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Shannon B. Olsson · Charles E. Linn Jr
Wendell L. Roelofs

The chemosensory basis for behavioral divergence involved in sympatric host shifts II: olfactory receptor neuron sensitivity and temporal firing pattern to individual key host volatiles

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Abstract The *Rhagoletis* species complex has been a key player in the sympatric speciation debate for much of the last 50 years. Studies indicate that differences in olfactory preference for host fruit volatiles could be important in reproductively isolating flies infesting each type of fruit via premating barriers to gene flow. Single sensillum electrophysiology was used to compare the response characteristics of olfactory receptor neurons from apple, hawthorn, and flowering dogwood-origin populations of *R. pomonella*, as well as from the blueberry maggot, *R. mendax* (an outgroup). Eleven volatiles were selected as stimuli from behavioral/electroantennographic studies of the three *R. pomonella* host populations. Previously, we reported that differences in preference for host fruit volatile blends are not a function of alterations in the general class of receptor neurons tuned to key host volatiles. In the present study, population comparisons involving dose–response trials with the key volatiles revealed significant variability in olfactory receptor neuron sensitivity and temporal firing pattern both within and among *Rhagoletis* populations. It is concluded that such variability in peripheral sensitivity and temporal firing pattern could influence host preference and contribute to host fidelity and sympatric host shifts in the *Rhagoletis* complex.

Keywords *Rhagoletis* · Speciation · Discrimination · Antagonism · Single sensillum electrophysiology

S. B. Olsson
Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14850, USA

S. B. Olsson (✉) · C. E. Linn Jr · W. L. Roelofs
Department of Entomology,
NYS Agricultural Experiment Station,
Cornell University, Geneva, NY 14456, USA
E-mail: SBB23@cornell.edu
Tel.: +1-315-7872337
Fax: +1-315-7872326

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

Introduction

The *Rhagoletis* species complex contains a series of monophagous host races, sibling species and species that have been cornerstones of the sympatric speciation debate over much of the latter part of the twentieth century. In particular, members of the *Rhagoletis pomonella* group are suggested to have arisen via sympatric host shifts (Bush 1969) after Walsh (1867) first cited the shift of *R. pomonella* (a true fruit fly) from its native host hawthorn (*Crataegus* spp.) to the new viable host, apple (*Malus pumila*), as the establishment of a new host race.

In North America, three distinct populations of *R. pomonella* occur: a hawthorn (*Crataegus* spp.) infesting host race, a domestic apple (*Malus pumila*) infesting host race, and an undescribed flowering dogwood (*Cornus florida*) infesting sibling species (see Berlocher 2000). *Rhagoletis* flies mate and oviposit directly on or near the fruit of their host plant (Prokopy et al. 1971). Consequently, variation in host preference serves as an effective premating barrier to gene flow among flies infesting different host species (Feder et al. 1994). Previous studies indicate that olfactory preference for host volatiles could play a key role in *Rhagoletis* host location (Linn et al. 2003). Flies infesting each host fruit preferentially flew upwind in flight-tunnel assays to unique volatile blends identified from each fruit type (Zhang et al. 1999; Nojima et al. 2003a, b; Linn et al. 2003, 2005b). In addition, flies from each host fruit displayed arrested flight when non-host volatiles were added to the host blend, indicating that volatiles can have both agonist and antagonist effects on fly foraging behavior (Linn et al. 2005a). Preference for a unique mix of volatiles, and recognition of non-host volatiles, constitutes a basis for host fidelity. Variation among individuals in a population provides a

potential source for the host shifting process (Linn et al. 2005b). Furthermore, a recent study indicating reduced olfactory host preference in *R. pomonella* hybrids could constitute an olfaction-based post-zygotic barrier to gene flow as well (Linn et al. 2004).

Olfactory preferences in the *Rhagoletis* species complex could diverge via alterations in the central processing centers of the brain (i.e., the antennal lobe or modifications in direct or indirect glomerular innervation of mushroom bodies, central complex, lateral accessory lobes and the lateral protocerebrum) and/or through variation in peripheral chemoreception. In a previous study, we showed that both *R. pomonella* (apple, hawthorn, and the undescribed flowering dogwood fly races/sibling species) and *R. mendax* (blueberry fly) populations possessed similar classes of olfactory receptor neurons (ORNs) responding to host and non-host volatiles (Olsson et al. 2005; for a list of volatiles see Table 1, Olsson et al. 2005). No population possessed significantly more ORNs tuned to one set of compounds than another group, and topographical mapping showed that ORN locations did not differ morphologically between fly taxa (Olsson et al. 2005). Therefore, differences in host-plant preference among these populations do not appear to be a function of altering receptor neuron specificity to host or non-host volatiles. However, differences in other ORN response characteristics, such as sensitivity and temporal firing pattern, could significantly alter the type of information being sent to the brain. Differences in sensitivity could develop through genetic alterations in the expression of receptor proteins or second messenger cascades within the ORNs (Menken and Roessingh 1998). Differences in temporal firing pattern are related to ORN adaptation/disadaptation rates (see Todd and Baker 1999 and references therein), which could result from alterations in the time course of cGMP formation. These alterations affect both the second messenger cascades and ion channels within the ORN (Stengl et al. 1999). It has also been suggested that differences in temporal firing pattern result from changes in ligand receptor binding or to differences in molecule inactivation/removal from the receptor membrane (Almaas et al. 1991).

Menken and Roessingh (1998) hypothesized that changes in peripheral sensitivity to phytochemicals can both restrict and promote host-plant shifts. They proposed that for insects with a limited range of chemoreception, host shifts that appear quite unrelated taxonomically could still be "sensorily conservative" as they utilize the same olfactory palette to perceive the new host. Thus, natural polymorphisms in a population involving either a gain in sensitivity to new host compounds and/or a loss in sensitivity to deterrents in the new host could facilitate a host shift. Though variation due to age or physiological state has been examined, natural polymorphisms in sensitivity have seldom been considered (Menken and Roessingh 1998). In *Yponomeuta* larvae, differences in gustatory sensitivity are suggested to facilitate host shifts, but correlation of these stimuli to adult oviposition preference has not been established (Menken and Roessingh 1998; Roessingh et al. 1999, 2000). Moreover, peripheral chemosensory systems have often been considered mere "relay stations" for olfactory processing, while true discrimination would occur at central processing centers.

