

Comparing Peripheral Olfactory Coding with Host Preference in the *Rhagoletis* Species Complex

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Abstract

Recent studies have shown that flies from sympatric populations of *Rhagoletis pomonella* infesting hawthorn, apple, and flowering dogwood fruit can distinguish among unique volatile blends identified from each host. Analysis of peripheral chemoreception in *Rhagoletis* flies suggests that changes in receptor specificity and/or receptor neuron sensitivity could impact olfactory preference among the host populations and their hybrids. In an attempt to validate these claims, we have combined flight tunnel analyses and single sensillum electrophysiology in F₂ and backcross hybrids displaying a variety of behavioral phenotypes. Results show that differences in peripheral chemoreception among second-generation adults do not provide a direct correlation between peripheral coding and olfactory behavior. We conclude that either the plasticity of the central nervous system in *Rhagoletis* can compensate for significant alterations in peripheral coding or that peripheral changes present subtle effects on behavior not easily detectable with current techniques. The results of this study imply that the basis for olfactory behavior in *Rhagoletis* has a complicated genetic and neuronal basis, even for populations with a recent divergence in preference.

Key words: coding, flight tunnel, hybrid, single sensillum electrophysiology, speciation, ORN

Introduction

The *Rhagoletis pomonella* species complex contains a group of monophagous tephritid flies believed to be undergoing sympatric speciation via shifts from one host plant to another (Bush 1966). In eastern North America, a number of different host races and sibling species of *R. pomonella* co-occur, including domestic apple (*Malus pumila*) and hawthorn (*Crataegus* spp.) infesting host races as well as an undescribed sibling species believed to be the sister taxon to *R. pomonella* that attacks flowering dogwood (*Cornus florida*; Berlocher 2000).

Host plant choice is a key barrier to gene flow between *R. pomonella* populations. *Rhagoletis* flies mate on or near the fruit of their respective host plants (Feder et al. 1994). Consequently, differences in host choice translate directly into mate choice and generate prezygotic reproductive isola-

tion between flies utilizing different plants. Evidence suggests that olfactory cues are important aspects of host location among *Rhagoletis* flies (Zhang et al. 1999; Nojima, Linn, Morris, et al. 2003; Nojima, Linn, and Roelofs 2003; Linn et al. 2004; Dambroski et al. 2005; Forbes et al. 2005; Linn, Dambroski, et al. 2005; Linn, Nojima, and Roelofs 2005; Forbes and Feder 2006). Studies utilizing gas chromatography coupled with electroantennographic detection (GC-EAD) and flight tunnel assays have identified several key host volatiles from apple, hawthorn, and flowering dogwood host fruits (Zhang et al. 1999; Nojima, Linn, Morris, et al. 2003; Nojima, Linn, and Roelofs 2003). In both flight tunnel and field analyses, members of the 3 host populations preferentially oriented to their own host fruit blends. In addition, flies in each population were antagonized by the

addition of nonhost volatiles to their host blend (Forbes et al. 2005; Linn, Nojima, and Roelofs 2005; Forbes and Feder 2006). However, in any population, a small but significant proportion of flies oriented both to their own host blend as well as the blend of another host population (Linn, Dambroski, et al. 2005). This variation in fruit odor acceptance could provide the basis for shifts to novel hosts or may alternately reflect low-level gene flow between populations.

Studies analyzing peripheral chemoreception in *R. pomonella* populations showed that although all taxa possessed the same number and classes of host volatile-responding olfactory receptor neurons (ORNs; Olsson et al. 2006a), significant variation in sensitivity to host compounds could impact their olfactory host preference (Olsson et al. 2006b). For example, ORNs recorded from apple- and dogwood-origin flies, 2 populations believed to have derived from the hawthorn race of *R. pomonella* via host-shifts (Berlocher 1998, 2000), showed a trend in being less sensitive to hawthorn fruit volatiles than hawthorn flies. ORNs from apple flies were also more sensitive to several apple volatiles than either hawthorn or dogwood flies. In contrast, dogwood flies were less sensitive to dogwood volatiles than the other 2 populations. Variation in sensitivity to host volatile components could potentially have important consequences for host preference and affect the propensity of *Rhagoletis* populations to shift from one host plant to another (Linn et al. 2003; Forbes et al. 2005; Forbes and Feder 2006).

Hybrid F_1 crosses between the 3 *R. pomonella* populations exhibited significantly reduced behavioral response in flight tunnel analyses (Linn et al. 2004). Hybrid flies did not respond to host blends at concentrations eliciting maximum levels of upwind flight in parent flies. In fact, upwind orientation occurred only when 10 \times doses (2000 vs. 200 μ g) of blends were added to the rubber septum dispensers or when parental host blends were combined. Both these conditions induced arrested upwind flight in the parent populations. The significant reduction in F_1 hybrid host volatile response could provide a postzygotic barrier to gene flow isolating *R. pomonella* flies, rendering them behaviorally sterile in locating potential host plants (Linn et al. 2004; Dambroski et al. 2005; Forbes et al. 2005; Feder and Forbes 2007, 2008).

Analysis of peripheral chemoreception in F_1 hybrids revealed a similarly unique result (Olsson, Linn, Michel, et al. 2006). In all F_1 hybrid crosses tested between apple, hawthorn, and dogwood host populations, distinct ORN response profiles were found that were absent from any parent population. Many parent generation cells responded to single host volatiles, whereas several F_1 hybrid cells responded to those compounds as well as other structurally unrelated host compounds. In addition, many hybrid cells responded to unique combinations of host volatiles unseen in parent responses. It was proposed that these changes in host volatile specificity could result from misexpression of multiple receptors in hybrid neurons due to genomic incom-

patibilities in receptor–gene pathways between parent populations. In turn, these peripheral alterations could be responsible for the reduced olfactory performance in F_1 hybrid flies in flight tunnel assays. Table 1 provides a summary of behavioral and physiological phenotypes for *Rhagoletis* populations and their hybrids.

A recent study of F_2 and backcross progeny between the 3 *R. pomonella* races/species revealed a variety of behavioral responses to host odors in second-generation hybrids. Although many F_2 and backcross flies did not orient to fruit volatiles in flight tunnel assays, a significant proportion (30–65%) of second-generation hybrids showed normal parental apple, hawthorn, and dogwood fly response phenotypes (Dambroski et al. 2005). The presence of both parental and hybrid response phenotypes in a single generation (see Table 1) provides the opportunity to test whether changes in ORN physiology underlie the reduced flight tunnel behavior seen in many hybrids.

