Rhagoletis sibling species and host races differ in host odor recognition

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Abstract

Electronantennograms (EAG) were recorded from the apple and hawthorn host race of the apple maggot fly, Rhagoletis pomonella (Walsh), and from the blueberry maggot fly, R. mendax (Curran) (Diptera: Tephritidae), in response to host fruit extracts and nine volatile host fruit odor compounds at six concentrations. Mean relative EAG response to apple odor is the same in both species, but in respect to blueberry odor, it is significantly stronger in R. mendax than in both host races of R. pomonella (P < 0.05), indicating that antennal sensitivity is selectively adapted to species specific host fruit odors. Differences in antennal response to several host fruit odor compounds were found between both species as well as between the host races. This indicates differences in antennal receptor cell types and/or numbers between species and host races. The flies had no prior host fruit experience which indicates that the measured differences are genetically based. Because Rhagoletis fruit flies are highly host specific parasites which meet and mate on their respective host plants, the results suggest that antennal sensitivity plays an important role in host shifts and speciation in this genus.

Introduction

Odors are among the primary cues used by many parasitic insects for locating and accepting hosts. Differential host preference between sibling species may often be due to differences in the odor recognition system either at the peripheral level, in the way the central nervous system processes the same peripheral information, or a combination of both. Several studies suggest that differential odor recognition may be due in part to peripheral differences and that only one or a few gene(s) is responsible for their inheritance (Falk & Atidia, 1975; Venard & Pichon, 1984; Arora et al., 1987; Lilly & Carlson, 1989). Because many parasites mate only on their hosts, odor cues serve as important reproductive isolating mechanisms. The underlying genetic mechanism for host odor recognition has, therefore, an important function in the speciation process in host specific parasitic insects.

Fruit flies of the genus Rhagoletis are host specific phytophagous insects. Males and females use the host plant as a rendezvous site for courtship and mating (Bush, 1969; Prokopy et al., 1971). R. pomonella larvae feed primarily on hawthorns (Crateagus spp.) and domestic apples (Malus pumila), whereas larvae of its closely related sibling species, R. mendax, feed on blueberries (Vaccinium spp.) and huckleberries
(Galyussacia spp.). The host shift of *R. pomonella* to introduced domestic apple occurred only about 150 years ago (Walsh, 1867) and resulted in the genetic differentiation of co-occurring partially reproductively isolated host races of *R. pomonella* feeding on apples and hawthorns (Feder et al., 1988; McPheron et al., 1988).

Host plant chemicals are among the most important specific cues for host plant finding and acceptance in the behavioral repertoire of *Rhagoletis* species (Prokopy et al., 1973; Fein et al., 1982; Carle et al., 1987; Averill et al., 1988). Bierbaum and Bush (1988, 1990) showed that *R. pomonella* laid significantly more eggs on artificial fruits treated with apple fruit extracts than on those treated with blueberry fruit extract and vice versa for *R. mendax*. In their experiments, oviposition by *R. pomonella* flies was also elicited by a blend of 7 apple esters (identified by Fein et al., 1982). Field experiments with *R. pomonella* showed that apple odors resulted in significantly higher trap catches in baited trees compared to trees without apple odor source (Prokopy et al., 1973). Further investigations revealed that this species utilizes chemical as well as visual stimuli during long-range orientation to the host plant, but that the flies use only chemical cues to discriminate between host and non-host fruit (Prokopy & Roitberg, 1984). Furthermore, Fein et al. (1982) demonstrated that electroantennogram (EAG) responses of *R. pomonella* to esters emitted by apples correspond to behavioral host finding responses in this fly measured in wind tunnels. Their results indicate that host odor perception through the antennae serves as a primary cue in host finding in *Rhagoletis* species.

