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Receptor expression and sympatric speciation: unique olfactory receptor neuron responses in F_1 hybrid *Rhagoletis* populations

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Summary

The *Rhagoletis pomonella* species complex is one of the foremost examples supporting the occurrence of sympatric speciation. A recent study found that reciprocal F_1 hybrid offspring from different host plant-infesting populations in the complex displayed significantly reduced olfactory host preference in flight-tunnel assays. Behavioral and electrophysiological studies indicate that olfactory cues from host fruit are important chemosensory signals for flies to locate fruit for mating and oviposition. The reduced olfactory abilities of hybrids could therefore constitute a significant post-mating barrier to gene flow among fly populations. The present study investigated the source of changes in the hybrid olfactory system by examining peripheral chemoreception in F_1 hybrid flies, using behaviorally relevant volatiles from the parent host fruit. Single-sensillum electrophysiological analyses revealed significant changes in olfactory receptor neuron (ORN) response specificities in hybrid flies when compared to parent ORN responses. We report that flies from F_1 crosses of apple-, hawthorn- and flowering dogwood-origin populations of *R. pomonella* exhibited distinct ORN response profiles absent from any parent population. These peripheral alterations in ORN response profiles could result from misexpression of multiple receptors in hybrid neurons as a function of genomic incompatibilities in receptor-gene pathways in parent populations. We conclude that these changes in peripheral chemoreception could impact olfactory host preference and contribute directly to reproductive isolation in the *Rhagoletis* complex, or could be genetically coupled to other host-associated traits.

Key words: host shift, hybridization, postzygotic isolation, single sensillum electrophysiology, discrimination, specificity.

Introduction

Sympatric speciation occurs in the absence of geographic isolation when ecological adaptation to different habitats (niche specialization) overcomes the homogenizing effects of gene flow and initiates population divergence. Sympatric speciation is one of the most controversial issues in evolutionary biology (Coyne and Orr, 2004) because the strong divergent ecological selection pressures and genetic architecture thought to be required for the process are more restrictive than the precondition of geographic separation facilitating allopatric forms of speciation. In order for speciation to occur, pre- and/or post-zygotic barriers must be sufficient to reproductively isolate the populations while the populations are at all times within 'cruising range' (Mayr, 1970) of each other. Sympatric speciation is theoretically intriguing, however, because in the absence of any physical barrier to gene flow the same traits differentially adapting populations to alternative habitats pleiotropically act as important pre- and/or postzygotic ecological barriers, reproductively isolating specialist populations. Elucidating the morphological, physiological and/or behavioral basis for these ecological barriers translates directly into understanding involved in the underlying processes speciation.

The *Rhagoletis pomonella* sibling species complex is often cited as an example of sympatric speciation in progress (Berlocher, 1998). In 1867, Benjamin Walsh first observed the shift of the apple maggot fly *Rhagoletis pomonella* (Walsh), from its native host hawthorn (*Crataegus* spp.) to introduced domestic apple (*Malus pumila* P. Mill) as the formation of a new host race. Bush later postulated that members of the *R. pomonella* group were established by sympatric speciation *via* host plant shifts (Bush, 1969). This complex contains a variety

of host-specific frugivorous races and sibling species (see Berlocher, 2000). Electroantennographic, field trapping and flight-tunnel studies have shown that flies from sympatric populations infesting hawthorn (*Crataegus mollis*), domestic apple (*Malus pumila*) and flowering dogwood (*Cornus florida*) fruit can distinguish among the unique volatile blends identified from each host fruit. These populations not only preferentially orient to their own natal host fruit volatiles (Zhang et al., 1999; Nojima et al., 2003a; Nojima et al., 2003b; Linn, Jr et al., 2003; Linn, Jr et al., 2005b), but avoid non-natal fruit blends (Forbes et al., 2005) and display arrested flight when non-natal volatiles are added to the host blend (Linn, Jr et al., 2005a; Forbes et al., 2005). Host fruit volatiles can thus exert both agonist and antagonist effects on foraging behaviors. Because *Rhagoletis* flies mate and oviposit directly on or near the fruit of their host plant (Prokopy et al., 1971), variation in olfactory host preference serves as a pre-mating barrier to gene flow among flies infesting different host species (Feder et al., 1994).

A recent study (Linn, Jr et al., 2004) showed that olfactory cues can also serve as a form of postzygotic isolation. F_1 hybrids from crosses between apple-, hawthorn- and dogwood-origin flies displayed significantly reduced orientation to host volatiles in flight-tunnel assays. Hybrid flies failed to respond to volatiles at concentrations generating maximum levels of upwind flight from parent flies. Upwind orientation could generally be elicited (in 50–60% of the flies tested) only when higher concentrations (ten times) of host volatiles were used. These high concentrations normally antagonize the upwind flight of parent flies (Dambroski et al., 2005), suggesting a shift in olfactory response threshold for hybrid flies. Furthermore, the few hybrid individuals that did orient at lower concentrations (~10%) typically flew only when the two parental blends were combined, indicating an alteration or relaxation in agonist/antagonist pathways in the olfactory system of hybrids. This response to the combined blends was also amplified at the higher concentrations. These observed changes in olfactory preference could significantly reduce hybrid fitness in the field by diminishing the ability of flies of mixed ancestry to locate hosts, and successfully mate and oviposit on host fruit. Reduced hybrid fitness therefore produces an additional barrier to gene flow among *Rhagoletis* populations by generating a form of olfactory-based, postzygotic isolation.

Here, we investigate a potential source for the changes in the hybrid olfactory system by examining peripheral chemoreception in F_1 hybrid flies to behaviorally relevant volatiles from the parent host fruit. Few studies have examined peripheral chemoreception in F_1 insect hybrids, and those that have were concerned only with pheromonal reception. These previous studies revealed four different characteristics of hybrid olfactory receptor neurons (ORNs): (1) hybrids can possess identical ORNs to parental populations, but in different proportions [*Ips pini* (Mustaparta et al., 1985)]; (2) hybrids can respond more similarly to one parent over the other [*Agrotis ipsilon* × *A. segetum* (Gadenne et al., 1997)]; (3) hybrid ORNs can generate intermediate spike amplitudes (Roelofs et al., 1987) and frequencies (Cossé et al., 1995); [two pheromone races of *Ostrinia nubilalis*]; and (4) hybrid ORNs can exhibit a variety of responses, some similar to the parents, and some 'atypical' responders [*Ctenopseustis obliquana* × *Ctenopseustis* sp. (Hansson et al., 1989); *Heliothis subflexa* × *H. virescens* (Baker et al., 2006)]. In this study, we test the hypothesis that *Rhagoletis* hybrid ORNs also exhibit unique characteristics from parent populations, and that these peripheral alterations can impact olfactory host volatile preference.