In the present study, F_2 and backcross hybrid individuals were first phenotyped for behavior via flight tunnel analyses (Dambroski et al. 2005) and the same individuals were subsequently assessed via single sensillum electrophysiology. We report that differences in peripheral chemoreception do not correlate directly with the behavioral responses of second-generation hybrids to host fruit odors. We discuss the implications of these results for understanding host preference, host fidelity, and host shifts in the *Rhagoletis* species complex.

Materials and methods

Details about insect origins, rearing, and flight tunnel analyses can be found in Dambroski et al. (2005). Methods concerning chemical stimuli and neurophysiological analyses were identical to those performed in Olsson, Linn, Michel, et al. (2006). We present a brief overview of these methods below.

Rhagoletis origins and rearing conditions

Apple and hawthorn flies were collected as larvae from fruit at Grant (MI), Fennville (MI), and Urbana (IL) during the 2002 field season, and dogwood flies were collected from Raccoon Lake (IN). All populations were reared to adulthood in the laboratory using standard *Rhagoletis* protocols as described in Linn et al. (2004) and Dambroski et al. (2005).

After overwintering fly pupae at 4 °C for 4–7 months, newly eclosing adult apple, hawthorn, and dogwood flies were mass crossed (i.e., multiple males of one host origin were crossed with multiple females from another host and vice versa) in Plexiglas cages held at constant temperature and humidity and supplied with water, food, and red delicious apples for oviposition (see Linn et al. 2004). F_1 fly puparia obtained from the apples were held at constant temperature and reared to adulthood without diapause.

Table 1 Summary of behavioral and physiological response to host volatiles among parent, F₁, F₂, and backcross hybrids in the *Rhagoletis* species complex

	Phenotype	Parent host populations	F ₁ hybrid	F ₂ and backcross hybrids
Flight tunnel behavior ^a	Response to single blend of own host volatiles	— ^b		— ^c
	Response to multiple host volatile blends	— ^d		— ^c
	Antagonism to mixtures of host blends	— ^e		— ^c
	No response to host blends at optimal parent concentrations		— ^f	— ^c
	Response to host blends at 10× optimal parent concentrations		— ^f	— ^c
	Response to mixtures of host blends		— ^f	— ^c
Peripheral physiology	5 ORN response classes among all host populations: Class A (1-octen-3-ol responders), Class B (hexyl butanoate/dihydro-β-ionone responders), Class C (4,8-dimethyl-1,3(E), 7-nonatriene/3-methylbutan-1-ol [with or without other compounds] responders), Class D (ester responders), and Class E (multiple compound responding ORNs)	— ^g	— ^h	This study
	Variation in ORN sensitivity	— ⁱ	— ^h	This study
	Unique response to combinations of volatiles found among the 5 ORN classes above		— ^h	This study

^aDefined as upwind anemotactic flight in the presence of test stimuli, see Materials and Methods for details.

^bZhang et al. (1999); Nojima, Linn, Morris, et al. (2003); Nojima, Linn, and Roelofs (2003).

^cDambroski et al. (2005).

^dLinn, Dambroski, et al. (2005).

^eLinn, Nojima, and Roelofs (2005).

^fLinn et al. (2004).

^gOlsson et al. (2006a).

^hOlsson, Linn, Michel, et al. (2006).

ⁱOlsson et al. (2006b).

Eclosing, nondiapause F₁ hybrid adults were then mated to each other or to individuals from the parent populations in single-pair crosses to produce F₂ and backcross progeny, respectively. Single-pair crosses were performed in Plexiglas cages under constant temperature and humidity, and flies were supplied with food, water, and an apple for oviposition. Resulting F₂ and backcross larvae and pupae were then reared to adulthood without diapause under constant temperature and humidity.

Sexually mature, odor-naïve F₂ and backcross adults aged 10–21 days were tested for their behavioral and electrophysiological responses to fruit volatiles. Of the 55 individuals tested in both flight tunnel and electrophysiological measurements, 49 were female. Flies can be used for behavioral analyses after 8–10 days of age, the age of reproductive maturity (Zhang et al. 1999; Nojima, Linn, Morris, et al. 2003; Nojima, Linn, and Roelofs 2003), and typically survive for up to 4 weeks. Thus, senescence was not suspected in flies up to 21 days of age. Additionally, both sexes have been found to respond similarly to fruit volatiles through numerous flight tunnel analyses regardless of age after sexual maturity, mating status, or exposure to other individuals (Linn CE Jr, unpublished observations). Previous electrophysiological

analyses also tested flies up to 20 days of age (Olsson et al. 2006a, 2006b).

Flight tunnel analyses

Details of the flight tunnel, protocols, and host volatile blends can be found in Zhang et al. (1999), Linn et al. (2003), Nojima, Linn, Morris, et al. (2003), and Nojima, Linn, and Roelofs (2003). Flight tunnel data from all F₂ and backcross hybrids used in this study were also presented as a portion of Dambroski et al. (2005) results. The flight tunnel tests were designed to behaviorally phenotype flies for quantitative trait loci analysis. All individuals were tested to host blends from both grandparents at 200 μg. For these assays, each fly from apple × hawthorn and apple × dogwood crosses was tested 6 times to both volatile blends over a 2-day period. Hawthorn × dogwood crosses were tested 9 times. A subset of apple × hawthorn crosses were also tested at a higher 2000 μg host blend concentration and a proportion of apple × hawthorn and apple × dogwood cross individuals to the combined host blends of the grandparents at the 2000-μg dose.

Flies were considered responders if they displayed upwind anemotactic flight over 1 m to the source in at least half of the trials. Nonresponders remained at the release point for the

1-min trial period and/or flew briefly in an undirected manner to the side of the tunnel for all trials. For the 2000- μg dose, only a single trial was conducted for each fly to the respective grandparent host blends or combined blends.

Parent *Rhagoletis* populations generally displayed 3 basic flight tunnel behaviors (see Table 1): 1) response to a single blend containing their own host volatiles (Zhang et al. 1999; Nojima, Linn, Morris, et al. 2003; Nojima, Linn, and Roelofs 2003), 2) response to both their own and another host volatile blend (Linn, Dambroski, et al. 2005), or 3) antagonistic response to mixtures of blends (Linn, Nojima, and Roelofs 2005). Conversely, F_1 hybrid crosses between parent populations displayed 1 of 3 alternate flight tunnel behaviors (Linn et al. 2004): 1) no response, 2) response to blends at high concentrations, and 3) response to mixtures of blends. F_2 and backcross hybrids studied here displayed one of the 6 basic types of parent or hybrid flight tunnel behaviors when presented with the various stimuli (Dambroski et al. 2005). Thus, second-generation hybrids could be classified dichotomously as “parent” or “hybrid” behavioral phenotypes.