Because there are clear qualitative and quantitative differences in the chemical composition of the fruit volatiles of blueberries and those of apples and hawthorns (P. Silk, pers. comm.; Parliment & Kolor, 1975) and to a lesser extent between apples and hawthorns (Carle et al., 1987) peripheral sensitivity to these odors may affect host recognition. In this paper, we establish that differences in peripheral sensitivity exist between closely related *Rhagoletis* sibling species and host races, and we suggest that genetically based changes in peripheral sensitivity have played an important role in the evolution of host races and species in this genus of flies.

**Material and methods**

We used a *Rhagoletis pomonella* laboratory strain that has been reared on apples for ca. 70 generations. Earlier studies revealed no significant differences between the laboratory strain and flies from field-collected pupae of *R. pomonella* in their electroantennogram (EAG) response to some esters extracted from apples (Averill et al., 1988; Frey, unpubl. data). The hawthorn host race of *R. pomonella* was reared from larvae infesting hawthorn bushes near East Lansing, MI, during summer 1987 and stored as pupae at −20 °C. *R. mendax* flies originated from field-collected infested blueberries of the summer 1987 harvest at a site near Sawyer, MI, and were also stored as pupae at −20 °C. After emergence, the adult flies were kept at 27 °C, 15L:9D photoperiod and provided with water and dried foodstrips of hydrolyzed yeast and brown sugar. For our experiments, we used females at the age of 9–18 days of both species and host races. Interspecific differences between the apple host race of *R. pomonella* and *R. mendax* were analyzed.

Methods for EAGs using *Rhagoletis* flies have been published by Roelofs (1977). Our experiments were conducted using a similar set-up with some modifications. We used head preparations which produced reliable results for at least 1 h. The preparations were fixed with the inner side of the right uncut antenna at a distance of 5 mm from the outlet of a glass tube (5 mm dia.) venting an air stream flowing at a constant rate of 750 ml/min over the preparation. Pure air from a compressed air tank passed through a gas purifier for cleaning and drying prior to application.

Disposable Pasteur pipettes containing a piece of filter paper (Whatman Nr. 1, 0.5 × 2 cm), to which the test compound has been applied, were used to introduce specific odors to the air stream. Each pipette was provided with a connector, a 3 cm long piece of plastic tubing (0.5 cm inner
dia.) containing a 1 cm long piece of glass tubing (0.5 cm outer dia., 0.1 cm inner dia.). This connector significantly reduced contamination and diffusion. Concentration loss from the pipettes was checked by applying ethyl pentanoate (10^{-2}, diluted vol/vol in paraffin oil), which elicits responses with a very short recovery time, at intervals of 20 s. In a series of 10 tests, a distinct decrease in response amplitude was not apparent after 25 applications and therefore, it was safe to use the pipettes for up to 15 applications. To correct for changes in the antennal responses during the experimental period, a standard (n-propyl hexanoate at a dilution of 10^{-4} vol/vol in paraffin oil) was applied at intervals of approximately 90 s.

For volatile application, we used an electronic valve (General Valve Corporation) with a pulse supply and a split air-flow system, where 70\% of the total air was rehumidified and lead through the main branch and 30\% was lead through the valve. Application of volatiles was conducted by shifting between different channels of the valve. By keeping the total amount of air in the system constant, this method eliminated the effects of mechanical and humidity stimulation. The standard application pulse in our experiments was 0.5 s. Data were sampled using a computer-based data-acquisition system with onscreen display and real-time data storage at a sampling rate of 10 Hz. The stored data were analyzed by a computer program developed by one of the authors (J.E.F.).

We analyzed the EAG responses to pentane extracts of apples and blueberries. To prepare extracts, 250 Jersey blueberries and 28 McIntosh apples, respectively, were rinsed for one hour in one liter of distilled pentane. The wash was rotary-evaporated to 100 ml and stored at -80 °C until use.