Previously, we found that ORNs from various parental *Rhagoletis* host populations demonstrated inter-population variation in ORN sensitivities and temporal firing patterns to an array of key host fruit volatiles (Olsson et al., 2006b). However, all populations tested possessed similar classes of ORNs responding with similar odor specificities (Olsson et al., 2006a). In the present study, we report a unique finding: crosses between apple, hawthorn and dogwood host populations of *R. pomonella* generated F_1 hybrids with distinct and diverse alterations in ORN response profiles absent from parent population ORNs. We discuss possible causes of these alterations in ORN specificity as well as their potential effect on oriented flight to host volatiles.

Materials and methods

Insect origins and rearing were as described (Linn, Jr et al., 2004). Chemical stimuli and neurophysiological analyses were similar to those previously published (Olsson et al., 2006a; Olsson et al., 2006b). We briefly overview these methods below.

Rhagoletis origins and rearing conditions

Apple and hawthorn *Rhagoletis pomonella* (Walsh) flies were collected as larvae from infested fruit at Grant and Fennville, Michigan, and Urbana, Illinois, USA during the 1999–2002 field seasons and reared to adulthood in the laboratory using standard *Rhagoletis* protocols (Feder at al., 1989). Dogwood flies were collected from Granger and Raccoon Lake, Indiana, USA from 2000–2002 and were treated in the same manner as the host races. After overwintering as pupae at 5°C for 4–7 months, eclosing adults were placed in a controlled environmental chamber (24°C, 15 h:9 h light:dark photoperiod, 60–70% relative humidity) and fed a diet of brewers yeast, brown sugar and molasses (Neilson and McAllen, 1965). Adults were mass crossed in mating cages containing at least 20 females and 20 males and supplied with water, food and four Red Delicious variety apples for female oviposition. Puparia were placed in small, clear plastic Solo cups™ containing moist vermiculite and held in a constant temperature chamber to allow flies to develop into adults.

Olfactory stimuli

Synthetic blends and sources of chemicals were the same as reported previously (see Olsson et al., 2006a). Stock solutions (1 $\mu g\ \mu l^{-1}$) 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 electrophysiological analyses (see Table 1) (Olsson et al., 2006a). Dilutions of each compound (1, 10 and 100 ng μl^{-1}, and 1 $\mu g\ \mu l^{-1}$) were prepared for dose response trials. 10 μl of each diluted compound or blend was pipetted onto filter paper (~5 mm×15 mm) in disposable Pasteur pipettes. Blank cartridges, containing only filter paper plus solvent, were also prepared.

Electrophysiological recording

Adult *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, serving as a ground electrode. Electrolytically sharpened tungsten microelectrodes were used to establish contact with the ORNs. The recording electrode was positioned using a preparation microscope with up to 200× magnification and an electrophysiological recording unit with combined joystick micromanipulators and amplifier (Syntech INR-5, Hilversum, The Netherlands). 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^{-1} (Syntech, CS-5, Hilversum, The Netherlands) through a metal tube protruding 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^{-1} during 0.5 s) through the pipette and replaced a complimentary air stream during that time period.

The analog signal originating from the ORNs was amplified (10×) (Syntech INR-05, Hilversum, The Netherlands), sampled (31746.0 samples s^{-1}) and filtered (200 Hz–3000 Hz with 50/60 Hz suppression) *via* USB-IDAC connection to a computer (Syntech, Hilversum, The Netherlands). Action potentials were extracted as digital spikes from the analog signal according to top-top amplitudes using Syntech Auto Spike 32 v. 1.1b–3.2 software. When co-located in the same sensilla, individual neurons were sorted manually for each recorded trace based on differences in the amplitude of their action potentials (spikes). Changes in amplitude due to excessive firing (i.e. 'pinching') were taken into account.

Table 1. *Volatiles used for electrophysiological analyses*

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

Compounds determined from host fruit through gas chromatography coupled with electroantennographic detection and behavioral assays (see Zhang et al., 1999; Nojima et al., 2003a; Nojima et al., 2003b).

In the event of a contact, ORNs were screened with the three fruit blends at 10 μg, and the blank (hexane). These stimuli were tested at least once at the beginning and, in nearly all cases, 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 were tested individually at a concentration of 10 μg. Those compounds eliciting responses were subsequently tested in dose–response trials (10 and 100 ng, 1 μg and 10 μg) to determine each cell's sensitivity to those chemicals.

Data analysis

For each ORN testing period, spike frequencies of the blank (hexane) were calculated every 500 ms for the 10.5 s recording period (including 1 s pre- and 9.5 s post-stimulus onset). In the majority of the cases, more than one blank trial was presented. Spike counts per 500 ms were then averaged across all blank trials. An increase in spike frequency for the 500 ms following stimulus presentation was considered a response if it rose >3 standard deviations (s.d.) above the blank mean. ORN spike increases below this level were not considered further. A response threshold was calculated as the lowest concentration eliciting a spike frequency increase >3 s.d. over the mean of the blank trials. In the few cases where threshold could not be determined (owing to incomplete dose–response trials or cell death), threshold was given as 75% of the lowest concentration eliciting a response <4 s.d. of the blank mean or 50% of a response >4 s.d. of the mean. Sensitivities were assigned as reciprocals of the threshold values (e.g. 10 ng threshold=10^4, 100 ng=10^3, 1 μg=10^2, and 10 μg threshold=10^1).

Each ORN response pattern (i.e. the array of biologically relevant host volatiles to which each ORN responded) to 10 μg of the 11 host compounds (Table 1) was first compared to the ORN response patterns to 10 μg of the same compounds in Olsson et al. (Olsson et al., 2006a). If an ORN responded to the same array of host compounds as ORNs found in the parent population study (Olsson et al., 2006a), it was considered 'parent-like' and labeled with the appropriate ORN class (A–E) determined by cluster analysis (Olsson et al., 2006a) (see Table 2). If the ORN responded to unique combinations of compounds not found in any parent classification, then it was designated as 'hybrid-like' and labeled with the lettered class or classes it most closely resembled. Cells in classes D and E were difficult to classify as 'parent-' or 'hybrid-like' because

Table 2. *Rhagoletis olfactory receptor neuron response profile classes determined through cluster analysis of parent population response to key host volatiles*

Class	Response profile
A	1-Octen-3-ol
B	Dihydro-β-ionone or hexyl butanoate
C	4,8-Dimethyl-1,3(*E*),7-nonatriene and/or 3-methylbutan-1-ol (with or without other compounds)
D	Long-chain esters (response to at least TWO esters and no response to 3-methyl butan-1-ol)
E	Multiple compound responders, including at least TWO long-chain esters

Based on data from Olsson et al. (Olsson et al., 2006a).

of the diversity and number of compounds to which they responded. To maintain the most conservative and accurate depiction of 'hybrid' cells, these cells were not classified as either category. This is also true for a small number of cells (N=6) in the first three classes (A–C) as well.