Electrophysiological analyses

Stock solutions (1 $\mu\text{g}/\mu\text{l}$) of individual key volatiles and blends (see Table 2) were prepared in hexane and 10 μl pipetted onto filter paper in disposable Pasteur pipettes. Blank stimuli containing 10 μl hexane and dilutions of each host compound at 1, 10, and 100 $\text{ng}/\mu\text{l}$ were also prepared.

An electrolytically sharpened tungsten electrode was used to contact ORNs and another sharpened electrode inserted in the eye as a ground. Recordings were performed with an electrophysiological recording unit containing micromanipulators and an amplifier (Syntech INR-5, Hilversum, The Netherlands).

Table 2 Volatiles used for electrophysiological analyses determined through GC-EAD and behavioral assays of host fruit (from Zhang et al. 1999; Nojima, Linn, Morris, et al. 2003; Nojima, Linn, and Roelofs 2003)

	Host volatiles	Abbreviation	Source
1	1-Octen-3-ol	O3OL	Dogwood (<i>Cornus florida</i>)
2	Dihydro- β -ionone	DBI	Hawthorn (<i>Crataegus</i> spp.)
3	Hexyl butanoate	HB	Apple (<i>Malus pumilla</i>)
4	4, 8-Dimethyl-1, 3(E), 7-nonatriene	DNT	Hawthorn
5	Propyl hexanoate	PrH	Apple
6	Butyl butanoate	BB	Apple
7	Pentyl hexanoate	PeH	Apple
8	Butyl hexanoate	BH	Apple/hawthorn
9	Ethyl acetate	EA	Hawthorn/dogwood
10	3-Methylbutan-1-ol	3MB	Hawthorn/dogwood
11	Isoamyl acetate	IAA	Hawthorn/dogwood

A constant flow of filtered and humidified air passed over the antenna via a stimulus air controller (Syntech CS-5). The test pipettes were attached to the controller, which generated 0.5-s stimulus puffs into the air stream. The analog signal from the ORNs was amplified, sampled, and filtered via USB-IDAC connection to a computer and action potentials extracted as digital spikes using Syntech Auto Spike v. 1.1–3.2 software. Each contacted ORN was first screened with the fruit blends and the blank at 10- μg stimulus loading. If the neurons responded to one or more of the blends, then all 11 components of the blends were tested individually at a 10- μg stimulus loading. Those compounds eliciting responses were subsequently tested in dose–response trials (10- and 100-ng and 1- and 10- μg stimulus loads) to determine each cell’s sensitivity to those chemicals.

Data analysis

A total of 189 ORNs from 55 individuals among the various F_2 and backcross populations were used for neurophysiological analyses (Table 3). Responses to host stimuli were determined from spike counts through statistical comparison to the blank trials as described in Olsson, Linn, Michel, et al. (2006) and Olsson et al. (2006a, 2006b). Response thresholds to host stimuli were determined by dose–response trials (10 ng–10 μg) and calculated as the lowest concentration eliciting a statistically significant spike frequency over the mean of the blank trials. Sensitivities were assigned as reciprocals of the threshold values (e.g., 10 ng threshold = 10 000, 100 ng = 1000, 1 μg [1000 ng] threshold = 100,

Table 3 Second-generation *Rhagoletis* hybrids used for coupled behavioral and electrophysiological analyses

Pedigree		<i>n</i>	ORNs sampled
Female	× Male		
Apple	Hawthorn × apple	2	5
Hawthorn × apple	Apple	2	8
Apple × hawthorn	Hawthorn	1	2
Hawthorn	Hawthorn × dogwood	3	11
Hawthorn	Dogwood × hawthorn	3	11
Hawthorn × dogwood	Hawthorn	1	3
Dogwood	Dogwood × hawthorn	1	3
Apple × hawthorn	Apple × hawthorn	5	17
Apple × hawthorn	Hawthorn × apple	11	34
Apple × dogwood	Dogwood × apple	1	5
Hawthorn × apple	Apple × hawthorn	10	31
Hawthorn × apple	Hawthorn × apple	5	19
Hawthorn × dogwood	Hawthorn × dogwood	7	28
Dogwood × hawthorn	Dogwood × hawthorn	3	12

and 10 μg [10 000 ng] threshold = 10). To statistically analyze sensitivities, Mann–Whitney tests were used to compare specific behavioral phenotypes with each other. Both were performed via SPSS version 11.0 and 12.0 software graphs were generated using SPSS version 11.0 and 12.0 software.

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 2) was compared with the ORN response patterns to 10 μg of the same compounds in Olsson et al. (2006a). This previous study found that parent population ORNs could be grouped into 5 major classes, ranging from single to multiple compound responding ORNs: Class A (1-octen-3-ol responders), Class B (hexyl butanoate/dihydro- β -ionone responders), Class C (4,8-dimethyl-1, 3(*E*),7-nonatriene/3-methylbutan-1-ol [with or without other compounds] responders), Class D (ester responders), and Class E (multiple compound responding ORNs). If an ORN responded to the same array of host compounds as ORNs found in this parent population study (Olsson et al. 2006a, Table 1), it was considered “parent like” and labeled with the appropriate ORN class (A–E). ORNs that exhibited response patterns similar to those in the hybrid study (Olsson, Linn, Michel, et al. 2006, Table 1) or displayed unique response patterns not found in any parent classification were determined to be “hybrid like” according to the statistical analyses outlined below.

For statistical comparison of ORN responses, parsimony networks depicting the interrelationship of single-cell response patterns for parents (Olsson et al. 2006a, 2006b), F_1 hybrids (Olsson, Linn, Michel, et al. 2006), and F_2 and backcross hybrids to the 11 volatile compounds tested in the study were constructed using the program TCS v 1.13 as in Olsson, Linn, Michel, et al. (2006). Parsimony networks establish a graphical comparison of phenotypes across an entire population, allowing for multidimensional comparison of response patterns between various populations. Significant connections between different response patterns based on parsimony were limited to 1 step due to the low number of sites (compounds). Each connector therefore signifies a difference in response pattern of one compound. Consequently, the graphical pattern created by the network can be compared among groups to determine the degree of variation within and between populations (see Olsson, Linn, Michel, et al. 2006 for detailed explanation). In order to connect all response patterns, the maximum number of connections was set to 4 and networks are shown without breaking reticulations.