Assessment of the compound composition of the fruit extracts was not possible prior to this study. It was performed in a follow-up study using a gas chromatograph with an outlet split to an EAG preparation. For each fruit extract some compounds eliciting strong EAG responses in either or both Rhagoletis species were found (J.E. Frey, T.J. Bierbaum and G.L. Bush, in prep.). Identification of these compounds is in progress and bioassays will reveal their behavioral significance.

We therefore used literature data to select nine different compounds known to contribute to the odors of either apples (Fein et al., 1982; Dimick & Hoskin, 1983), hawthorns (Carle et al., 1987) or blueberries (P. Silk, pers. comm.; F.N. Lugemwa et al., 1989) for the analysis of EAG responses to single odor compounds. These include the esters butyl-2-methyl butanoate, hexyl butanoate, hexyl propanoate, methyl pentanoate, ethyl pentanoate, butyl hexanoate and propyl hexanoate, the alcohol 2-nonanone and the ketone 2-nonanone. Butyl-2-methyl butanoate, hexyl propanoate and methyl pentanoate are known from apples, hexyl butanoate, butyl hexanoate and propyl hexanoate from both apples and hawthorns. Ethyl pentanoate and 2-nonanol were found in both apples and blueberries, whereas 2-nonanone was only reported from blueberries. Six concentrations (from 10^{-6} to 10^{-1}, diluted vol/vol in paraffin oil) of all compounds were prepared and also stored at -80 °C until use. For the experiments, 25 µl of each concentration to be tested was applied to a filter paper placed in a disposable pasteur pipette and closed with the connector as described above.

The response to the fruit extracts is expressed as the ratio (in mV) between the blueberry and the apple response. These data were analyzed by the Mann-Whitney U test. To quantify the EAG responses to identified odor compounds, we used the ratio between the amplitude of the response to the compound and the amplitude of the response to the standard applied before and after that compound (Guerin & Visser, 1980). Data were square-root transformed and analyzed by multi-way analysis of variance (MANOVA) for differences in response to all compounds over all concentrations and by two-way analysis of variance (ANOVA) for interspecific differences in response to single compounds over all concentrations. Furthermore, differences at each concentration of each compound were analyzed using the nonparametric Kolmogorov-Smirnov test on...
the untransformed data (sample numbers in brackets).

Results

Significant differences in the response ratio to host fruit extracts exist between *R. pomonella* and *R. mendax* ($U = 240$, $p = 0.008$), indicating that antennal sensitivity to blueberry odors is relatively higher in *R. mendax* than in *R. pomonella*. However, there is considerable interindividual variation in both species (*R. pomonella* $0.63 \pm 0.12$, $N = 15$; *R. mendax* $0.76 \pm 0.13$, $N = 21$). Analysis of the frequency distribution of the EAG responses reveals that the interspecific differences are due to differences in the population means of antennal sensitivity between *R. pomonella* and *R. mendax* (Fig. 1). Furthermore, some individuals of each species show response ratios typical for their sibling species, resulting in a considerable overlap of response pattern (Fig. 1).

Analysis of EAG responses to all nine compounds at the whole concentration range revealed that interspecific differences are highly significant ($F = 31.14$, $p < 0.001$). Four out of the nine tested compounds showed no interspecific differences in dose response curves between the apple host race of *R. pomonella* and *R. mendax*: hexyl propanoate ($F = 0.66$, n.s.; Fig. 2a), methyl pentanoate ($F = 0.55$, n.s.; Fig. 2b), ethyl pentanoate ($F = 0.78$, n.s.; Fig. 2c), and propyl hexanoate ($F = 0.10$, n.s.; Fig. 2d). Significant response differences were found, however, to butyl-2-methyl butanoate ($F = 24.45$, $P < 0.001$; Fig. 2e), hexyl butanoate ($F = 7.48$, $P = 0.007$; Fig. 2f), butyl hexanoate ($F = 23.33$, $P < 0.001$; Fig. 2g), 2-nonanone ($F = 6.82$; $P = 0.011$;
Fig. 2a–i. Dose response curves of the apple host race of *R. pomonella* and of *R. mendax* to specific odor compounds, expressed as the ratio between the response to the odor and the response to the standard.