For statistical comparison of ORN responses, parsimony networks depicting the interrelationship of single cell sensillum response patterns for parents (Olsson et al., 2006a; Olsson et al., 2006b) and F_1 hybrids to the 11 volatile compounds tested in the study were constructed using the program TCS v. 1.13 (Clement et al., 2000). These networks provided a graphic overview of ORN response relationships for the entire sample population, allowing for multi-dimensional characterization of similarities and differences in the patterns of variation for parents and hybrids. Each of the compounds, except for pentyl hexanoate and butyl hexanoate, was considered to represent the equivalent of a different nucleotide base position in a DNA sequence, with + and 0 neuron responses coded as if they were alternate base pairs for a genetic polymorphism (e.g. A and T). Pentyl hexanoate and butyl hexanoate were recoded as a single volatile for network construction and branch length calculation because of the high positive correlation in neuron response observed between the two compounds (parent population r=0.697, P<0.0001, 76 d.f.; F_1 hybrid population r=0.812, P<0.0001, 117 d.f.). Significant connections between different response patterns based on parsimony were limited to one step because of the low number of sites (compounds). In order to connect all response patterns, the maximum number of connections was set to four and networks are shown without breaking reticulations.

Nearest neighbor distances (NNDs) were calculated as a metric to describe the degree of similarity/difference in neuron response patterns observed among and between parental and F_1 hybrid flies. NND measures the dispersion of a population sample. As estimated in the current study, NNDs represented a summary statistic quantifying in a single value the overall similarity or difference in response patterns of ORNs depicted in the parsimony networks within and between parent and hybrid populations. To determine the NND for a neuron, the neuron's response to the suite of compounds tested was coded as a series of 1s and 0s depending upon whether the volatiles did (1) or did not (0) induce a statistically significant neuronal response, with pentyl hexanoate and butyl hexanoate again considered a single volatile (see above for statistical determination of a response). The resulting response vector for the reference neuron was then compared with each of the ORN responses in a chosen comparison population (parent or F_1 hybrid), with pairwise response distances calculated as the absolute value of the total difference between the reference and comparison response vectors. The neuron in the comparison population displaying the fewest number of differences to the reference neuron was considered the nearest neighbor and the difference the NND for the reference neuron. In cases in which the reference neuron population and comparison population were the same, the reference neuron was excluded from the comparison population when NND values were calculated. In the results section, we present the mean and histograms of the distributions of NNDs for comparisons of parent (reference) *vs* parent (comparison) populations, parent *vs* F_1 hybrid, and F_1 hybrid *vs* parent.

Mean NND values were assessed for statistical significance by parametric bootstrapping based on Monte Carlo generated probability distributions. For the parent *vs* F_1 hybrid and F_1 hybrid *vs* parent comparisons, this involved randomly drawing computer generated parent (N=77) and F_1 hybrid (N=118) simulated data sets with replacement from a combined neuron response sampling pool of parent and hybrid patterns. Mean NND values were then calculated between the simulated hybrid and parent data sets and a probability value (P value) estimated from the simulated distribution of NND values as the number of times in 10 000 trials that a simulated NND value as great or greater than the observed value was obtained. For the parent to parent analysis, the P value instead represents the proportion of randomly drawn data sets (N=77) sampled with replacement from the F_1 hybrid population that had a mean NND to the actual parent population the same or less than the observed parent to parent mean NND value in 10 000 trials. This latter analysis determined whether any observed difference between parent and hybrid response patterns could be caused by biased sampling of parent neurons due to smaller sample sizes in parent (N=77) *vs* hybrid (N=118) populations.

Results

Olfactory receptor neurons (N=118) from 39 individuals among the various populations were used for neurophysiological analyses. These included: 12 individuals of apple (female) × hawthorn (male) origin (ORN N=39),

15 individuals of hawthorn (female) × apple (male) origin (ORN *N*=48), 5 individuals of apple (female) × dogwood (male) origin (ORN *N*=15), and 7 individuals of dogwood (female) × apple (male) origin (ORN *N*=16). All data for parent comparisons were taken from Olsson et al. (Olsson et al., 2006a; Olsson et al., 2006b).

Fig. 1 illustrates typical parent and hybrid ORN response profiles for three basic classes of *Rhagoletis* ORNs, as described by Olsson et al. (Olsson et al., 2006a) (see Table 2). As Fig. 1 shows, although hybrid ORNs still responded to the same compounds as the parent ORNs, they also responded to other, sometimes structurally unrelated, compounds.

Fig. 2 depicts response profiles from 63 of the total 118 responding ORNs for each hybrid population with their respective threshold sensitivity to each of the 11 tested host compounds. ORNs were classified and organized from left to right according to Olsson et al. (Olsson et al., 2006a) into the five major ORN classes representing increasing levels of complexity with respect to the number of compounds eliciting a response (Table 2). An additional 55 cells were categorized as Class D or E. Owing to the diverse response profiles of cells in classes D and E (i.e. response to at least two long-chain esters and/or several host volatiles) these cells were not labeled as either parent- or hybrid-like and are not graphically represented. The y axis depicts sensitivities [i.e. reciprocal thresholds displayed as log(sensitivity)] to each host volatile as determined by dose–response trials. 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.

The graphs in Fig. 2 show that some cells in each hybrid cross responded with ORN response profiles identical to those found for parent populations (Olsson et al., 2006a). These cells were classified as parent-like in the first columns of classes A, B and C. Of the 118 recorded ORNs, 6.7%, 9.3% and 12.7% of the cells could be categorized as parent-like class A, B or C cells, respectively. Several ORNs from hybrid individuals responded exclusively to 1-octen-3-ol (class A: one ORN from an apple × hawthorn hybrid, two from hawthorn × apple, three from apple × dogwood, and two from dogwood × apple), dihydro-β-ionone (class B: two ORNs from apple × hawthorn hybrids, three from hawthorn × apple, and one ORN from a dogwood × apple hybrid) or hexyl butanoate (Class B: one ORN each from apple × hawthorn, hawthorn × apple, and dogwood × apple hybrids), as in parent class A and B ORNs. Two of the class B apple × hawthorn hexyl butanoate responders also responded to propyl hexanoate as was found in one instance of the parent population recordings. Several cells also responded with class C response profiles (response to 4,8-dimethyl-1,3*(E)*,7-nonatriene and/or 3-methylbutan-1-ol, either exclusively or with one or more other compounds): five ORNs from apple × hawthorn hybrids, six ORNs from hawthorn × apple, two from apple × dogwood, and two from dogwood × apple.