Although *R. pomonella* populations possess the same olfactory palette with which to perceive host and non-host stimuli (Olsson et al. 2005), differences in ORN sensitivity or temporal firing pattern to these volatiles could have significant impact on *Rhagoletis* host choice, and also on shifts from one host to another. We, therefore, conducted a comparative study of peripheral chemoreception in four closely related *Rhagoletis* taxa: two host races (apple and hawthorn origin), a sibling species (flowering dogwood origin), and the most closely related confirmed species (*R. mendax* Curran, the blueberry maggot) (Berlocher 2000), using biologically relevant olfactory stimuli. The relevant host volatiles for *R. pomonella* had been determined by gas chromatography/electroantennographic (GC-EAD) and flight-tunnel behavioral studies of *R. pomonella* host fruit (Zhang et al. 1999; Nojima et al. 2003a, b). Through the use of single sensillum electrophysiology, we demonstrate that variation in olfactory receptor neuron threshold sensitivities and temporal firing patterns among the populations could influence host preference and contribute to sympatric host shifts.

Materials and methods

Insect origins, rearing, chemical stimuli, and electrophysiological recording setup are identical to those found in Olsson et al. (2005). The following is a brief overview of these methods.

Rhagoletis origins and rearing conditions

Female *Rhagoletis* flies were selected from lab-reared or field-collected populations as in Olsson et al. (2005). Field-collected flies were gathered as larvae from fruit at the site of origin, shipped as post-diapause pupae (Feder et al. 1994), and kept in an environmental chamber at 23–24°C temperature, 16L:8D photoperiod, and 55–60% relative humidity. Adults were maintained on an artificial diet (Fein et al. 1982). Lab-reared flies were maintained on red delicious apples (Neilson and McAllen 1965) and adults kept in an environmental chamber at 23°C temperature, 50% relative humidity, 16L:8D photoperiod. Flies used for neurophysiological analyses were between 0 and 20 days of age, but in most cases were between 0 and 7 days old because of the increased vigor of younger flies.

Olfactory stimuli

Synthetic blends and sources of chemicals were the same as reported previously (Olsson et al. 2005). Stock

solutions (1 μg/μl) of individual key fruit volatiles and specific fruit blends in hexane were prepared according to the key volatiles determined for each fruit through behavioral and/or electrophysiological analyses (see Table 1, Olsson et al. 2005). Dilutions of each host volatile (1, 10, and 100 ng/μl) were also prepared for dose–reponse trials. Ten microliters of each diluted compound or blend was pipetted onto filter paper (ca. 5 mm×15 mm) in disposable Pasteur pipettes. Blank cartridges, containing only filter paper plus solvent, were also prepared. In order to prevent evaporation and contamination, the 10 μg stimulus load cartridges were not used after 2.5 h. For dose–response trials, the 10, 100 ng, and 1 μg stimulus load cartridges were filled approximately 5 min before use to prevent evaporation and fresh cartridges were prepared for each new contacted ORN and set of dose–response trials.

Electrophysiological recording

Female *Rhagoletis* were confined in the tapered, cut end of a 100 μl pipette tip with only their heads protruding and immobilized with dental wax. A sharpened tungsten wire was inserted into the right eye as a ground electrode. Electrolytically sharpened tungsten microelectrodes 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).

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) through a metal tube protruding approximately 10 mm from the antenna. 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 a complementary 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 Hz–3,000 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 co-located, individual neurons were separated based on differences in the amplitude of their action potentials (spikes).

In the event of a contact, ORNs were first screened with the three fruit blends at the 10 μg stimulus load (Table 1, Olsson et al. 2005), and the blank (hexane). These stimuli were tested at least once at the beginning and, in nearly all cases, at the end of each recording period. 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 response), then all 11 components of the three blends were tested individually at a 10 μg stimulus load. The compounds eliciting responses were subsequently tested in dose–response trials (10 and 100 ng, 1 and 10 μg stimulus loads) to determine each cell's sensitivity to those chemicals. The stimulus loads were presented once in increasing order of stimulus load with time allowed for resumption of baseline firing between stimulations.

Data analysis

For each ORN testing period, spike frequencies with the blank (hexane) were calculated every 600 ms for a 10.8 s recording period (including 1 s pre- and 9.8 s post-stimulus onset). In the majority of the cases, more than one blank trial was presented. Spike counts per 600 ms were then averaged across all blank trials. An increase in spike frequency for the 600 ms following stimulus presentation was considered a response if it rose >3 SD above the blank mean firing rate. ORN spike increases below this level were not considered further. Responses were calculated from 600 ms bins of the blank as this time frame encompassed the entire 500 ms stimulus period and an additional 100 ms as stated in the previous study.

A response threshold was calculated as the lowest stimulus loading eliciting a spike frequency increase >3 SD over the mean spike frequency of the blank trials. In the few cases (6.4% of all recordings) where threshold could not be determined (due to incomplete dose–response trials or cell death), threshold was given as 75% of the lowest stimulus loading eliciting a response <4 SD of the blank mean response or 50% of the lowest stimulus loading inducing a response >4 SD of the mean value for the blank response. Sensitivities were assigned as reciprocals of the threshold values [e.g., 10 ng = 10,000, 100 ng = 1,000, 1 μg (1,000 ng) = 100, and 10 μg (10,000 ng) threshold = 10].

To determine temporal firing pattern, spike frequencies were measured for the 10 μg screening trials with host stimuli. The percent change in spike frequency was calculated as: (2nd 600 ms–1st 600 ms following stimulus onset) / 1st 600 ms × 100. The baseline firing frequency (600 ms prior to stimulus exposure) was subtracted from each of the values in the calculation. A rapidly adapting cell with a high percent change in spike frequency was characterized as having a "phasic" response, whereas a slowly adapting cell with a percent change close to zero was considered a "tonic" response.

Because the data comprised a non-normal distribution (Kolmogorov–Smirnov $p < 0.001$), Kruskal–Wallis H tests were used to compare all fly types. To compare specific fly taxa to each other, Mann–Whitney tests were employed. Both tests were performed via SPSS version 11.0 software. All graphs were generated using SPSS version 11.0 or 12.0 software.