Fig. 2h) and 2-nonanol (*F = 9.96, *P* = 0.002; Fig. 2i).

The interspecific differences in EAG response are concentration dependent. Significant response differences were found at one concentration of hexyl propanoate (*10^{-4}*: *D_{8,10} = 48, *P* < 0.05; Fig. 2a) and of 2-nonanone (*10^{-6}*: *D_{8,6} = 64, *P* < 0.001; Fig. 2h), at two concentra-
tions of 2-nonanol \(10^{-6}: D_{8,9} = 46, P < 0.05; D_{8,9} = 56, P < 0.05; \) Fig. 2i), and at three concentrations of butyl hexanoate \(10^{-6}: D_{8,9} = 47, P < 0.05; 10^{-4}: D_{8,10} = 52, P < 0.05; 10^{-3}: D_{8,10} = 52, P < 0.05; \) Fig. 2g and of butyl-2-methyl butanoate \(10^{-3}: D_{8,10} = 48, P < 0.05; 10^{-2}: D_{8,10} = 70, P < 0.001; 10^{-1}: D_{8,9} = 56, P < 0.01; \) Fig. 2e).

The shape of the dose response curves of most compounds are similar for both species. However, interspecific differences clearly occur, mostly either at the upper or the lower end of the concentration scale (e.g., butyl-2-methyl butanoate, Fig. 2e; 2-nonanone, Fig. 2h; 2-nonanol, Fig. 2i).

The analysis of inter-host race differences in

\[\text{Fig. 3a-e. Dose response curves of the apple and the hawthorn host race of } R.\text{ pomonella expressed as the ratio between the response to the odor and the response to the standard.}\]
EAG response to five host fruit odor compounds at six concentrations revealed significantly different peripheral sensitivity between the apple and the hawthorn host race of *R. pomonella* (F = 29.31, P < 0.001). Significant inter-host race differences were found in butyl-2-methyl butanoate (F = 7.77, P = 0.007; Fig. 3a), hexyl butanoate (F = 7.58, P = 0.007; Fig. 3b), hexyl propanoate (F = 6.80, P = 0.011; Fig. 3c) and propyl hexanoate (F = 7.46, P = 0.008; Fig. 3d). EAG responses to butyl hexanoate were not different (F = 1.29, n.s.; Fig. 3e). Differences were again concentration dependent. Significant differences in dose response curves between the host races of *R. pomonella* were found in one or two concentrations of all five tested compounds: butyl-2-methyl butanoate (10⁻⁵: D₆,₈ = 40, P < 0.01; 10⁻²: D₆,₈ = 42, P < 0.01; Fig. 3a); hexyl butanoate (10⁻⁶: D₆,₈ = 34, P < 0.05; Fig. 3b); hexyl propanoate (10⁻⁶: D₆,₈ = 40, P < 0.01; Fig. 3c); propyl hexanoate (10⁻⁶: D₆,₈ = 42, P < 0.01; 10⁻²: D₆,₈ = 40, P < 0.01; Fig. 3d); butyl hexanoate (10⁻⁵: D₆,₈ = 36, P < 0.05; Fig. 3e). However, all dose response curves parallel each other and overlap over broad concentration ranges.

**Discussion**

Our data indicate differences in antennal sensitivity to host plant odors between *Rhagoletis* species and host races. The flies used in our experiments had no prior host fruit experience indicating that the peripheral differences have a genetic basis. *R. mendax* is relatively more sensitive to the odor of blueberries, its specific host plant, than to the odor of apples and vice versa for *R. pomonella*, indicating that antennal sensitivity may be adapted to the species specific host fruit odors. This pattern is, however, not reflected in the responses to the single odor compounds we used in this study. The relative EAG responses of *R. pomonella* are mostly higher than those of *R. mendax* suggesting that synergistic effects may be responsible for the interspecific differences in response to fruit extracts. Alternatively, we may not have used those odor compounds that are most critical for host fruit determination.