Other ORNs from hybrid individuals showed response patterns that were not observed in any of the parent flies. Because these response patterns were never found for any parent population ORNs, these cells were classified as 'hybrid-like' and are shown in Fig. 2 in the right columns of classes A, B and C. For example, hybrid-like class A ORNs recorded from apple × hawthorn individuals (black bars) responded to 1-octen-3-ol (an alcohol) as did parent population ORNs, but also responded to dihydro-β-ionone (a ketone), 4,8-dimethyl-1,3*(E)*,7-nonatriene (a hydrocarbon), propyl hexanoate, pentyl hexanoate, butyl hexanoate (all esters), isoamyl acetate (an acetate) and/or ethyl acetate (acetate). These response profiles were never recorded from any parent population class A cells. Likewise, class B hybrid-like hawthorn × apple ORNs (white bars) responded to dihydro-β-ionone or hexyl butanoate as in parent population ORNs, but also responded to 4,8-dimethyl-1,3*(E)*,7-nonatriene, and/or ethyl acetate, 3-methylbutan-1-ol or isoamyl acetate. Several other examples are listed with their corresponding response profiles in Fig. 2, classes A–C. Note that cells with profiles resembling multiple classes are repeated for each class they resembled and indicated by asterisks on the *x* axis. In total, 29/63 (46%) of the cells exhibiting characteristics of parent class A, B, or C cells responded to unique combinations of volatiles absent from parent population recordings. Furthermore, both hybrid- and parent-like ORN profiles were observed in the same individual in 14 of 39 hybrid individuals tested (36%; six apple × hawthorn individuals, four hawthorn × apple, two apple × dogwood, and two dogwood × apple individuals), and twice in the same sensillum (one each from an apple × hawthorn and hawthorn × apple individual).

Fig. 2 also provides response threshold sensitivities (i.e. reciprocal dose–response thresholds) for each contacted ORN. The figures show a great deal of variation in ORN sensitivity both within and between individuals. Mann–Whitney tests found very few significant differences ($P>0.05$) in host volatile sensitivity between hybrid populations or between hybrids and parents for any tested volatile. Only the dogwood parent population (Olsson et al., 2006b) *vs* its reciprocal hybrid crosses was significantly different ($P<0.05$) with respect to sensitivity to 4,8-dimethyl-1,3*(E)*,7-nonatriene.

Parsimony networks were constructed for parent and F_1 hybrid ORN responses depicting the relationships of neuron response to the 11 fruit volatile compounds tested in the study (Fig. 3). Unlike the graphs above, which group general classes (A–E) of response patterns based upon cluster analysis (Table 2) (Olsson et al., 2006a), parsimony networks statistically compare ORN response profiles for exact matches, with each node representing a different profile. For example, a neuron responding to hexyl butanoate, propyl hexanoate and butyl hexanoate (compounds 3, 5 and 8 from Fig. 2) would be pooled only with other neurons having that same profile. Thus, each node in the network represents a unique response profile and each single straight line between nodes reveals a one step difference in response (i.e. response to one different volatile) between the two profiles. Each subsequent line reveals another single compound difference in response profile up to a

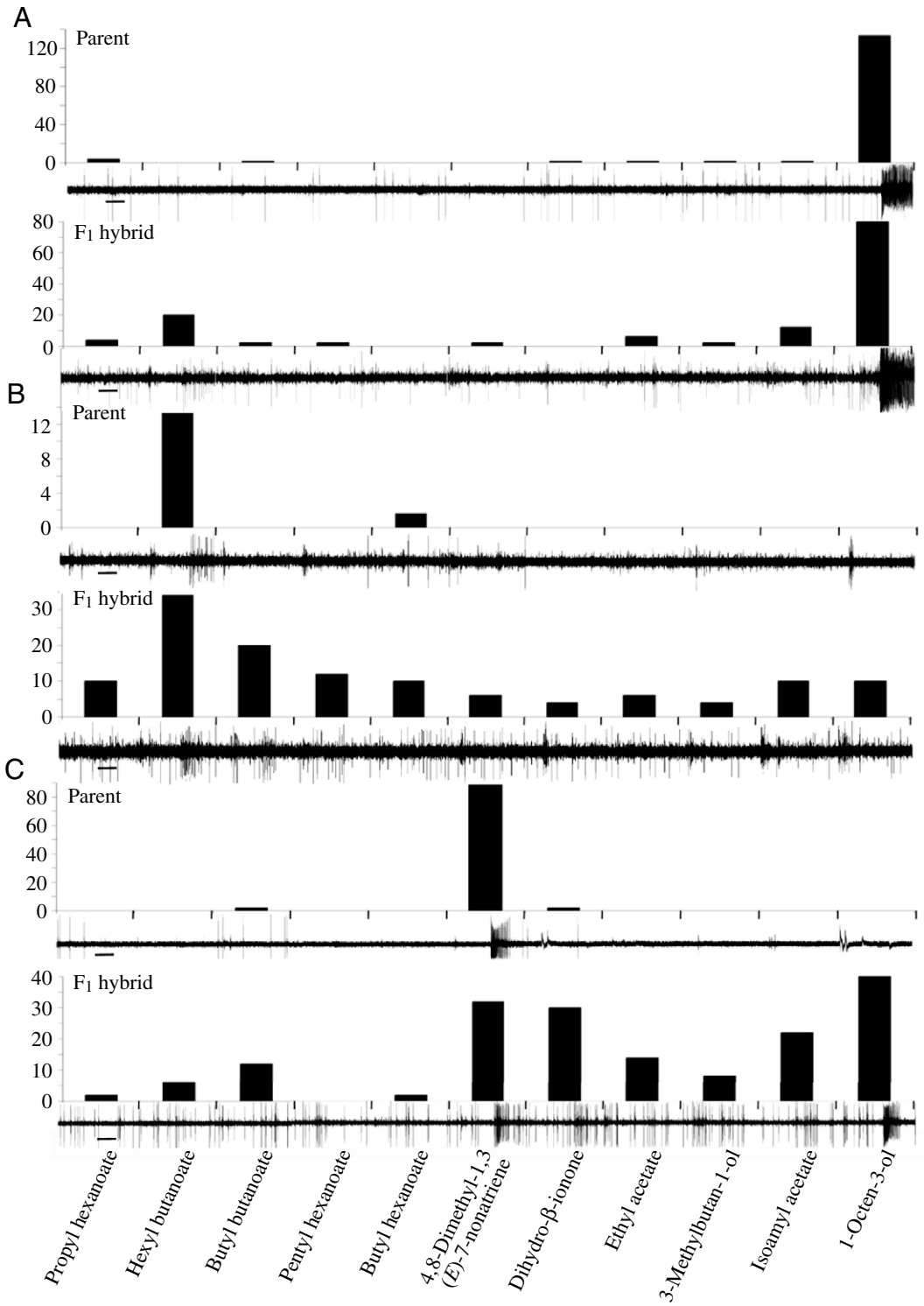

Fig. 1. Comparison of typical response profiles for *R. pomonella* parent and hybrid ORNs from (A) class A (1-octen-3-ol responders) (B) class B (hexyl butanoate or dihydro-β-ionone responders) and (C) class C (4,8-dimethyl-1,3*(E)*,7-nonatriene responders). Parent data was obtained previously (Olsson et al., 2006a). Bar charts indicate spike frequencies (spikes s^{-1}) for the 500 (hybrid) and 600 ms (parent) following initial exposure to 10 μg of each volatile listed at the bottom of the figure. The first 2 s of the 10.5 s recordings from which these frequencies were obtained is shown below each chart with chart ticks demarcating corresponding traces. Lines under the first trace indicate location of stimulus delivery for subsequent traces. In A, the parent ORN is specific to 1-octen-3-ol, whereas the hybrid ORN (dogwood × apple) responds to 1-octen-3-ol along with hexyl butanoate and isoamyl acetate. The B parent cell is specific to hexyl butanoate whereas the hybrid cell (apple × hawthorn) also responds to butyl butanoate. Finally, in C, the parent ORN is specific to 4,8-dimethyl-1,3*(E)*,7-nonatriene, whereas the hybrid ORN (dogwood × apple) also responds to dihydro-β-ionone, isoamyl acetate and 1-octen-3-ol.