Results

Olfactory receptor neurons (ORNs, $n = 99$) from 38 individuals among the various 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 sensitivity

Figure 1 illustrates typical response profiles and dose–response curves for three basic classes of *Rhagoletis* ORNs: single-compound, longer chain ester (i.e., not acetates; henceforth referred to simply as "esters"), and multiple compound responders (described in Olsson et al. 2005). Significant responses and threshold values were statistically calculated using comparisons with blank spike activity as described in the methods. Therefore, thresholds were not selected, as in other studies, from the point of steep ascent in the dose–response curve, but rather appear at the first point in the curve that attains a statistically significant spike increase. Figure 1a shows an ORN responding exclusively to a 1-octen-3-ol stimulus with a response threshold of 1 µg. Figure 1b shows an ORN responding to three esters (propyl hexanoate, pentyl hexanoate, and butyl hexanoate), with 10 , 10 , and 1 µg thresholds, respectively. Figure 1c shows an ORN responding to eight compounds: propyl hexanoate, hexyl butanoate, pentyl hexanoate, butyl hexanoate, 4,8-dimethyl-1,3*(E)*, 7-nonatriene, 3-methylbutan-1-ol, isoamyl acetate, and 1-octen-3-ol, all at a 10 ng threshold. Note that dose–response trials for this last ORN show a gradual decrease in spike frequency as opposed to the traditional sinusoidal curve for all but the 4,8-dimethyl-1,3*(E)*, 7-nonatriene stimulus. It is possible that response saturation had already been reached for these compounds at the 10 ng stimulus load and increasing concentrations would not induce higher firing rates. Note that the lowest 10 ng loading does give a statistically significant response.

Figure 2 depicts host volatile sensitivities for all ORNs contacted in each population. In effect, Figure 2 is a composite of 99 graphs similar to those in Fig. 1, with each dose–response graph reduced to one bar and the circled threshold translated to Log (sensitivity) (see methods for description of sensitivity). The lowest threshold responses (i.e., 10 ng) are shown as the highest sensitivity bars [Log (10,000) = 4]. The highest thresholds (i.e., 10 µg or 10,000 ng) are the lowest bars [Log (10) = 1]. Each vertical line for the 11 compounds represents a single ORN, and the response profile is preserved from top to bottom for each contacted cell. ORNs are horizontally organized by single, ester, and multiple-compound responders as described in Fig. 1. Percentage values above each graph provide a comparative measure of sensitivity by listing the percent of cells with high sensitivity (i.e., threshold < 1 µg).

Figure 2 reveals significant variability both within and among *Rhagoletis* populations for each tested volatile. Sensitivities range from 1 (10 µg or 10,000 ng threshold) to 4 (10 ng threshold)–a 1,000-fold difference. However, when the responses of each population and volatile are examined as a whole, several points are evident. First, both wild and lab apple race ORNs as a whole displayed high sensitivity (i.e., > 50% cells with high sensitivity) to their own apple volatiles (esters), as well as to 1-octen-3-ol (a key volatile in the dogwood blend and antagonist to apple flies). ORNs from wild apple race flies exhibited high sensitivity to several esters (hexyl butanoate, propyl hexanoate, butyl butanoate, pentyl hexanoate and butyl hexanoate), whereas lab apple race ORNs were sensitive only to butyl hexanoate, a key behavioral component in the apple blend (Zhang et al. 1999). Wild apple fly ORNs also exhibited high sensitivity to 4,8-dimethyl-1,3*(E)*, 7-nonatriene, which is a component of the hawthorn blend. Second, hawthorn race ORNs displayed high sensitivity to several of their own hawthorn volatiles (such as 4,8-dimethyl-1,3*(E)*,7-nonatriene, butyl hexanoate, 3-methylbutan-1-ol, and isoamyl acetate), as well as to hexyl butanoate (from the apple blend). Third, dogwood fly ORNs were highly sensitive to three apple volatiles (propyl hexanoate, pentyl hexanoate, and butyl hexanoate), but were markedly less sensitive to some of their own key host volatiles (e.g., 1-octen-3-ol and 3-methylbutan-1-ol). Finally, blueberry fly ORNs (the outgroup) were less sensitive to several of the host volatiles (such as 1-octen-3-ol, propyl hexanoate, butyl butanoate, pentyl hexanoate, butyl hexanoate, 3-methylbutan-1-ol, and isoamyl acetate) and were only sensitive to 4,8-dimethyl-1,3*(E)*,7-nonatriene.

During host location in the field, *Rhagoletis* flies do not detect one host volatile at a time, but are confronted with an entire bouquet of host or non-host odors concurrently. Thus, it is important to examine ORN sensitivity to the composite of all relevant host stimuli in each host blend. Figure 3 displays a series of box plots depicting sensitivities for the three field-collected *R. pomonella* fly populations. Each plot contains all ORN response sensitivities to every host volatile tested from each host fruit (Table 1, Olsson et al. 2005). Thresholds were statistically compared by Mann–Whitney and Kruskal–Wallis tests as indicated by asterisks and bars above the graphs. As was the case with individual compounds, there was considerable variation in sensitivity within and among populations as evidenced by the breadth of the box plots. However, some significant differences exist. Dogwood flies were the least sensitive of the populations to the dogwood blend [apple (wild) $p < 0.05$; hawthorn $p < 0.05$]. Hawthorn flies, the proposed ancestral population, were significantly more