Nearest neighbor distances (NNDs) were calculated as a metric to describe the degree of similarity between neuron response patterns for parent and hybrid flies (see Olsson, Linn, Michel, et al. 2006 for detailed methodology). NNDs measure the degree of dispersion within a population for a particular phenotype. Thus, low NND values indicate a high degree of similarity in response patterns within a particular population. Data for parent and F_1 response profiles

were obtained from Olsson, Linn, Michel, et al. (2006) and Olsson et al. (2006a), respectively. To determine the NND for a neuron, the neuron’s response to the suite of compounds tested was coded as a series of 1’s and 0’s depending upon whether the volatiles did (1) or did not (0) induce a statistically significant neuronal response. 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.

Mean NND values were assessed through Monte Carlo simulated parametric bootstrapping (Olsson, Linn, Michel, et al. 2006). Mean NND values were then calculated between the simulated data sets and a probability value (*P* value) estimated as the number of times in 10 000 simulation trials that an NND 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 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.

Results

F_2 and backcross flight tunnel behavior

Each of the 55 second-generation hybrids (Table 3) could be classified dichotomously as displaying parent-like or hybrid-like behavioral phenotypes (see Materials and Methods). Supplementary Table 1 online lists the corresponding flight tunnel behavior and response profiles for the 189 ORNs recorded from these 55 F_2 and backcross hybrid individuals.

Comparison of parent, first-, and second-generation ORN response profiles

F_2 and backcross ORNs displayed profiles similar to the 5 basic parent classes (Olsson et al. 2006a), as well as diverse profiles comparable to those found in F_1 hybrid individuals (Olsson, Linn, Michel, et al. 2006). Parsimony networks were constructed for second-generation ORN responses depicting the relationships of neuron response patterns to the 11 fruit volatile compounds tested in the study (Figure 1). Separate networks were established for ORN responses from flies exhibiting parent (Figure 1A) or hybrid (Figure 1B) flight tunnel behaviors in order to graphically compare differences in response patterns between the 2 behavioral phenotypes. Importantly, the analysis shows that F_2 and backcross flies exhibiting both behavioral phenotypes possessed ORNs with response profiles similar to the 5 basic parent classes (note the presence of large A–D and “P” labeled nodes in both Figure 1 diagrams), as well as diverse profiles comparable

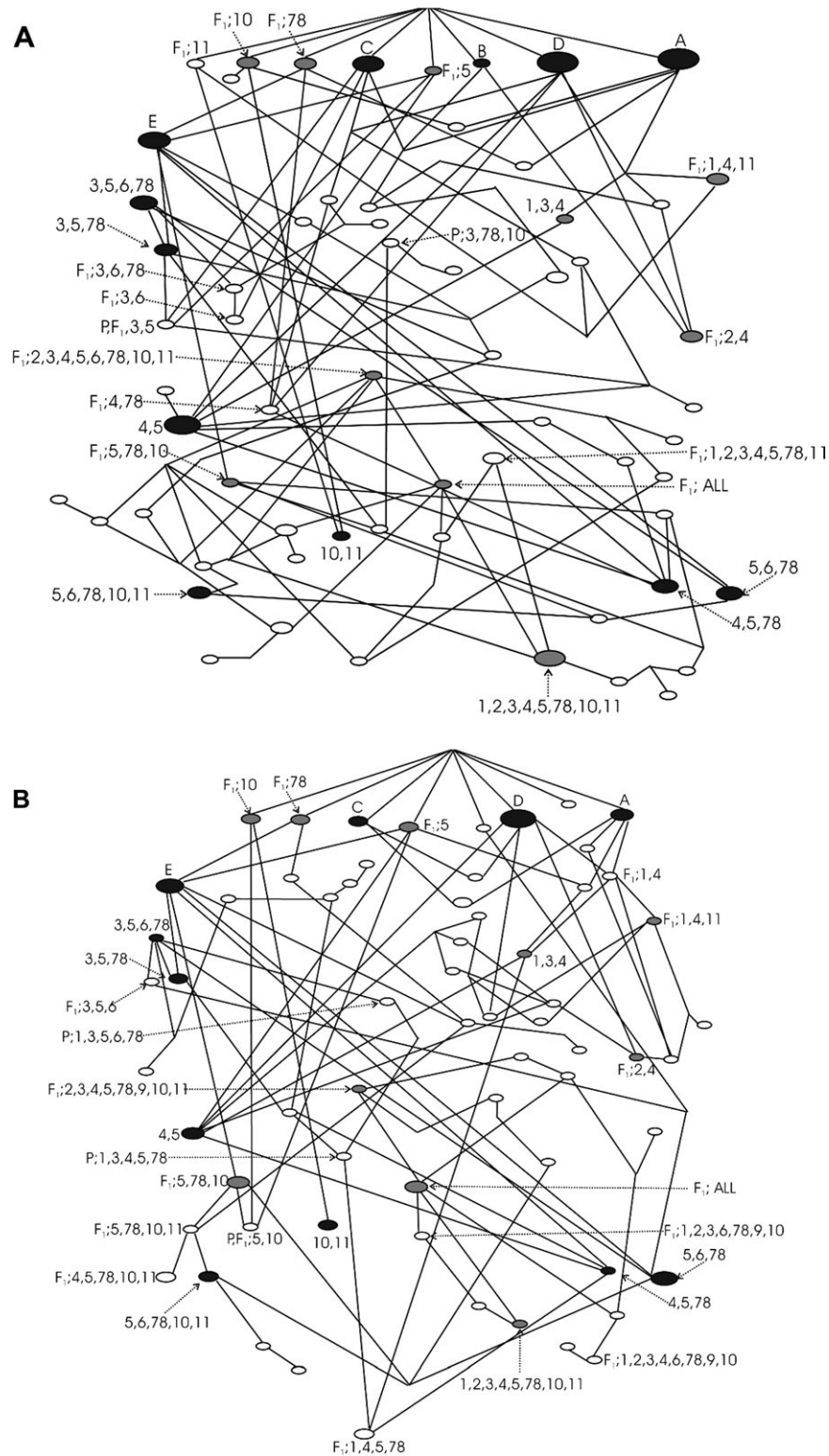


Figure 1 Most parsimonious TCS network depicting the relationships among ORN response patterns to the 11 tested fruit volatile compounds for **(A)** $F_2/$ backcross parent-like flight tunnel behavior (F_2/BC P) and **(B)** $F_2/$ backcross hybrid-like flight tunnel behavior (F_2/BC H) populations of flies. Each oval node represents a different response pattern observed in the F_2/BC P or F_2/BC H neuron population. Dark colored nodes indicate response patterns shared among parent, F_1 hybrid, F_2/BC P, and F_2/BC H populations of flies. The 5 dark nodes at the top of each diagram designated with upper case letters A–E represent the 5 general response categories identified in the parent population (Olsson et al. 2006a). Gray shaded nodes indicate a response pattern shared between F_2/BC P and F_2/BC H flies. White nodes without a letter designation are unique to either the F_2/BC P (A) or F_2/BC H (B) population. White nodes designated with P