maximum of four differences. The TCS network method allows for more direct statistical comparison of the breadth in response pattern variation between parent and hybrid populations. The networks highlighted some similarities and several major differences in the patterns of neuron response between parent and hybrid populations. For example, a number of parent neurons (41/77=53.2%) fell into clusters A–D (see dark nodes at the top of Fig. 3A). These neurons characteristically responded to just one or a few of the 11 compounds tested, and are similar to the classes previously determined by cluster analysis (Table 2). Clusters A–D were also evident in the hybrid population. However, the proportion of hybrid neurons constituting these five categories was significantly lower (28/118=23.7%) than the proportions observed for the parents (53.2%; $P<0.0001$, two-tailed Fisher exact test). Another major difference was that the hybrid population contained many neurons that responded to one compound in addition to those characteristic of clusters A–D (Fig. 3B).

Parent neurons not belonging to the five clusters responded to varying combinations of multiple compounds; apart from the five clusters, no parental neurons responded to only one compound (notice lack of labeled white nodes, Fig. 3A). Several of the multiple response neurons had shared counterparts in the F_1 hybrid population (10/30=33.3%, black nodes Fig. 3A,B). By contrast, the F_1 hybrid population contained a higher proportion of unique, multi-compound response patterns not seen in the parent population (56/67=83.6% unique to F_1 hybrids *vs* 66.7% unique to parents, unlabelled white nodes, Fig. 3A,B).

Nearest neighbor distances (NNDs) were calculated as summary statistics to quantify the difference between parent and F_1 hybrid population ORN responses relative to the variation present within parent flies (see Fig. 4A-C for histograms of NND values for parent *vs* parent, parent *vs* F_1 hybrid, and F_1 hybrid *vs* parent comparisons). The mean NND calculated for the F_1 hybrid *vs* parent comparison (0.831) was highly significant ($P<0.0003$) and much larger than the mean NND values estimated for the parent *vs* parent or parent *vs* F_1 hybrid analyses (mean NND=0.493 and 0.338, respectively). The high NDD value for the F_1 hybrid *vs* parent comparison reflects the large proportion of unique neuron response patterns present in the F_1 hybrid population that were not seen in parent flies. By contrast, the relatively low and non-significant mean NND for the reciprocal parent *vs* F_1 hybrid comparison (0.338; $P=0.326$) was due to the majority of neuron response patterns measured in the parent population having counterparts or close companions in the hybrid population. Finally, the low mean NND for the parent *vs* parent comparison reflects the high proportion of parental neurons with shared response patterns and the relatively few neurons having unique response patterns.

The parsimony networks and NND values imply that the parent neuron population represents a limited subset of F_1 hybrid response patterns, with the parent population being largely composed of high numbers of shared ORN response profiles and relatively few 'unique' ORN responses. It is conceivable, however, that this difference could have resulted from biased sampling of parent neurons and not from an inherent difference between parent and hybrid neuron populations. The sample size of the parent neurons measured in the study was smaller ($N=77$) than that for F_1 hybrids ($N=118$) and by chance we may have measured a non-representative set of parent neurons. To assess this possibility, we performed Monte Carlo parametric bootstrapping, randomly drawing 10 000 samples of 77 neuron response patterns with replacement from the F_1 hybrid population and calculating mean NND values for these samples relative to the observed parent population. Given that the F_1 hybrid population represents the true underlying distribution of parents, then the proportion of trials in which we derived a mean NND equal to or below the value calculated for the actual parent *vs* parent comparison (0.493) would indicate the probability of us having measured by chance a biased subset of parent neurons. The P value for the Monte Carlo parametric bootstrapping of simulated parental populations was 0.0004, implying that non-random sampling of parent neurons was not the likely cause for the observed difference between parent and hybrid populations; the two populations of neuron responses are inherently different.

Discussion

Previous flight-tunnel studies of *Rhagoletis* hybrids found both a shift in response threshold to host volatiles as well as a relaxation in agonist/antagonist host volatile processing (Linn, Jr et al., 2004). Although a small proportion of parents responded to multiple blends (Linn, Jr et al., 2005a), most flew only to their own natal host blend (Zhang et al., 1999; Nojima et al., 2003a; Nojima et al., 2003b; Linn, Jr et al., 2005b), and were antagonized by the presence of non-natal host volatiles (Linn, Jr et al., 2005a). Hybrids, on the other hand, did not respond to host volatile blends at concentrations eliciting maximum response in the parental studies. The small proportion that did respond did so only to combined blends from both parent hosts. Additionally, if the concentrations of the volatiles were increased tenfold, approximately 50–60% of the flies flew upwind to both single and/or combined blends of the parents. These high concentrations arrested the upwind flight of parents.

The unexpected results of our study indicate that peripheral chemoreception is also significantly altered and potentially compromised in F_1 hybrids between apple, hawthorn and dogwood host populations of *R. pomonella*. In a previous study of the three parent populations, all recorded ORNs could be grouped into five distinct classes with distinguishing characteristics (Olsson et al., 2006a) (Table 2). Although 54% of the 63 Class A-C ORNs from hybrids could also be classified into one of the first three parent ORN classes, 46% possessed unique and highly divergent response profiles not observed in any parent population (Figs 1 and 2). In fact, some of the hybrid ORN response profiles appeared to be combinations of profiles found in various parental ORN classes. Still others possessed completely unique profiles. Parsimony networks and nearest