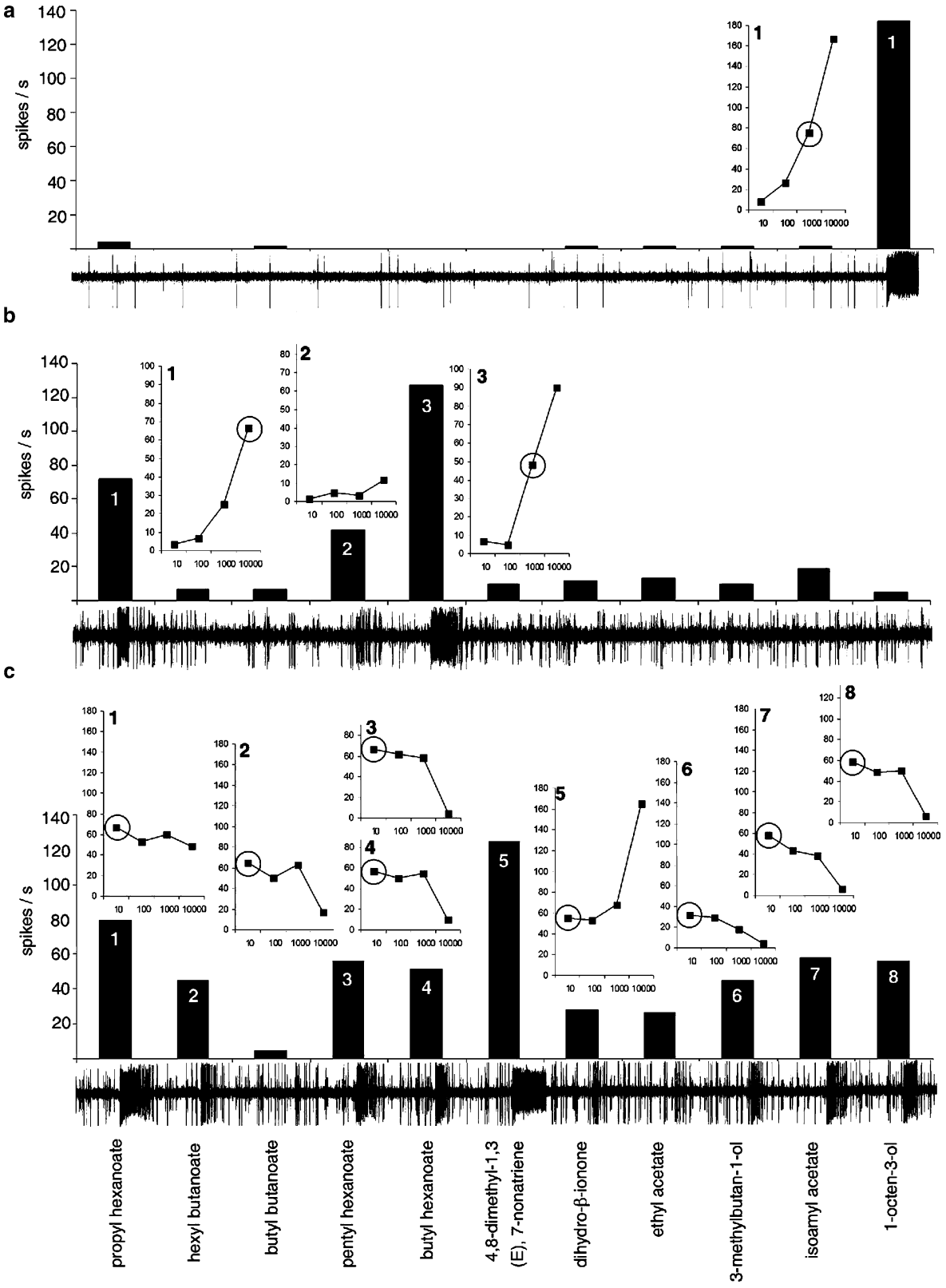

Fig. 1 Typical response profiles and dose–response curves for single compound (**a**), ester (**b**), and multiple compound (**c**), classes of ORNs. The ORNs correspond to a hawthorn, wild apple, and a hawthorn ORN respectively. *Bar charts* indicate spike frequencies for the 600 ms following initial exposure to 10 μg of each volatile listed at the bottom of the figure. The 2 s spike traces corresponding to these frequencies are shown directly below each graph. *Numbered bars* indicate statistically significant responses (see methods), with dose–response trials (10 and 100 ng, and 1 and 10 μg) for each response shown as *line graphs* next to the bar. *Circled points* indicate the statistically calculated threshold for that stimulus. Note: **b** Bar 2 lacks a circled threshold as no dose–response stimulus loading elicited sufficient spike frequency to warrant a statistical response