to those found in F_1 hybrid individuals. The gray nodes in Figure 1 indicate a number of response profiles shared between the 2 F_2 and backcross behavioral phenotypes. The figure suggests significant overlap in ORN host volatile response among flies regardless of their flight tunnel behavior. Furthermore, there was no significant difference in the percentage of parent-like ORN profiles from second-generation hybrids exhibiting parent-like versus hybrid-like behavioral phenotypes. The mean number of parental type ORNs for hybrid-like second-generation hybrids was 35.2% (32/91) compared with 38.8% (38/98) for parent-like F_2 and backcross flies (G -heterogeneity = 0.264, $P = 0.608$, 1 degree of freedom [df]). The distribution of the number of parental type ORNs for all behavioral classes of F_2 and backcross hybrid flies for which 4 or more neurons were measured did not deviate significantly from a binomial distribution with mean 0.3705 ($\chi^2 = 3.64$, $P = 0.457$, 4 df). F_2 and backcross flies exhibiting either parent or hybrid behavioral phenotypes also displayed similar distributions in the number of parental ORNs in individuals for which 4 or more neurons were assayed (G -heterogeneity for difference in behavioral categories = 1.79, $P = 0.62$, 3 df).

Mean NND values (Figure 2) calculated as a comparison between parent versus second-generation hybrid population ORNs for both behavioral phenotypes (0.878 and 0.835, respectively) were highly significant ($P \leq 0.0001$) and much greater than values estimated in the reverse direction between second-generation hybrids versus parents (mean NND = 0.429 and 0.416, respectively). The higher NND value for the parent versus F_2 /backcross hybrid generation comparison reflects the large proportion of unique neuron response patterns present in F_2 /backcross flies that were not seen in parent flies. In contrast, the relatively low and nonsignificant mean NNDs for the reciprocal second-generation versus parent comparisons were due to the majority of neuron response patterns measured in the parent population having counterparts or close companions in second-generation hybrid populations displaying both behavioral phenotypes.

The mean NND was also significant ($P \leq 0.05$; Figure 3) for comparisons between F_1 versus F_2 /backcross hybrids for both parent and hybrid second-generation behavioral phenotypes (0.490 and 0.506, respectively), as well as between the 2 second-generation behaviors themselves (0.582). These results imply that a significant proportion of ORN response profiles were unique to each of these categories of flies and neither shared between F_1 and F_2 /backcross populations

nor between F_2 /backcross parent versus hybrid behavioral phenotypes.

Comparisons of F_2 and backcross ORN sensitivities with behavior

Considerable variation in ORN threshold sensitivity was observed for each type of flight tunnel response (Figure 4). There were few significant differences in ORN host volatile sensitivity between second-generation flies exhibiting parent or hybrid behavioral phenotypes (see Materials and Methods for complete list of behaviors). Only flies exhibiting response to high concentrations (a hybrid behavioral phenotype; last box plot) were significantly more sensitive to hawthorn volatiles than flies responding to the hawthorn blend (a parent behavioral phenotype; third box plot). However, second-generation hybrids that responded to the apple blend (second box plot) were significantly more sensitive to apple volatiles than flies that responded to the hawthorn blend (third box plot). Flies that responded to both hawthorn and apple blends (fifth box plot) were significantly less sensitive to apple volatiles than flies that displayed all other possible behaviors. Finally, flies that responded to both hawthorn and dogwood blends (the sixth box plot) were significantly less sensitive to both hawthorn and dogwood volatiles than flies that exhibited other behaviors.

Discussion

There is mounting evidence for a direct link between olfactory host fruit location and speciation in the *R. pomonella* complex. Yet, critical questions concerning the physiological basis for this behavior remain. In the present study, we were able to couple physiological analyses with behavioral assays for individual flies. F_2 and backcross hybrid populations possessed ORNs with response profiles identical to cells contacted in the apple, hawthorn, and dogwood parent populations (Olsson et al. 2006a), as well as cells that responded with unique profiles similar to those observed for F_1 hybrids (Olsson, Linn, Michel, et al. 2006). In addition, we found no significant difference in the proportion of ORN phenotypes among flies exhibiting parent- or hybrid-like behaviors in flight tunnel behavior assays (G -heterogeneity for difference in behavioral categories $P = 0.62$, not significant, see Results). As a result, the presence of parent-like cells does not automatically ensure normal, parent population behavior. Nevertheless, the presence of hybrid-like cells with broad

and/or F_1 indicate a response pattern shared by a second-generation hybrid with the parent and/or F_1 hybrid population of flies, respectively. Numbers associated with the nodes indicate the ORN response profile according to the numbered compounds listed in Table 2. We give these response profiles only for ORN response patterns shared among populations. The sizes of oval nodes reflect the relative proportions of the different neural response patterns observed in the test population ($n = 98$ neurons recorded for F_2 /BC P flies, $n = 91$ for F_2 /BC H flies). Shared neurons are anchored in the same positions in A and B to provide reference points for comparing the networks. The number of straight-line segments connecting 2 nodes indicates 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 due to the high positive correlation in neuron response between the 2 compounds.