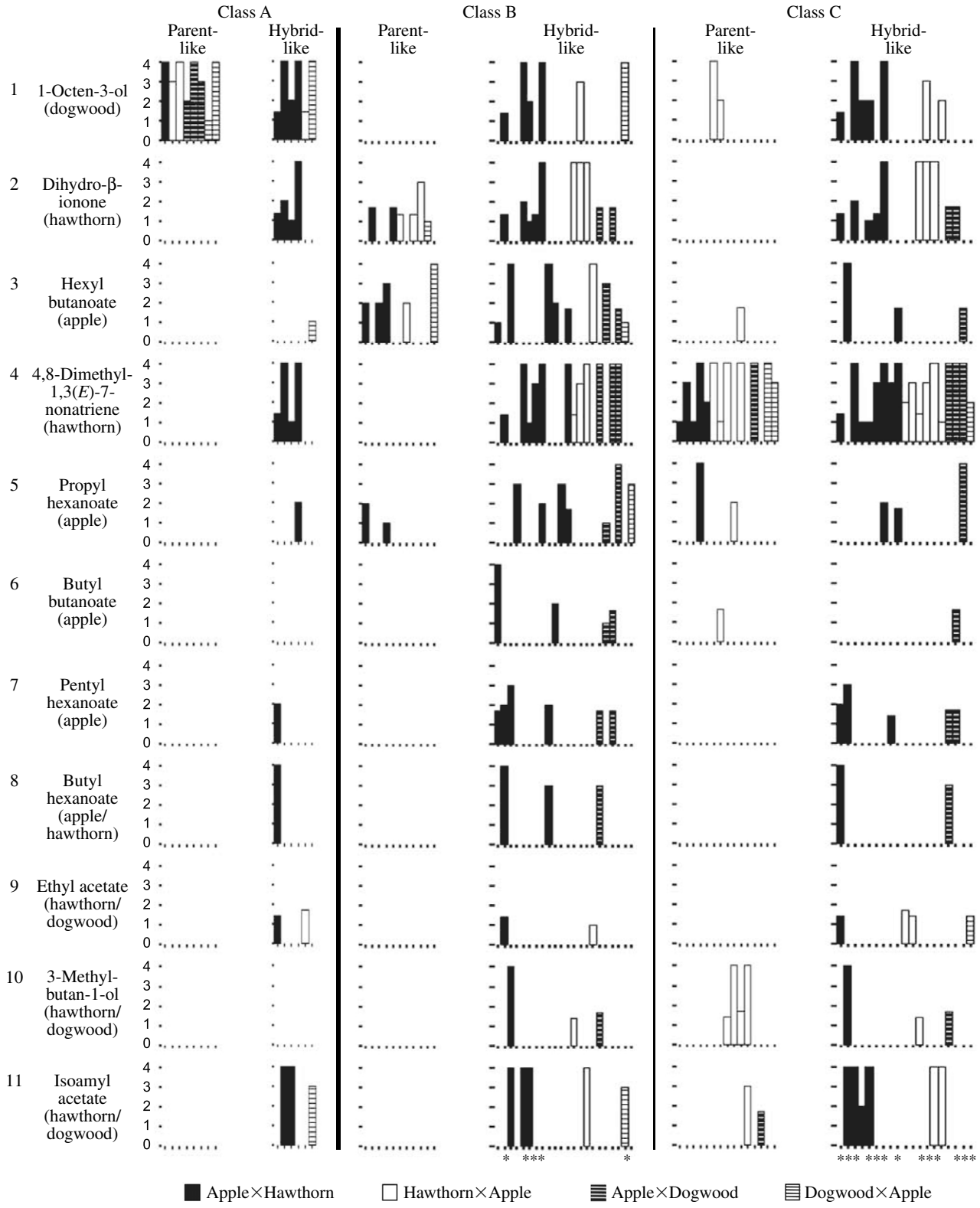

Fig. 2. Bar graphs depicting olfactory receptor neuron (ORN) response profiles and sensitivities for *Rhagoletis* hybrid populations. Cells are grouped by receptor neuron class and host volatile. The *x* axes list contacted ORNs and bar pattern indicates the hybrid population. ORNs are arranged from left to right according to cluster analysis classes (Olsson et al., 2006a). Sensitivities (*y* axes) are depicted as log(sensitivity). Parent-like and Hybrid-like indicate hybrid ORNs from classes A–C that responded with parent- or hybrid-like response profiles, respectively (see Materials and methods). Asterisks below certain ORNs indicate that the response profile for that ORN corresponded to multiple classes and was listed for each class it resembled.

neighbor distances (Figs 3 and 4) were used to statistically summarize differences in the response profiles within and between parent and F_1 hybrid neurons. These analyses reflect the diversity of ORN response profiles in the F_1 hybrid population as compared to the more homogeneous parent ORNs. Monte Carlo parametric bootstrapping shows that these unique ORN response profiles are an inherent characteristic of hybrid neurons, and are not simply due to experimental sampling bias in the parent population.

Contrary to previous studies in which F_1 hybrid ORN responses generally resembled one parent or a mixture of both parents, hybrid *Rhagoletis* ORNs in hybrid individuals appeared to have unique response profiles completely absent in any parent population. Abnormalities in the peripheral system of F_1 hybrids, therefore, cannot simply be the result of the inheritance of traits from both parents. Rather, the unique profiles indicate some form of breakdown in the development and function of the ORNs. Although it is not surprising that each host-related taxon responds to its own set of unique host volatile cues (Zhang et al., 1999; Nojima et al., 2003a; Nojima et al., 2003b), it is surprising that these hybrid abnormalities occur in spite of the fact that the different host-related taxa are morphologically indistinguishable and lack any fixed allozyme or nuclear and mtDNA sequence differences (Berlocher, 2000; Feder et al., 1988; Feder et al., 2003; Feder et al., 2005).

There are several potential explanations for the altered neurophysiology in *Rhagoletis* F_1 hybrids. It is possible that genomic incompatibilities between parent *Rhagoletis* populations result in physical deformities in the structure of olfactory sensilla and alter the response properties of the ORNs they house. In *Drosophila*, the dominant mutation *Sco* causes specific alterations in chemoreception (Dubin et al., 1995), as well as a severe reduction in expression of specific antennal odor receptors and malformations in certain basiconic sensilla that may result in the loss of responsiveness to certain chemicals (A. Ray, personal communication). However, in *Rhagoletis* hybrids, any obvious deformities in sensilla were never observed. We also did not discern any significant difficulties in contacting ORNs, which might be assumed if those sensilla were malformed.

Alternatively, each recorded antennal sensillum in *Rhagoletis* housed between one and three neurons discriminated by differences in amplitude. It is possible that genomic incompatibilities governing neuron-to-sensilla