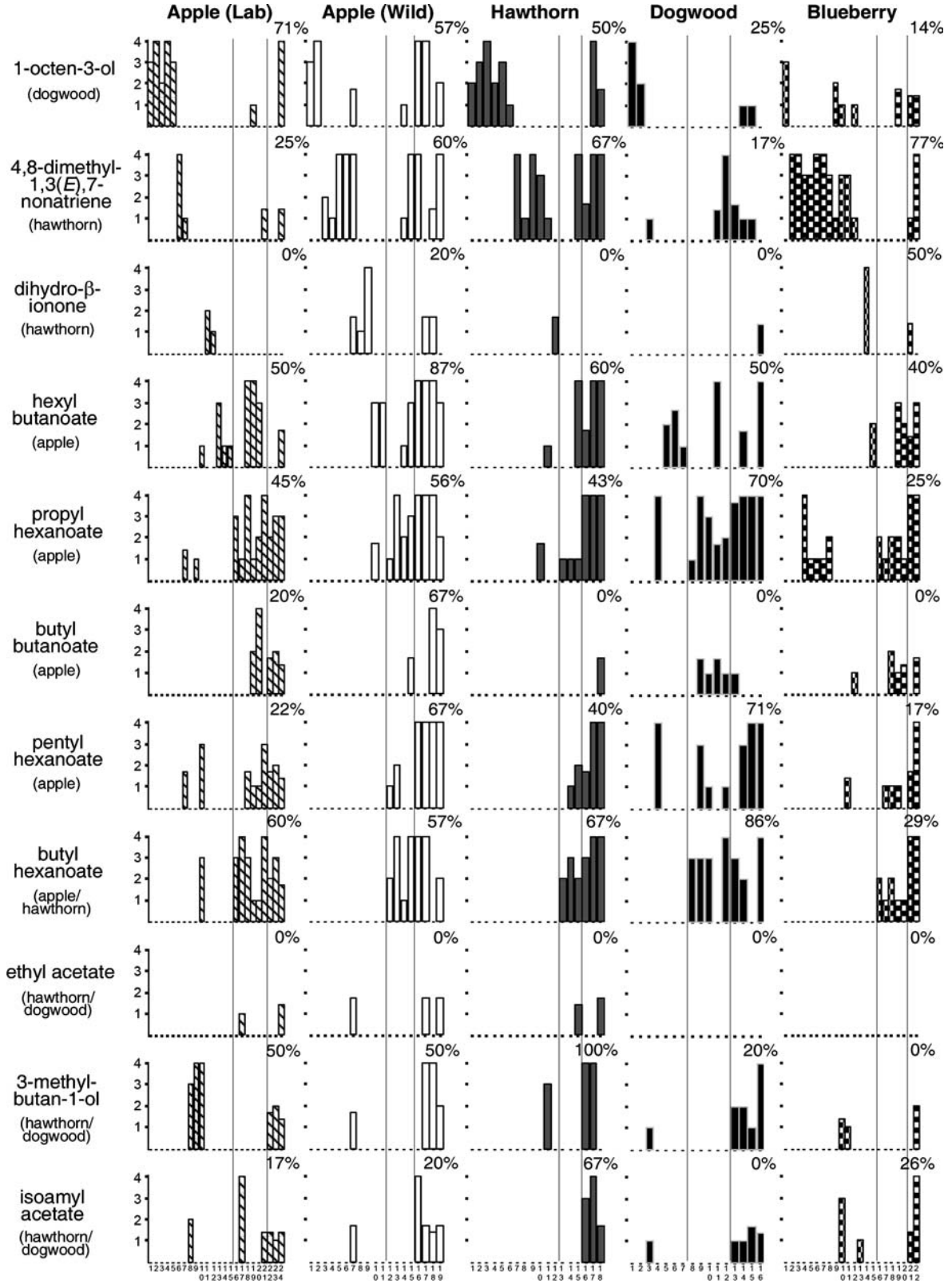

Fig. 2 Bar graphs depicting ORN response sensitivity for all *Rhagoletis* populations. Cells are graphed by population and host volatile. X-axes list contacted ORNs for each population. Sensitivities are graphed as log(sensitivity) and *numbers* to the upper right of each graph indicate the percentage of sensitive ORNs (threshold < 1 μg) for each population and chemical. ORNs are arranged from left to right in order of receptor neuron class according to Olsson et al. (2005) and as represented in Fig. 1. Classes are indicated by *faint lines* separating the cells. The first group contains single compound responding ORNs and the second and third contain ester responders and multiple compound responders, respectively. Volatiles are arranged vertically in a similar fashion with chemicals stimulating single compound ORNs at the top followed by esters and finally compounds eliciting responses mainly from multiple compound ORNs at the bottom of the figure

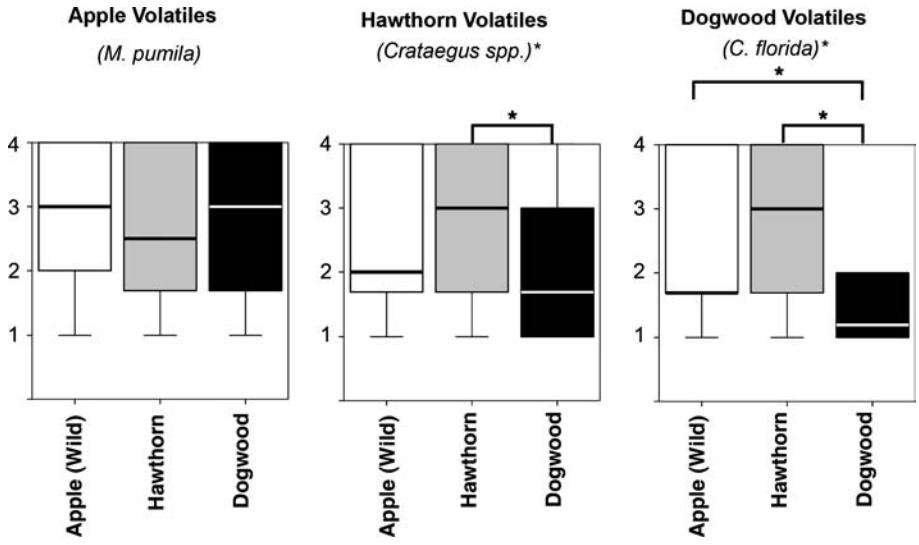

Fig. 3 Box plots with whiskers depicting threshold sensitivities of the three field-collected *R. pomonella* populations to the three volatile groups used in the study (see methods). Sensitivities are graphed as log (sensitivity). Bars above each graph indicate significant differences ($p < 0.05$). *Asterisks* after graph titles indicate significant differences across all populations

sensitive to their own blend than dogwood flies ($p < 0.05$).

ORN temporal firing pattern

While recording ORN responses to host stimuli, it was observed that some cells responded with slow adaptation to a compound (''tonic''), whereas other cells adapted quickly during/following stimulus exposure to return to baseline firing rate (''phasic''). This temporal firing pattern can be quantified as the % drop in spike frequency following stimulus exposure (see methods). Figure 4 illustrates the typical response patterns of a highly tonic (4% drop in spike frequency), moderately