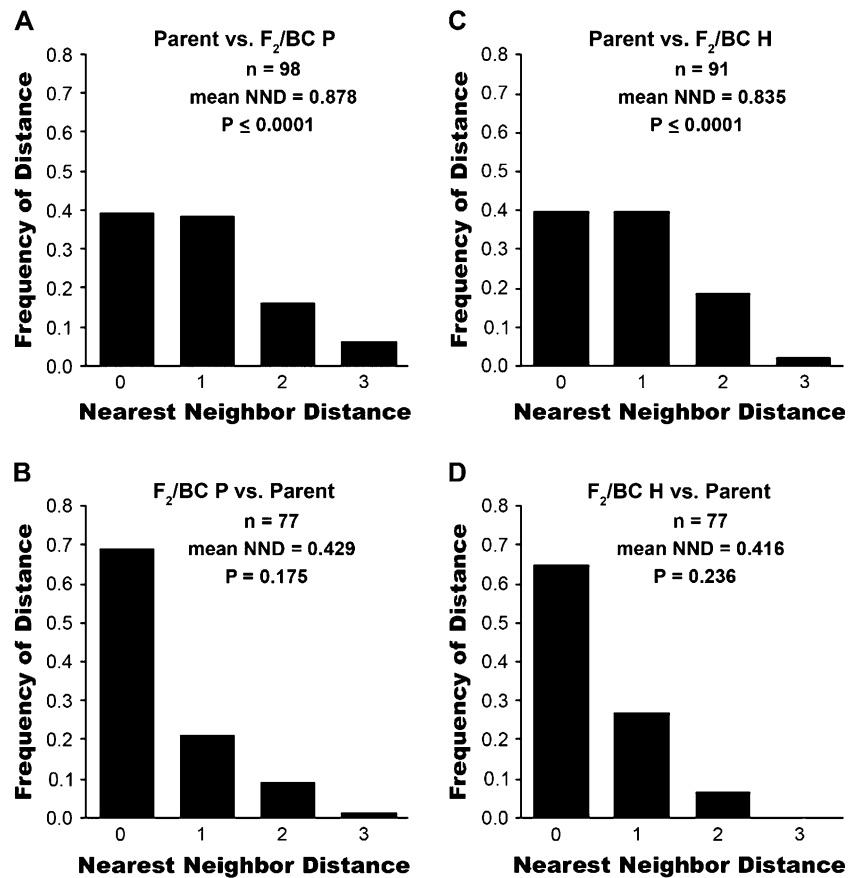


Figure 2 Histograms of NNDs calculated between neuron response patterns observed in reference (first) versus comparison (second) populations for (A) parent versus F₂ and backcross flies with parent-like flight tunnel behaviors (F₂/BC P), (B) F₂/BC P versus parent flies, (C) parent versus F₂ and backcross flies with hybrid-like flight tunnel behaviors (F₂/BC H), and (D) F₂/BC H versus parent flies. Also given are mean NNDs for each comparison (mean NND) and the probability level (*P* value) for the mean NND as determined by Monte Carlo parametric bootstrapping. *P* values indicate the proportions of randomly drawn parent and F₂ 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.

specificity was suggested to be a key factor contributing to reduced olfactory performance in hybrid flies (Olsson, Linn, Michel, et al. 2006). If this is true, then any fly possessing hybrid-like cells should be olfactorily compromised and be unable to elicit normal, parental behaviors. In the present study, however, we found a number of second-generation individuals that displayed normal parental behavior despite significant alterations in peripheral coding as compared with parent generations (Figures 1–3).

The presence of such diverse ORN profiles in *Rhagoletis* hybrids and relative lack of behavior in F₁ hybrids (Linn et al. 2004) indicate a significant departure from prior studies of the inheritance of chemical communication systems. Previous studies have revealed 4 basic characteristics of hybrid pheromone receptor neurons (Olsson, Linn, Michel, et al. 2006): 1) hybrids can possess different proportions of parental ORN types (*Ips pini*; Mustaparta et al. 1985); 2) hybrid ORNs can resemble a single parent (*Agrotis ipsilon* × *Agrotis segetum*; Gadenne et al. 1997); 3) hybrid ORNs can possess intermediate amplitudes (Roelofs et al. 1987) and spike frequencies

from parent ORNs (Cossé et al. 1995) (2 pheromone races of *Ostrinia nubilalis*); and 4) hybrid ORNs can possess a variety of parent and also “atypical” responses (*Ctenopseustis obliquana* × *Ctenopseustis* spp., Hansson et al. [1989]; *Heliothis subflexa* × *Heliothis virescens*, Baker et al. [2006]). In both *Ostrinia* (Roelofs et al. 1987) and *Ctenopseustis* (Foster S in Löfstedt 1990; Foster et al. 1996), second-generation flight tunnel behavior and ORN response to pheromone components indicated a segregation of alleles for behavior and physiology between parent populations. In *Agrotis*, both first- and second-generation hybrids followed dominance for one parent, with physiological and behavioral studies corresponding to that of a single-parent population (Gadenne et al. 1997). The lack of correlation between physiology and behavior among *Rhagoletis* generations indicates a complex genetic and neuronal basis for host volatile preference.

The most straightforward explanation for the appearance of aberrant ORN profiles in second-generation *Rhagoletis* flies exhibiting normal, parental behaviors is that aberrant response profiles have no effect on behavior. Yet, some of