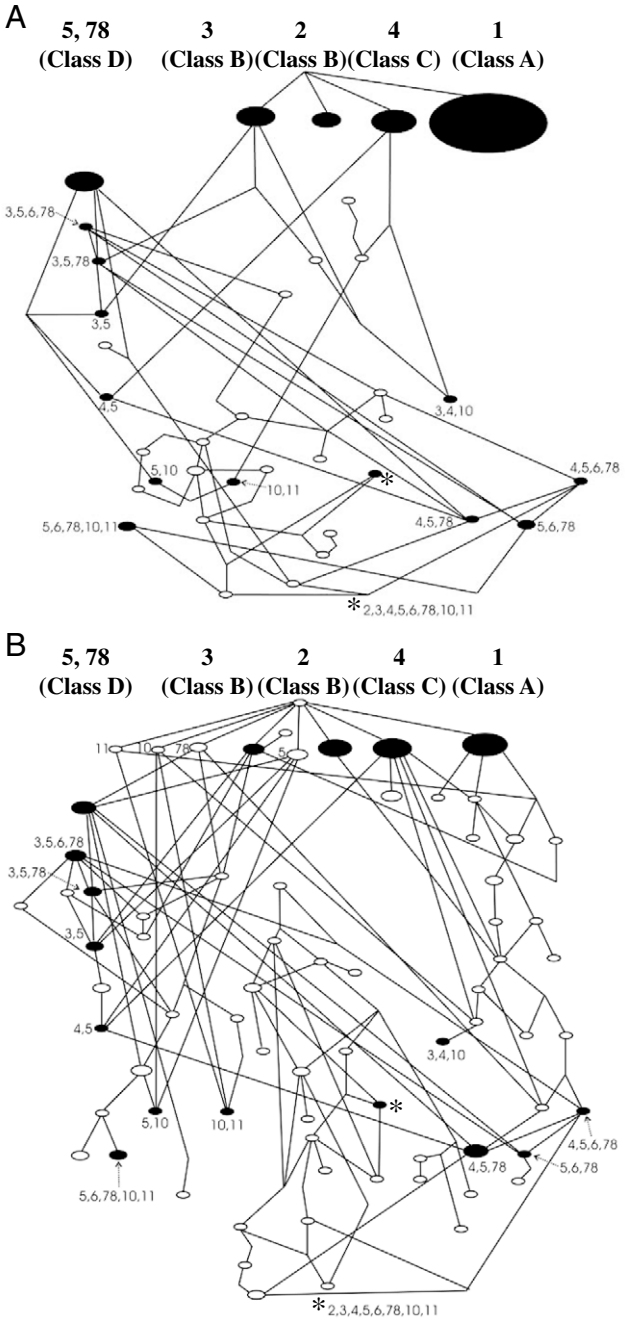

Fig. 3. Most parsimonious TCS network depicting the relationships among ORN response patterns to the 11 tested fruit volatile compounds (see Fig. 2) for (A) parental apple, hawthorn and dogwood flies, and (B) F_1 hybrids. Each oval node represents a different response pattern observed in the parent or hybrid neuron population, with numbers above or below nodes indicating the ORN response profile according to the numbered compounds listed in Fig. 2. The five black nodes at the top of each diagram designate the five general response categories identified in the parent population. These response categories are similar to the cluster analysis classes (Olsson et al., 2006a) (Table 2) listed above each node. The sizes of oval nodes reflect the relative proportions of the different neural response patterns observed in the test population (N=77 neurons recorded for parents, N=118 for F_1 hybrids), with black nodes indicating shared response patterns seen in both parents and hybrids and white nodes unique response patterns. Furthermore, white labeled nodes represent neurons responding to only the respective compound shown (mono-response patterns). Shared neurons (black nodes) are anchored in the same positions in A and B to provide reference points for comparing the parent and hybrid networks, and are labeled by their response pattern. The number of straight-line segments connecting two nodes indicate the difference in the number of compounds that the neurons responded to. Compounds 7 and 8 (pentyl hexanoate and butyl hexanoate) were considered to represent a single volatile for network construction and branch length calculation owing to the high positive correlation in neuron response between the two compounds.

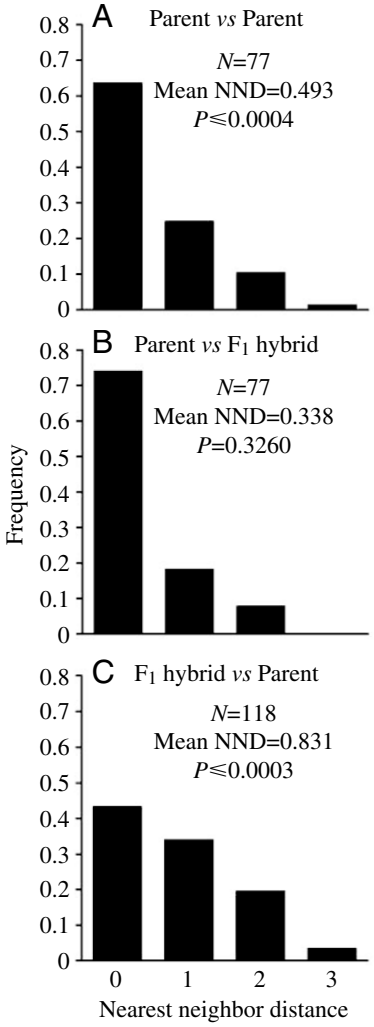

Fig. 4. Histograms of nearest neighbor distances calculated between neuron response patterns observed in reference (first) *vs* comparison (second) populations for (A) parent *vs* parent, (B) parent *vs* F_1 hybrid, and (C) F_1 hybrid *vs* parent analyses. Also given are mean nearest neighbor distances for each comparison (mean NND), and the probability level (*P* value) for the mean NND as determined by Monte Carlo parametric bootstrapping. For the parent *vs* F_1 hybrid and F_1 hybrid *vs* parent analyses (B,C), *P* values indicate the proportions of randomly drawn parent and F_1 hybrid data sets sampled with replacement from a combined neuron response sampling pool that had mean NND as great or greater than the observed value in 10 000 trials. For the parent to parent analysis, the *P* value represents the proportion of randomly drawn data sets of 77 neurons sampled with replacement from the F_1 hybrid population that had a mean NND to the actual parent population the same or less than the observed parent to parent value in 10 000 trials.

mapping caused parent ORNs to become co-located in hybrid sensilla. These multiple ORNs then possessed identical amplitudes that could not be distinguished by the methods in this study, and appeared as single ORNs with unique response profiles. However, during the diminished amplitude (pinching) that often occurs with a high frequency response to an odorant (see Fig. 1C parent), one might be able to distinguish an unaffected ORN. Additionally, 15 of the 29 hybrid-like cells were already found co-located with ORNs easily discriminated by amplitude. It is unlikely that all of these hybrid-like ORNs were the result of co-located ORNs that were never distinguished.

The most parsimonious explanation for the observed changes in hybrid *Rhagoletis* neurophysiology involves the ORNs themselves. Studies of *Drosophila* have shown that odorant receptors confer the entire odorant response spectrum of an olfactory neuron (Elmore et al., 2003; Dobrista et al., 2003; Hallem et al., 2004). Therefore, any changes in response profile seen in hybrid ORNs are most likely the result of alterations in the olfactory receptors themselves. Olfactory receptor neurons have traditionally been thought to express only one type of olfactory receptor conferring the entire response profile of that neuron (Jacquin-Joly and Merlin, 2004). However, a recent study (Goldman et al., 2005) found multiple, naturally expressed and completely functional odor receptors co-expressed within a single neuron.

A recent study of chemoreception in hybrids between two closely related moth species, *Heliothis virescens* and *H. subflexa*, found that some hybrid ORNs responded to unique combinations of pheromone components absent from either parent species (Baker et al., 2006). Combined with both flight tunnel assays (Vickers, 2006a) and antennal lobe recordings (Vickers, 2006b), Baker et al. (Baker et al., 2006) postulated that the "*broad yet specific tuning of certain ORN types*" suggests that multiple olfactory receptors were expressed in the dendritic membranes of the olfactory neurons. In congruence with the hypothesis by Baker et al. for *Heliothine* hybrids (Baker et al., 2006), it is also possible that *Rhagoletis* hybrid ORNs express multiple receptors, in combinations absent from parent ORNs. This could produce the diverse response profiles witnessed in hybrid ORN responses. For instance, some hybrid ORNs responded to combinations of 1-octen-3-ol, 4,8-dimethyl-1,3*(E)*,7-nonatriene, hexyl butanoate and/or dihydro-β-ionone concurrently (Figs 1 and 2). In the parent populations we found separate ORNs specific to these single components at the concentrations tested in the current study (Olsson et al., 2006a) (Fig. 1, Table 2). If parent populations possessed separate receptor types for each of these compounds, then it is possible that hybrid ORNs expressed multiple receptors for these compounds in a single neuron.