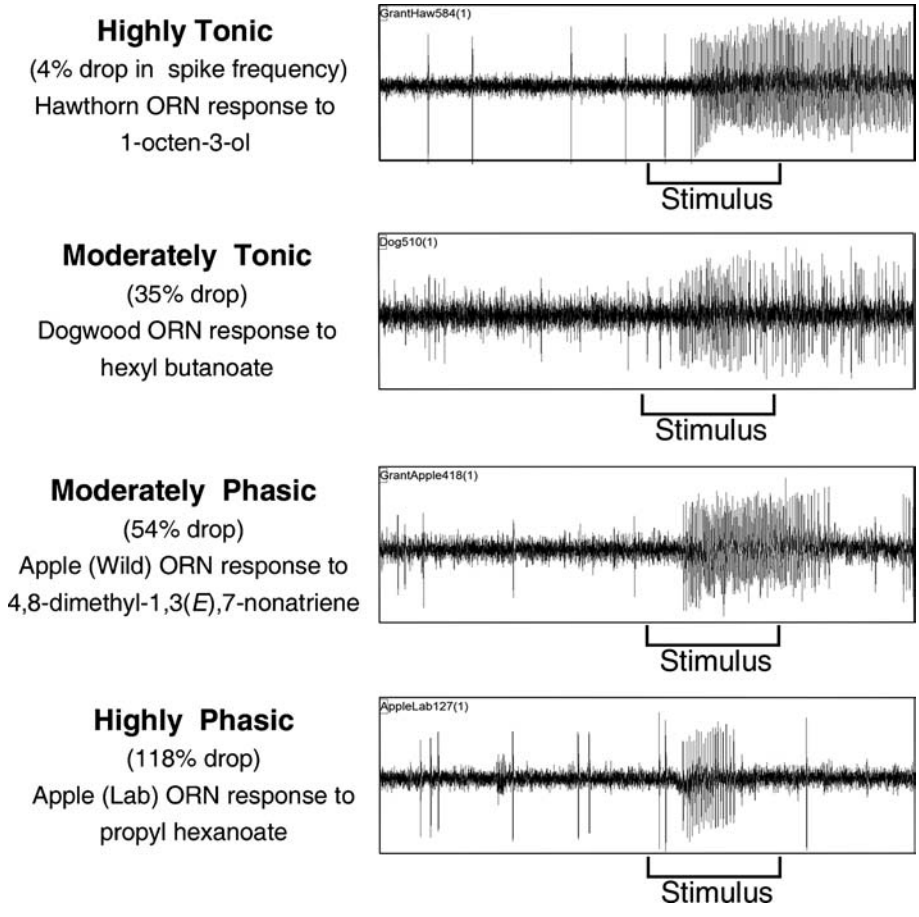

Fig. 4 Two second spike traces depicting various types of temporal firing patterns in response to 10 μg stimulus loads

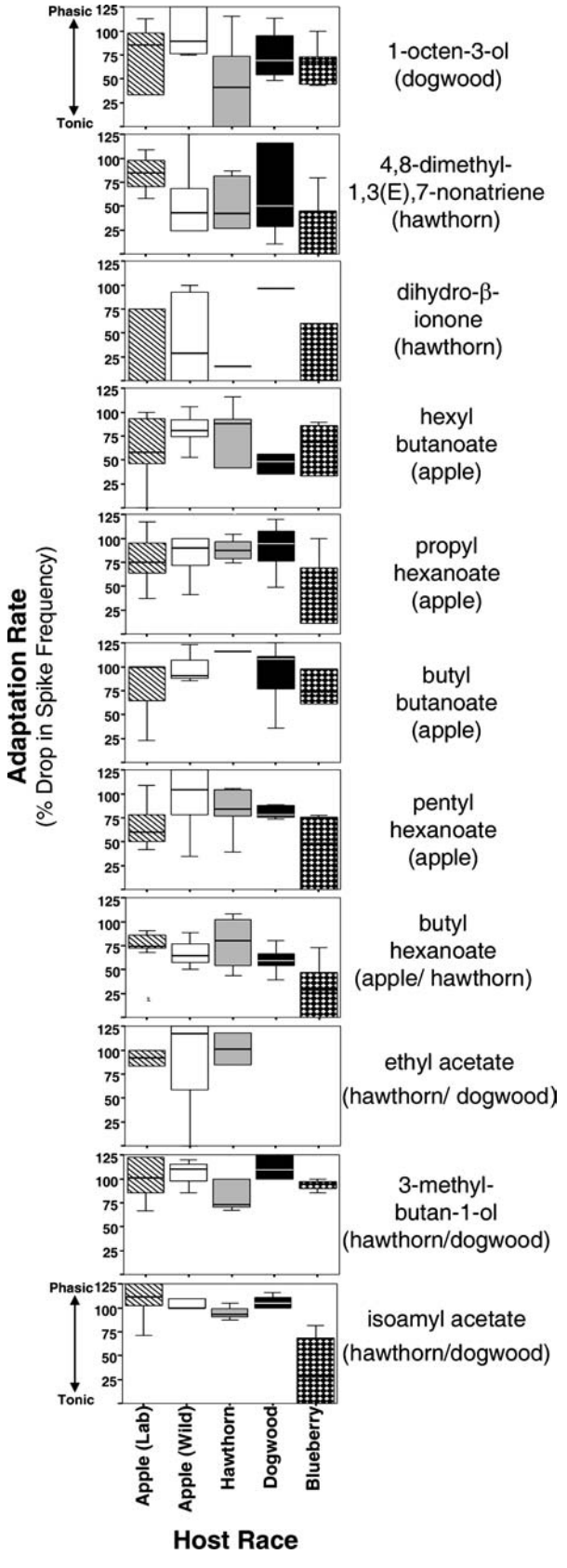

Fig. 5 Box plots with whiskers indicating ORN temporal firing rate for each population and volatile. Responses to 10 μg test stimuli were graphed as % drop in response frequency for the 600 ms following stimulus exposure

tonic (35% drop), moderately phasic (54% drop) and highly phasic (118% drop in spike frequency) ORN cell from *Rhagoletis* populations. All were responding to a 10 μg stimulus loading to compounds listed to the left of each spike trace.

Figure 5 displays box plots with whiskers of temporal firing patterns for all ORNs contacted in each of the fly populations to a single host volatile. As with sensitivity, there was significant variability in temporal firing pattern within and among the tested populations. However, certain tendencies are present. First, hawthorn ORNs generally gave more tonic responses (lower % drop in spike frequency) to 1-octen-3-ol and 3-methylbutan-1-ol than did the other populations, whereas dogwood ORNs responded more tonically to hexyl butanoate than all populations except blueberry. Second, apple (lab) ORNs responses were more phasic to 4,8-dimethyl-1,3*(E)*,7-nonatriene and butyl hexanoate than the other populations and more tonic to hexyl butanoate and pentyl hexanoate than their wild counterparts. Finally, blueberry ORN responses were more tonic to several of the compounds (i.e., 4,8-dimethyl-1,3*(E)*,7-nonatriene, propyl hexanoate, pentyl hexanoate, butyl hexanoate, and isoamyl acetate).