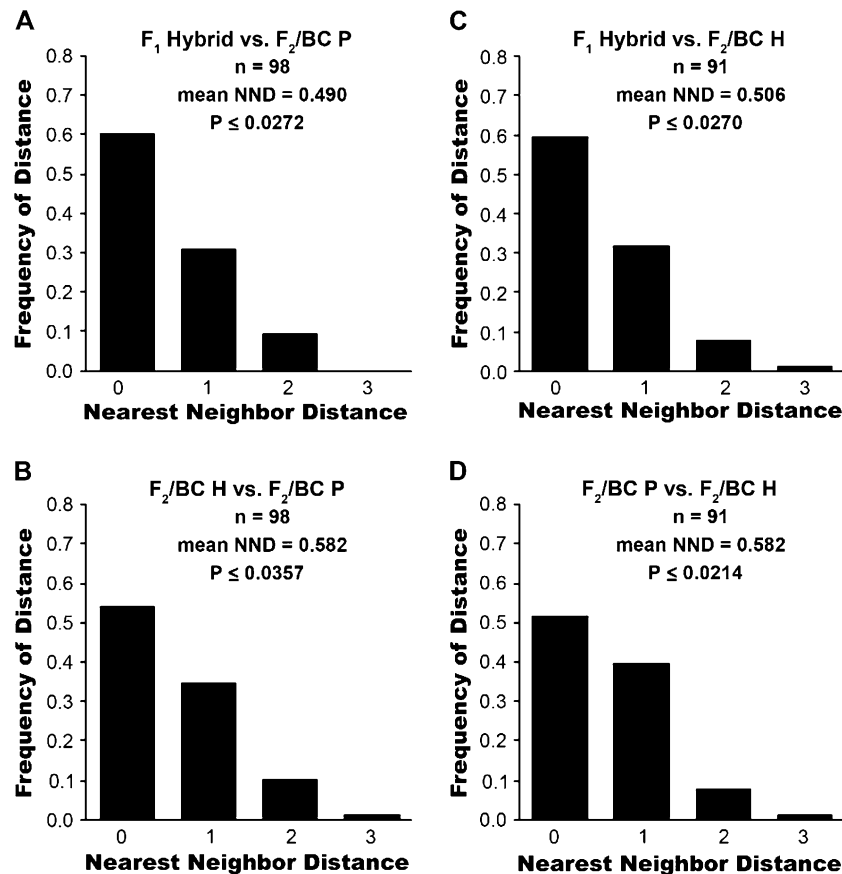


Figure 3 Histograms of NNDs calculated between neuron response patterns observed in reference (first) versus comparison (second) populations for (A) F₁ hybrid versus F₂ and backcross flies with parent-like behaviors (F₂/BC P), (B) F₂/BC H versus F₂/BC P flies, (C) F₁ hybrid versus F₂ and backcross flies with hybrid-like behaviors (F₂/BC H), and (D) F₂/BC P versus F₂/BC H. Also given are mean NNDs for each comparison (mean NND) and the probability level (*P* value) for the mean NND as determined by Monte Carlo parametric bootstrapping. See Figure 2 and Materials and Methods for details concerning calculation of *P* values.

the ORNs with broadened specificities responded to both antagonistic and agonistic compounds concurrently. How could ORNs responding to volatiles with conflicting behavioral signatures have no impact on the processing of host odorants and behavior?

With a convergence of approximately 2000:1 from peripheral to central cells (Shepherd 1993), it is conceivable that the central nervous system can integrate the diverse signals received by hybrid cells and still recognize the appropriate signal provided that a fly possesses more than some limited, threshold number of “normal” parent-type ORN response profiles. The replication of signaling pathways for specific compounds among several ORNs is considered an important aspect of olfaction. Redundancy may compensate for loss or injury to peripheral cells (Shepherd 1993), may buffer against olfactory mutations and allow for adaptation (Fishilevich et al. 2005), and may contribute to “hyperacuity,” where a weak signal can be recognized in a noisy environment (Ramen and Stopfer 2007). It has been shown that insects can still exhibit appropriate behavior to olfactory signals even with a significantly altered detection system. For example, *Drosophila lar-*

vae with only a single functional neuron could still chemotax toward a number of stimuli (Fishilevich et al. 2005), and *Manduca sexta* females with significantly altered antennal lobes were still able to perform anemotactic upwind flight to host volatiles (Willis et al. 1995). The peripheral redundancy of host volatile signals in *Rhagoletis* second-generation hybrids may allow them to exhibit appropriate host preference even with significant alterations in peripheral coding. Interestingly, a high proportion of second-generation *Rhagoletis* flies did not display a significant reduction in sensitivity during flight tunnel tests (Dambroski et al. 2005), even though they possessed significantly altered ORN response profiles. Host location in the face of relatively massive alterations in ORN response patterns may indicate the necessity to alter current dogmas regarding the relationship between ORN physiology and olfactory behavior.

If these peripheral alterations have no impact on behavioral response to host volatiles, then why were first-generation *Rhagoletis* hybrids unable to orient to host volatiles at concentrations eliciting maximal parent fly behavior (Linn et al. 2004)? One possibility suggests that the physiological

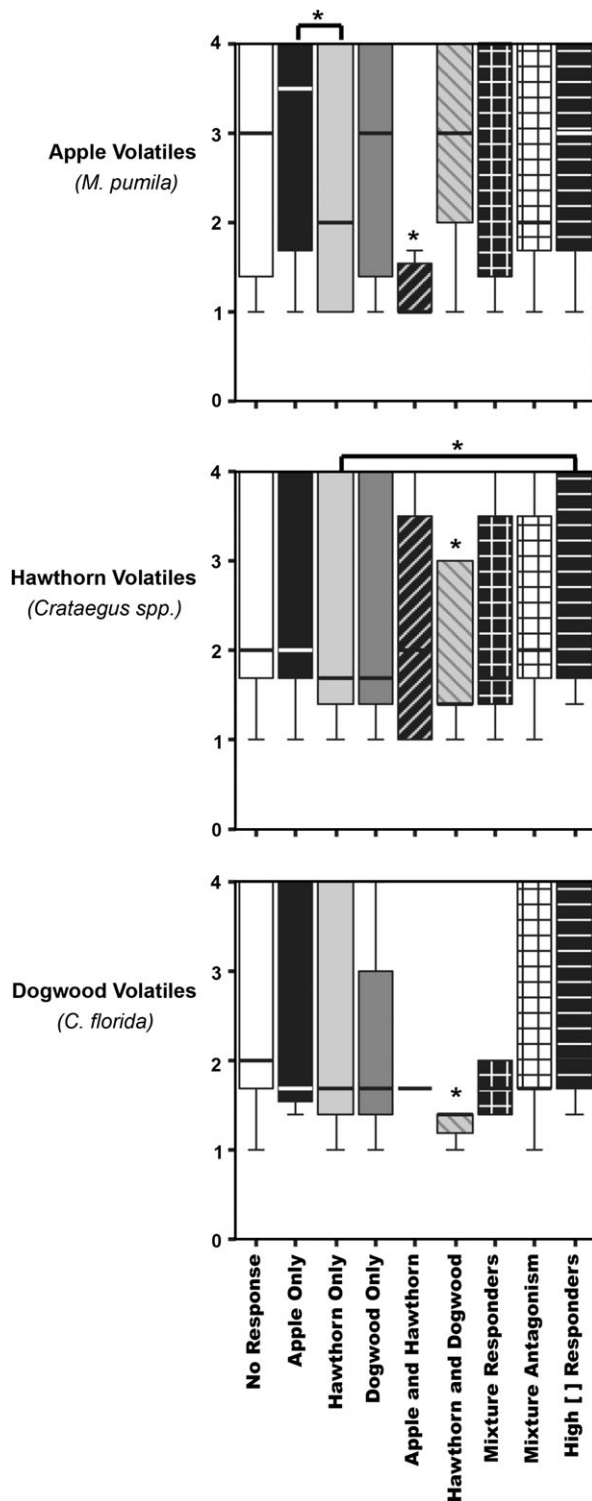


Figure 4 Box plots with whiskers depicting F_2 and backcross ORN sensitivities versus flight tunnel behavior for the 3 volatile blends used in the study (Table 2). ORN threshold responses are divided on the basis of individual flight tunnel response to host volatiles, regardless of pedigree, and each graph compares ORN sensitivities from individuals displaying the behaviors listed on the x axis. Sensitivities are depicted as the log reciprocal of the threshold + 1 (see Materials and Methods). Bars above each graph indicate significant differences ($P < 0.05$).