Laboratory experiments showed that *R. pomonella* and *R. mendax* can hybridize and produce fertile F₁, F₂ and backcross progeny (J. E. Frey, pers. obs.). However, Feder et al. (1989) showed that these species do not interbreed in nature. Because *Rhagoletis* flies meet and mate on their host plant, strong premating isolating barriers must exist to prevent interspecific hybridization. Long range pheromones are not important sexual attractants for the two species (Boller & Prokopy, 1976) and cannot be considered as premating isolating barriers. Body coloration and wing pattern, which are believed to be species recognition cues during courtship of several *Rhagoletis* species (Bush, 1966), are almost identical for *R. pomonella* and *R. mendax* and, because hybridization can occur in the laboratory, they cannot be considered as strong ethological isolating barriers (Feder et al., 1989). Populations of *R. pomonella* and *R. mendax* co-occur for over a month at sites where both hosts are growing side by side, which indicates that allochronic isolation only plays a minor role as interspecific premating barrier (Feder et al., 1989). Therefore, differential host odor recognition is likely to be an important factor in reducing a gene flow between *R. pomonella* and *R. mendax*.

There is considerable intraspecific variation in EAG responses to host fruit extracts (Fig. 1). In each species, some individuals show response patterns that are typical for their sibling species. This indicates that antennal sensitivity to host fruit odors may only be one of several factors involved in host recognition. Prokopy and Roitberg (1984) found that visual as well as olfactory stimuli are involved in host finding by *Rhagoletis*. Host conditioning through prior adult experience can affect host preference of *R. pomonella* (Prokopy et al., 1982). Furthermore, we established differences at the peripheral level only. Interspecific differences may also exist at higher levels of integration. Even though some *R. mendax* flies show an EAG typical for *R. pomonella* flies (and vice versa), the interpretation by their
central nervous system may be different from that in *R. pomonella*. These and possibly other factors, such as visual cues, play together to make host recognition sufficiently precise. However, plasticity in host recognition caused by variation in these factors may be a necessary condition to allow flies of the *R. pomonella* group to colonize new host plants.

The between species and between host race differences in dose dependent response curves indicate differentiation at the antennal receptor cell level. Initial experiments with whole fruit flies, decapitated preparations and excised antennae revealed essentially the same EAG response. Therefore, the measured EAG differences are a peripheral phenomenon suggesting that one of the basic mechanisms for differential host recognition is dependent upon differences in antennal receptor cells. Because an EAG is the sum of the potentials of all stimulated receptor cells (Schneider, 1962; Mayer et al., 1984), differences in response to the same odor can be due to different numbers of the same receptor cells of the antenna, if receptor cell numbers are species or host race specific. Alternatively, they can be due to differences in receptor cell types between species or host races. In this case, a specific odor may stimulate a receptor cell type only present in one species or host race. As a third possibility, they can be due to a combination of both.

In a preliminary genetic analysis of host odor perception, we found that several genetic elements may be responsible for determination of EAG responses to different odor compounds and that sex-linkage is likely to be involved (Frey & Bush, unpubl.). Although peripheral sensitivity seems to be a polygenetically inherited trait, our data suggest that the number of genetic factors determining the EAG response to single odor compounds may be fairly limited.

The results outlined in this paper indicate that genetically based differences in antennal sensitivity may contribute to differential host preference in *Rhagoletis* species and host races. Modifications in host preference caused by minor genetic changes affecting the number or odor specificity of a specific receptor cell type may thus be an important mechanism promoting host shifts and speciation. Divergence based on this mechanism may have occurred in absence of geographic isolation under sympatric conditions.

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**Zusammenfassung**

*Rhagoletis* Schwesternarten und Wirtsrassen – Unterschiede in der Wirtsdufterkennung


References