Discussion

ORN variation in sensitivity

In the previous study, we showed that *Rhagoletis* flies infesting apple, hawthorn, dogwood and blueberry fruit possessed similar ORN classes in response to the 11 host volatiles used in the current study (Olsson et al. 2005). Consequently, any differences in peripheral chemoreception affecting host preference must be found in the response characteristics of the ORNs themselves, and not merely in the presence or lack of response to a particular volatile. In the present study, we found large and significant (1,000-fold) differences in sensitivity within and among *Rhagoletis* populations to the tested volatiles.

The breadth of variation in sensitivity observed among *Rhagoletis* populations is present across all volatiles and populations, and thus appears to be a unique function of the ORNs themselves. Variation in sensitivity could effectively increase the dynamic range of response to a particular volatile (de Bruyne et al. 2001). It is conceivable that such heterogeneity in sensitivity is a natural phenomenon in these populations, and might also contribute to their propensity to host shift. An ancestral population possessing a broad range of sensitivities would possess a greater palette from which changes in response to a unique blend and behavioral functionality could emerge, facilitating the acceptance and subsequent colonization of a new host. Indeed, this variability might also contribute to the genetic and behavioral variation currently witnessed in the different

fly populations. Also, in any *Rhagoletis* population, a small but significant percentage of flies are attracted to the blend of a non-host (Linn et al. 2005b). Perhaps the peripheral variation seen in the present experiments contributes to such behavior.

Dose–response and sensitivity studies of non-pheromonal stimuli have been performed on several other Dipteran species, including: tsetse flies (Bogner 1992; Den Otter and van der Goes van Naters 1992; Voskamp et al. 1999), Australian sheep blowflies (Park and Cork 1999), sandflies (Dougherty et al. 1999), Queensland fruit flies (Hull and Cribb 2001), houseflies (de Weerdt and Kelling 2001; Kelling et al. 2002), mosquitoes [*C. pipiens*: (Bowen 1990, 1992) and *A. gambiae*: (Meijerink and van Loon 1999; Meijerink et al. 2001)] and *Drosophila* (Clyne et al. 1997; de Bruyne et al. 1999, 2001; Stensmyr et al. 2003). However, no study has revealed the breadth of variation observed here with *Rhagoletis* flies, both within and among populations. Indeed, most of the studies using Dipteran species averaged spike counts from several ORNs to generate dose–response curves, and did not examine differences between individuals or populations. One study of pre- vs. post-diapause responsiveness in *C. pipiens* did report a nearly tenfold difference in lactic acid sensitivity, but this difference was fairly consistent for several tested neurons (Bowen 1990).

One source for the lack of variability in other studies could lie in the much higher stimulus loadings used, from 1–1,000 μg in blowflies (Park and Cork 1999), 0.001–10 mg in houseflies (de Weerdt and Kelling 2001; Kelling et al. 2002) and 0.005–20 mg in tsetse flies (Den Otter and van der Goes van Naters 1992). However, a recent study by Stensmyr et al. (2003) used as little as 100 pg dosages and still maintained consistent dose–response curves for several neurons. It is also possible that some of the variation in our study could be due to the unique statistical methods used in calculating response threshold. However, it is unlikely that this could fully account for the 1,000-fold differences observed among the ORNs.

ORN sensitivity and host plant shifts

Despite the extensive variation observed in ORN sensitivity among *Rhagoletis* flies, certain trends in the data indicate that differences in sensitivity to host volatiles might have a significant impact on host fidelity and the host shifting process. In our study, apple and dogwood fly ORNs showed a trend in being less sensitive to hawthorn volatiles than hawthorn fly ORNs (Fig. 3), the suggested ancestral population (Berlocher 2000). Additionally, hawthorn fly ORNs displayed higher sensitivity to several compounds from their own blend (including butyl hexanoate which is present in both hawthorn and apple blends), as well as hexyl butanoate, but were not notably sensitive to other apple or dogwood volatiles (Fig. 2). Menken and Roessingh (1998) suggested that host plant shifts could be facilitated by a gain in sensitivity to stimulants or a loss of sensitivity to deterrents (see also references therein). It is conceivable that less sensitive hawthorn flies were more accepting to a new host, and subsequently developed behavioral preferences and other isolating traits, such as differences in eclosion time (Feder and Filchak 1999). Once on a new host, heightened sensitivity to host compounds (as is the case with apple flies, Fig. 2), could also be rapidly selected as a component of host fidelity. And, in any *Rhagoletis* population, a small percentage of flies possess broad behavioral response profiles to multiple blends (Linn et al. 2005b). Based upon the results of this study, it would be interesting to test for correlations between broad response and variation in ORN sensitivity.

Although other studies have not compared divergent populations, they have also implied a link between host volatile sensitivity and behavior. For instance, post-diapause increase in peripheral sensitivity to the host attractant lactic acid correlates to a resumption in host-seeking behavior in *C. pipiens* mosquitoes (Bowen 1990). Doses of ammonia used in electrophysiological studies of *An. Gambiae* correlate to those attracting the mosquitoes in the flight tunnel (Meijerink et al. 2001 and references therein). And finally, a recent study of *Drosophila* by Stensmyr et al. (2003) shows that peripheral sensitivity and selectivity to relevant host stimuli can be correlated to behavioral activity.

In addition to olfactory preference for host volatiles, *Rhagoletis* populations are also behaviorally antagonized by certain non-host compounds. Increased sensitivity to these antagonistic compounds might also contribute to host fidelity and act to preserve recently established populations on the new host. Dogwood fly ORNs, though relatively insensitive to their own host volatiles (Fig. 3), were particularly sensitive to esters found in the apple blend (Fig. 2). Interestingly, these esters are significant behavioral antagonists for dogwood flies when added to the dogwood blend (Linn et al. 2005a). Likewise, apple fly ORNs were particularly sensitive to 1-octen-3-ol, and half were also sensitive to 3-methylbutan-1-ol (Fig. 2). Both of these compounds are dogwood volatiles and known antagonists in flight-tunnel assays for this fly (Linn et al. 2005a). Finally, hawthorn fly ORNs were also sensitive to butyl hexanoate (Fig. 2), which, though found in their own blend, is a behavioral antagonist when presented at the elevated levels found in the apple volatile blend (Linn et al. 2005a). Equipped with an olfactory palette capable of detecting both host and non-host odors that differ in functionality, increased sensitivity to non-host compounds could rapidly be selected for in a new host population as a means to enhance recognition and discrimination of non-host volatiles and facilitate avoidance of these cues during foraging behavior.