alterations of F_1 hybrids might be unrelated to their behavioral deficits or may be a side effect of other alterations. Comparison of central physiology between first- and second-generation hybrids could reveal significant alterations in antennal lobe connectivity in F_1 hybrids that was resolved in F_2 and backcross hybrids exhibiting normal behavior. Changes in connectivity may lead to corresponding changes in host volatile functionality that could significantly alter agonist/antagonist pathways.

It is also possible that the broadened specificities witnessed in *Rhagoletis* hybrid ORNs could affect behavior in some individuals, whereas leaving host location in other individuals remains intact. In *Rhagoletis*, several behaviorally relevant host volatiles are detected by multiple ORN classes, suggesting that behaviorally active blends are also perceived in a combinatorial fashion (Olsson et al. 2006a). Because multiple volatile combinations are used, it is possible that only certain combinations of volatiles affect the perception of host blends and subsequent behavior. If genomic incompatibilities in receptor–gene pathways between parent *Rhagoletis* host populations induce random, multiple receptor expression in hybrids (Olsson, Linn, Michel, et al. 2006), then certain receptor combinations with conflicting behavioral relevance may interrupt combinatorial coding in the central nervous system and contribute to the drastic reduction in behavior seen in F_1 hybrids (Linn et al. 2004). In other words, combinations of certain key compounds with direct antagonistic or agonistic properties (i.e., butyl hexanoate, 1-octen-3-ol) could affect behavior, whereas other combinations, though unique to hybrid crosses, are benign. In particular, combinations of chemicals with contrasting behavioral functionality and distinct ORN response profiles in the parent populations (i.e., esters with 4,8-dimethyl-1,3(*E*),7-nonatriene, dihydro- β -ionone, and/or 1-octen-3-ol) might produce confused input to central processing centers when they stimulate the same ORNs in hybrid individuals. Other combinations may not correlate to altered behavior because ORNs responding to these compounds do not innervate contrasting behavioral pathways. Thus, their combination in a single ORN would not produce conflicting input in hybrid individuals. Further testing, including a greater survey of the hybrid antenna as well as physiological testing of central processing in both parents and hybrids is required to test this hypothesis.

Another characteristic distinguishing *Rhagoletis* ORNs is their threshold sensitivity to host components. Studies in other Dipterans, including *Culex* and *Anopheles* mosquitoes as well as *Drosophila* flies, have suggested a link between peripheral sensitivity and behavior (Olsson et al. 2006b). Here, we found few significant differences in ORN sensitivity between flies exhibiting parent or hybrid flight tunnel behaviors (Figure 4). Nevertheless, there was a significant correlation between ORN sensitivity and behavior among second-generation flies that corresponded to previous findings from parent populations. ORNs from second-generation hybrid

flies attracted to the apple blend in flight tunnel analyses were significantly more sensitive to apple volatiles than ORNs from flies responding to the hawthorn blend (Figure 4). This result concurs with the study of apple and hawthorn-origin parent populations, where apple-origin flies displayed a tendency toward higher sensitivity to apple volatiles than hawthorn flies (Olsson et al. 2006b). Figure 4 also shows that ORNs from dogwood blend responders were sensitive to apple volatiles and rather insensitive to dogwood volatiles (although this was not significant). This is similar to what was found with dogwood-origin flies in the parent population study (Olsson et al. 2006b). Finally, ORNs from flies responding to multiple blends (i.e., apple and hawthorn or hawthorn and dogwood) were significantly less sensitive to certain host volatiles, a phenomenon predicted in the parent population study (i.e., that less sensitive flies might be more accepting to alternate hosts; Olsson et al. 2006b). This loss in sensitivity could be a source for the host-shifting process and allow sympatric speciation to ensue among these populations. However, sensitivities cannot explain the reduced behavioral response of F_1 hybrids nor those second-generation hybrids that did not orient to fruit blends in the flight tunnel. The box plots further show a wide range of sensitivities as found in all previous studies, indicating that a narrow range of sensitivity is not obligatory for behavioral response.

Conclusions

The present study endeavored to validate previous claims concerning the effect of peripheral chemoreception on *Rhagoletis* olfactory host location. Our results did not support a significant correlation between alterations in ORN response profiles among second-generation flies and flight tunnel behavior. Further studies examining the morphology and physiology of antennal lobe neurons are imperative to understand not only how host volatiles are processed but also if these varied inputs from the periphery affect olfactory behavior. Additionally, the identification of olfactory receptor genes in *Rhagoletis* will allow us to examine the expression and/or misexpression of these receptors at the periphery. The results of this study imply that the basis for olfactory behavior and divergence in preference between various *Rhagoletis* populations has a complicated genetic and neuronal basis that cannot be classified through the sampling of 10s, or in this case even 100s, of peripheral cells. Our study also emphasizes the fascinating and complex basis for the evolution of host-specific chemoreception. Even rapid alterations in behavior, such as those in *Rhagoletis*, may be due to complex and multimodal changes in physiology.

Supplementary material

Supplementary Table 1 can be found at <http://www.chemse.oxfordjournals.org/>.

Funding

National Science Foundation (DEB-9977011, DEB-04-45353, DEB-06-14252, Integrated Research Challenges); U. S. Department of Agriculture (National Research Initiative); state of Indiana 21st Century fund (to J.L.F.); Cornell Science Inquiry Partnerships (to S.B.O.).

Acknowledgements

We thank K. Filchak, R. Harrison, C. Musto, R. Oakleaf, K. Pelz, K. Poole, H. Reissig, J. Roethele, C. Smith, L. Stelinski, U. Stolz, F. Wang, F. Wang Jr, B. Westrate, J. Wise, X. Xie, the Niles (MI) United State Department of Agriculture facility, and the Geneva Fly Rearing Center at the New York State Agricultural Experiment Station.

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Accepted August 4, 2008