. ORN responses were mapped on a photograph of the ventral portion of the left apple fly antenna (Fig. 2). In the majority of cases, the left antenna was used for recording. Colors indicate the cluster or cell class from the dendrogram (Fig. 1) to which that ORN belongs. Cells represented with a hollow center indicate multiple ORNs that responded concomitantly (though to different compounds) within the same recording. These three "rare" cells were removed for statistical analyses. The figure illustrates that there is little localization of ORN response to specific chemicals. A multivariate analysis of variance showed that the effect of response profile was insignificant for the X coordinate of ORN location, $F(4,80) = 1.53$, $P = 0.20$, NS, and slightly significant for the Y, $F(4,80) = 2.79$, $P = 0.032$. Post hoc analyses using the Tukey post hoc criterion indicated that this significance was due entirely to a difference in location of pink class A and blue class B ORNs: $P = 0.043$ for the X coordinate and $P = 0.026$ for the Y. However, neither Pillai's Trace nor Wilks Lambda multivariate tests found any effect of response profile on location: $F(8,160) = 1.68$, $P = 0.11$, NS, and $F(8,158) = 1.69$, $P = 0.10$, NS, respectively. Thus, ORN locations do not differ significantly with respect to response classification.

The total map overlay (Fig. 2, bottom) reveals that multiple fly varieties responded at topographically similar locations to the same stimuli. Furthermore, a mul-

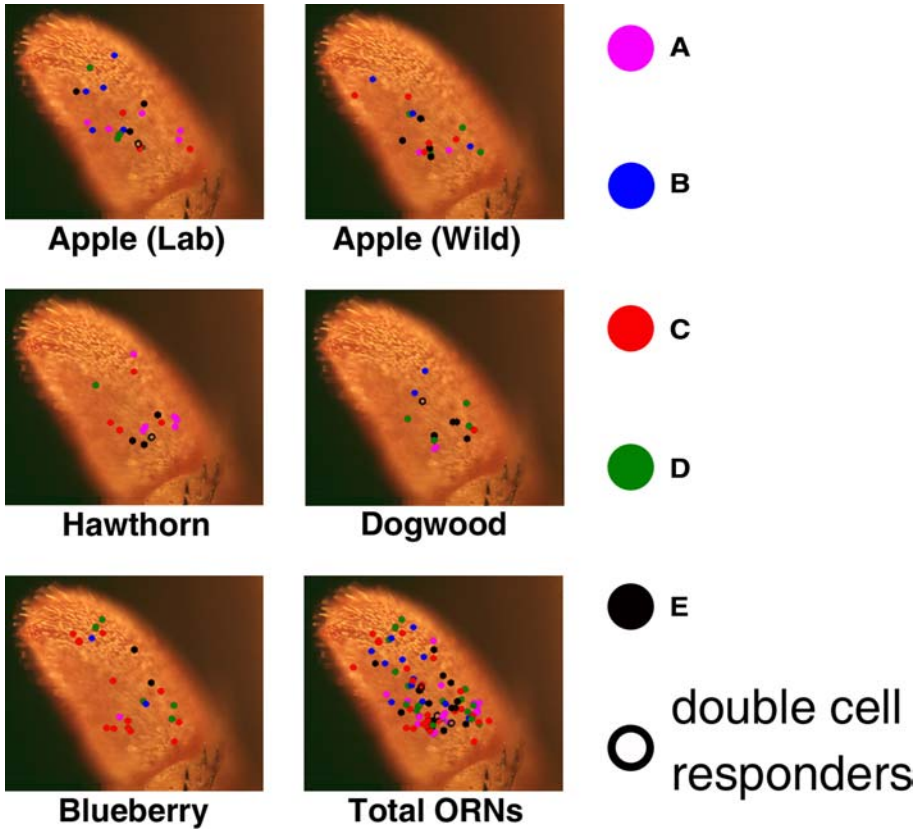

Fig. 2 Topographical maps indicating locations of recorded ORNs for each population and an overlay of all *Rhagoletis* ORNs. *Dots* indicate specific ORNs and *color* indicates cell class as determined in Fig. 1

tivariate analysis of variance showed that neither the effect of fly taxa, nor the interaction of fly taxa and response class were significant for location; *X* coordinate: fly taxa $F(4,80) = 1.10$, $P = 0.36$, NS, interaction $F(15,65) = 0.76$, $P = 0.71$, NS, *Y* coordinate: fly taxa $F(4,80) = 1.646$, $P = 0.17$, NS, interaction $F(15,65) = 0.55$, $P = 0.90$, NS. Pillai's Trace and Wilks Lambda multivariate tests concurred with this result: fly taxa, Pillai's: $F(8,160) = 1.39$, $P = 0.21$ NS, Wilks: $F(8,158) = 1.37$, $P = 0.21$, NS, interaction, Pillai's: $F(30,130) = 0.79$, $P = 0.76$ NS, Wilks: $F(30,128) = 0.79$, $P = 0.76$, NS. Thus, ORN locations do not vary by fly taxa, or for response class between taxa.