The presence of "antagonist" ORNs at the periphery has also been examined in a study of tsetse flies (Voskamp et al. 1999). Results showed that "repellent" compounds stimulated both ORNs specific to the

"repellents", suggesting a labeled line neural pathway for behavior, as well as ORNs of a great variety of cell types, suggesting that repellents "simultaneously activate many receptor types so that any olfactory information specific to host-finding is lost in the resulting barrage of sensory input". Voskamp et al. (1999) concluded that cells coding for attractants or repellants might produce a unique response profile to inform the central processing centers about the odors. A similar situation could be occurring in *Rhagoletis*. Known behavioral antagonists, such as 1-octen-3-ol and esters, are found to activate both ORNs exclusive to these compounds and more "generalist" cells responsive to several compounds at once (Fig. 1). Further studies examining the neurophysiology of non-pheromonal attractants and antagonists are necessary to understand how behaviorally relevant olfactory information is coded at the periphery.

To conclude the discussion of sensitivity, two observations should be noted. First, it is interesting that *R. mendax* [the most closely related confirmed species (Berlocher 2000)] was, relative to the other tested populations, much less sensitive to all compounds except 4,8-dimethyl-1,3*(E)*,7-nonatriene (Fig. 2). The distinctiveness of *R. mendax* ORN sensitivity supplements its recognition as the only separate species of the four populations tested (Berlocher 2000). Second, apple (lab) flies were generally less sensitive to host compounds (esters) than their wild counterparts (Fig. 2). A study of peripheral sensitivity in lab and natural strains of houseflies yielded similar results (de Weerdt and Kelling 2001). It was concluded that several generations of rearing with food and water readily available could have reduced the necessity for maintenance of an optimal sensory olfactory system for feeding, and through selection and adaptation peripheral changes became manifest in the flies. It is possible that a similar situation is occurring with host location in our flies, which have been reared in the lab for over 30 generations (S. Olsson, personal observation). However, these changes have not yet impacted behavioral response of lab apple flies to volatiles (Zhang et al. 1999; Linn et al. 2003, 2005a, b).

ORN variation in temporal firing pattern

As with sensitivity, ORN temporal firing patterns showed significant variation for each stimulus both within and among populations. Some cells responded to host compounds with a very short burst of activity (phasic), whereas others responded with a sustained train of pulses often lasting several seconds (tonic) (Fig. 4). Similar variation in ORN response to non-pheromonal compounds has been found in other Dipteran studies: tsetse flies (Den Otter and van der Goes van Naters 1992), *A. gambiae* (Meijerink and van Loon 1999), *Drosophila* (de Bruyne et al. 2001), Queensland fruit flies (Hull and Cribb 2001) and houseflies (Kelling et al. 2002), among others. An explanation for variation in ORN adaptation rate has not been confirmed. One study suggests that receptor neurons possess differential adaptation as a response to the pulsatory nature of an odor plume (Grant et al. 1997). Studies also suggest that phasic responses inform the insect about frequency and strength of odor bursts (Den Otter and van der Goes van Naters 1992) or rapid changes in concentration (de Bruyne et al. 2001; Kelling et al. 2002), whereas tonic responses function as a form of neuronal "memory" (Almaas et al. 1991) to sustain upwind flight in times where an odor plume is disrupted (Almaas et al. 1991; Den Otter and van der Goes van Naters 1992; de Bruyne et al. 2001).

In *Rhagoletis*, it is possible that differences in temporal firing pattern reflect the behavioral response of flies to certain compounds. ORNs from apple and dogwood flies both responded phasically to 1-octen-3-ol and 3-methylbutan-1-ol (Fig. 5), which are attractants for dogwood flies and antagonists for apple flies. The phasic nature of these ORN responses could allow the flies to quickly "reset" their neurons and follow or avoid the odor plume in nature. This is also the case with esters, which are behaviorally active and elicit a phasic response in all three populations (Fig. 5). However, a link between behavioral preference and temporal firing rate does not explain why hawthorn flies respond tonically to 4,8-dimethyl-1,3*(E)*,7-nonatriene, a behavioral attractant, nor why dogwood flies respond more tonically to the behavioral antagonist ester, hexyl butanoate (Fig. 5). Furthermore, the range of temporal firing rates is quite vast for each volatile and population, and trends for one type of pattern or the other are tenuous at best.

It is possible that phasic and tonic responses provide different types of information for different stimuli. Thus, a variable pattern of sensitivity and firing rate helps to distinguish time and intensity patterns for stimuli (Heinbockel and Kaissling 1996). Differences in temporal firing patterns might not only aid in the identification of volatiles (de Bruyne et al. 2001), but a range of response patterns could allow the insect to encode more information about its environment (Hull and Cribb 2001). Having a range of response characteristics, including sensitivity and temporal firing pattern, allow ORNs to filter the "spatially and temporally variable odor signals present in the natural environment" (Meijerink and van Loon 1999). Thus, while differences in temporal firing rate exist at the periphery, they would not represent a consistent pattern for each population or volatile.

Conclusions

The results presented in this study reveal significant variability in peripheral chemoreception among *Rhagoletis* populations that could impact their reception and discrimination of host volatiles. The breadth of variation

in ORN sensitivity provides the heterogeneity required for some members of a population to complete a host shift, and the lack of sensitivity in host shifting populations to ancestral host volatiles suggests that a loss in sensitivity might facilitate the exploitation of new and introduced hosts. Once on a new host, heightened sensitivity to certain host and non-host compounds could be rapidly selected for to facilitate the agonist/antagonistic effects of these cues during foraging behavior. Moreover, differences in temporal firing pattern could provide an additional dimension for central processing centers to decipher the signal. The level of receptor variability in *Rhagoletis* populations lends credence to the idea that variation in peripheral chemoreception can impact olfactory preference and contribute to the host shifting process. However, 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 response characteristics observed here and confirm that any variation observed is not simply an artifact of sample size. Additionally, further studies analyzing host volatile processing in the antennal lobe are imperative for a more complete understanding of the mechanisms by which divergent olfactory preferences can be established.

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