Unfortunately, "*remarkably little is known*" about how neurons determine which receptors to express (Hallem and Carlson, 2004). In *Drosophila*, Acj6, a POU-domain protein transcription factor, has been suggested to impact receptor gene choice (Clyne et al., 1999). In Acj6 mutant flies, a percentage of ORNs responded normally, a percentage were unresponsive, and another subset had unique response profiles that were absent from wild-type flies. This pattern is similar to what occurred in *Rhagoletis* hybrids. It is possible that crosses between parent *Rhagoletis* populations result in genomic incompatibilities that affect POU-domain transcription factors and produce receptor misexpression at the periphery. This, in

turn, could leave some ORN response profiles intact while others become distorted, gaining or losing response to certain odorants by altering the type of receptor proteins they express. Many (36%) of the tested individuals possessed both parent- and hybrid-like ORNs concurrently. In fact, two individuals possessed both parent- and hybrid-like cells in the same sensillum. The relationship between transcription factors and OR genes has been suggested as a pathway for the unique responses found in *Heliothine* hybrids (Baker et al., 2006).

However, unlike the *Heliothine* study where the hybrid ORNs displayed a stereotypical, though unique, set of responses from the parents, the *Rhagoletis* hybrid ORN response profiles were much more diverse, and almost random in appearance. We observed ORN response profiles with virtually every combination of the 11 host volatiles tested (note hybrid A, B, and C columns of Fig. 2). This reshuffling and randomization of receptor expression indicates a fundamental, and likely early, breakdown in odor receptor gene choice. Recent work in *Drosophila* has identified both positive and negative *cis*-regulatory elements that act in combinatorial code to dictate organ- and neuron-specific expression of individual receptor genes (Ray et al., 2005). It is possible that a breakdown in this combinatorial code early in development caused by genomic incompatibilities in parent host populations could cause the changes in receptor expression proposed for the *Rhagoletis* hybrids.

In the *Heliothine* study, Baker et al. suggested that because the regulation of agonist/antagonist behaviors are believed to occur primarily in the antennal lobe and higher brain centers, a simple change in receptor expression at the periphery could allow a shift in the agonistic/antagonist properties of a compound (Baker et al., 2006). Olfactory receptor neurons expressing one type of olfactory receptor target a single glomerulus in the antennal lobe of the brain (Vosshall et al., 2000; Gao et al., 2000), and ORNs will target the same glomeruli regardless of normal receptor expression (Dobrista et al., 2003). If an ORN targeting a glomerulus that is part of an agonistic pathway now expresses receptors for antagonistic compounds, it could drastically alter the behavioral relevance of those compounds. It is this type of shift in receptor expression that could have allowed hawthorn-origin flies to shift to apples and develop antagonistic responses to former host compounds. A subtle shift in receptor expression from neurons targeting agonist pathways to neurons targeting antagonist pathways could promote a shift from one host to another. A plasticity in receptor expression could also promote the variation in behavior witnessed in parent populations (Linn, Jr et al., 2005a).

If host populations with contrasting behavioral responses to host volatiles differ as to their expression of olfactory receptors, then hybridization could introduce the genomic incompatibilities discussed above. Formerly attractant compounds whose receptors are now expressed in multiple neurons could stimulate multiple glomeruli, sending conflicting input to the CNS and causing hybrids to respond to a combination of host blends as observed in behavioral trials (Linn, Jr et al., 2004). Furthermore, although hybrid ORN sensitivities were not significantly lower to host volatiles than parent ORNs, hybrids could still require higher concentrations in order to respond behaviorally because the hybrid antennal lobe requires a much higher concentration to process the confused input from the periphery. It is known that antennal lobe neurons are much less sensitive than receptor neurons [Hansson and Christensen (Hansson and Christensen, 1999) and references therein]. Alternatively, there are still some 'parental type' ORNs present in F_1 hybrids (Figs 2 and 3). These parent-like ORNs were often found in conjunction with hybrid-like ORNs on the same individual. Perhaps F_1 hybrid individuals can only detect host blends at significantly higher concentrations because there are fewer ORNs that still respond 'normally' to the host components. Fig. 1 shows that in several crosses roughly half of the cells tested possessed these altered, 'hybrid-like' response profiles.

These observed changes in hybrid ORN response profiles could have significant impacts on hybrid olfactory behavior. By all other accounts, the hybrid crosses are viable and fertile (Linn, Jr et al., 2004). However, olfaction has been shown to be an important factor in host selection (Zhang et al., 1999; Nojima et al., 2003a; Nojima et al., 2003b; Linn, Jr et al., 2003; Linn, Jr et al., 2005a). Chemosensory dysfunction in hybrids could significantly hinder their ability to locate hosts (Linn, Jr et al., 2004). This, in turn, would reduce their prospect of finding a mate and ovipositing onto host fruit, given that *Rhagoletis* flies mate and oviposit on or near the fruit of their host (Prokopy et al., 1971). Furthermore, the lack of sensitivity to high concentrations of volatiles in F_1 hybrids could reduce their ability to avoid overripe, substandard fruit for oviposition preference (Linn, Jr et al., 2004). Together, these factors could significantly reduce hybrid reproductive fitness in the field, and prevent further gene flow between hybrids and other individuals. Thus, reduced chemosensory ability in *Rhagoletis* F_1 hybrids can serve as an effective postzygotic barrier to gene flow between parent populations. More testing, including mark–release–recapture field tests with F_1 hybrids, are required to confirm the impact of reduced chemosensory ability on hybrid host location (Linn, Jr et al., 2004).

The results of the present study indicate that the reduced ability of *Rhagoletis* hybrids to orient to host volatiles may be due, in part, to significantly altered specificity in hybrid ORNs. The results also suggest that subtle differences in receptor-neuron targeting in the parents could account for differences in agonist/antagonist response to host volatiles and could lead to genetic incompatibilities and receptor misexpression in the hybrid offspring. Further testing involving the analysis of second generation F_2 and backcross hybrids is required to determine whether these incompatibilities have direct impacts on olfactory behavior in the hybrid populations. Alternatively, differences in olfactory behavior may be due to alterations at higher brain centers, and the changes in ORN response seen here may instead be the pleiotropic consequences of other phenotypes under host-associated selection, such as developmental timing (Feder and Filchak, 1999). Nevertheless,

the abnormalities found in *Rhagoletis* raise several new questions regarding the development of peripheral olfactory pathways and their impacts on behavior and speciation, which can only lead to exciting new discoveries in the study of chemical senses and evolutionary biology.

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