Discussion

Previous behavioral studies indicate that olfactory preference for fruit volatiles could play a key role in *Rhagoletis* host location (Linn et al. 2003). Although foraging includes both close- and long-range behaviors involving olfactory, visual, and tactile cues (Bush 1969; Fletcher and Prokopy 1991, and references therein), preference for a unique mix of volatiles, evidenced by upwind flight occurring only to that mixture, could constitute a basis for host fidelity. Variation among individuals in a population therefore provides a potential source for the host-shifting process.

It has been proposed that variation in *Rhagoletis* olfactory preference could be due to differences in antennal receptor neuron specificities and/or numbers of receptors expressed in the antenna among the various host taxa (Frey and Bush 1990). Cluster analysis similar to that used in the present study has been used to identify ORN classes in *Drosophila* (de Bruyne et al. 2001) as well as to compare various species to each other (Stensmyr et al. 2003). The latter study found a shift in ORN specificity among different species of *Drosophila* as well as a complete loss of a sensillum type in one species. One might expect changes in ORN specificity among *Rhagoletis* host populations to be even more likely than in the polyphagous *Drosophila* because monophagous *Rhagoletis* flies rely on a specific set of host compounds that differ among populations. Host-shifting populations of *Rhagoletis* (e.g., apple and dogwood) could express receptor proteins that differ from the ancestral hawthorn race, or mutations could have occurred that generated completely new types of receptors. In turn, these changes could alter the ability of flies within that population to detect certain volatiles.

Nevertheless, our analysis reveals that each fly population was capable of detecting all of the tested volatiles, regardless of host species. Furthermore, all populations possessed comparable numbers of single and multiple compound- responding ORNs, some specific to only one compound of the 11 tested (i.e., 1-octen-3-ol, hexyl butanoate, dihydro-β-ionone, and 4,8-dimethyl-1,3*(E)*, 7-nonatriene). Topographical mapping emphasizes that members of the *Rhagoletis* populations not only possessed the same basic ORN classes, but that ORN *XY* coordinate locations did not vary significantly among fly taxa or ORN response profiles, unlike the ORN localization found in *Drosophila* (de Bruyne et al. 2001). Finally, excitatory responses were observed for all 99 ORNs recorded for *Rhagoletis*. Therefore, it appears that differential host preference among *Rhagoletis* populations is not a function of alterations in the general classes of receptor neurons responding to host and non-host volatiles.

This is not to say, however, that all populations possessed identical ORNs. Indeed, the variety of recordings found for each class (Table 2) indicates that multiple ORN types could be present. Studies in *Drosophila* have described 18 functional classes of ORNs in eight different classes of basiconic sensilla, with each sensillum class containing different combinations of ORN types (reviewed in Hallem and Carlson 2004). The likely presence of different sensillum types among the *Rhagoletis* populations (such as only apple and dogwood populations containing ORNs responding to 1-octen-3-ol housed with a non-responding ORN) might indicate the presence of unique ORN types. Because we have only tested a small number of compounds and could not ensure that co-localized cells were housed in the same sensillum, we did not attempt to label these ORNs as certain "types" as in other studies of this nature (such as *Drosophila*; Hallem and Carlson 2004). Furthermore, although cluster analysis allows more objective categorization than hand sorting, it admittedly has limitations with smaller sample sizes or rare occurrences. Specifically, it may have misclassifed some of the rare ORNs as evidenced by the "exceptions" to classes reported in the Results. This might be alleviated with higher sample sizes. However, even if there are multiple ORN types represented in the data set, the cells in each class are similar in the context of behavioral relevance and compounds important for olfactory host preference.

Given the short time span since the hawthorn/apple divergence (~150 generations), the lack of significant differences in ORN specificity among *Rhagoletis* populations is not surprising. The presence of similar ORN responses in the dogwood fly and *R. mendax*, the most closely related species to the *R. pomonella* group, further indicates that altering receptor neuron specificity is neither inevitable nor essential to maintaining host fidelity, even in separate species. Furthermore, it does not appear that host fidelity is simply a result of possessing more ORNs tuned to particular volatiles, as all populations possessed similar numbers of receptor neurons tuned to host and non-host volatiles.

Instead, our results indicate that host-shifting populations already possessed the ability to detect novel host volatiles. These compounds could have been part of an ancestral host cue or, more likely, part of the broad repertoire of the olfactory palette. New hosts could subsequently have been colonized by more "broadly tuned" individuals capable of exploiting the new host (Linn et al. 2005b). Alternatively, changes in central

neural processing patterns could have generated preference for a new host in a few individuals from the ancestral race. Further studies analyzing host volatile sensitivity and adaptation at the periphery (see the following study, Olsson et al. 2005), as well as host volatile processing in the antennal lobe, are imperative for a full understanding of the mechanisms by which these flies have initiated and preserved their host shifts. In addition, a more concentrated study using only two of the host populations (such as apple and hawthorn) and recording from much larger numbers of ORNs could clarify some of the ambiguity in ORN classes observed here and confirm that there is indeed no difference in ORN host volatile specificity between the host populations. Finally, peripheral studies of F_1, F_2 and backcross progeny between these populations could reveal how hybridization affects host volatile chemoreception. Nevertheless, our study suggests that the key to host-shifts and speciation in *Rhagoletis* lies not in adding new pieces to the board, but simply changing the strategy of the game.

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