

# Specialized Feeding Behavior Influences Both Ecological Specialization and Assortative Mating in Sympatric Host Races of Pea Aphids

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**ABSTRACT:** Not only is ecological specialization a defining feature of much of Earth's biological diversity, the evolution of specialization may also play a central role in generating diversity by facilitating speciation. To understand how ecological specialization evolves, we must know the particular characters that cause organisms to be specialized. For example, most theories of specialization in herbivorous insects emphasize physiological trade-offs in response to toxic plant chemicals. However, even in herbivores, it is likely that other characters are also involved in resource specialization. Knowing the causes of ecological specialization is also crucial for linking specialization to speciation. When the same character(s) that cause specialization also influence assortative mating, speciation may occur particularly rapidly because specialization and reproductive isolation become coupled in a positive feedback that speeds the evolution of both. Indeed, a central hypothesis in the study of ecological speciation is that specialization in recently diverged taxa may often be due to characters that also produce assortative mating. We test this hypothesis by evaluating the causes of ecological specialization among host-associated populations of an herbivorous insect, the pea aphid (*Acyrtosiphon pisum*). These populations are highly specialized on different host plants (alfalfa or clover; "alternate hosts"), and the races are partially reproductively isolated. Here, we identify key characters responsible for host plant specialization. Our results suggest that the major proximal determinant of host specialization is the behavioral acceptance of a plant rather than the toxicity of the food source. Pea aphids rapidly assess alfalfa and clover and reject the alternate host based on chemical cues that are perceived before the initiation of feeding. This rapid behavioral rejection of the alternate

host by a given race has two consequences. First, unrestrained aphids quickly leave the alternate host and search for other plants. Because pea aphids mate on their host plants, divergence in host acceptance among ecologically specialized races leads to congregation on the favored host. This results in de facto assortative mating when sexual forms are produced in late summer. Second, specialized aphids that are held on the alternate host will not feed in a 7.2-h trial, even in the face of starvation. Thus, a complex trait, behavioral acceptance of a plant as host, influences both reproductive isolation (through host-associated assortative mating) and ecological specialization (because of low nutritional uptake on the alternate host). This dual influence of feeding behavior on both assortative mating and resource specialization is central to the maintenance of these divergent races, and it may also have been involved in their origin.

**Keywords:** insect-plant interactions, host races, local adaptation, trade-offs, ecological speciation, sympatric speciation.

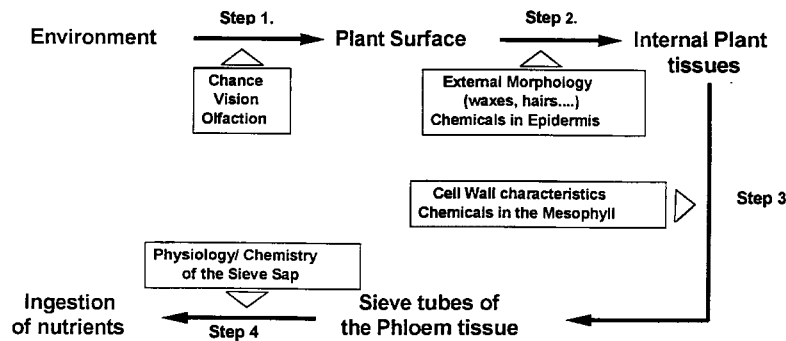
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Ecological specialization is widespread among species, and the ecological and genetic mechanisms that produce specialized resource or habitat use are receiving ongoing scrutiny (e.g., Berenbaum 1996). However, many open questions remain. How does specialization evolve, and in particular, does it involve fundamental genetic trade-offs in the use of different resources? What links specialization and speciation? Under what circumstances are specialized populations likely to diverge to the extent that they become separate species? To fully understand the evolution of ecological specialization and its link to speciation, it is important to identify the characters involved in specialized habitat or resource use. Ecological specialization and the possibility that it will lead to speciation hinges on the evolution of these physiological, morphological, or behavioral traits.

What types of characters contribute to the evolution of ecological specialization? Futuyma and Moreno (1988) suggested that behavior has a special importance in the study of specialization, on the grounds that behavioral changes often initiate the use of a new environment and that selection can only then act on the morphological and physiological characters expressed there. This is the basis

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**Figure 1:** The four steps of host plant acceptance in aphids. At each step, the plant can be either accepted (aphid proceeds to the next step) or rejected (aphid leaves the plant). Acceptance depends on a variety of plant characteristics. The final target is the sieve elements of the phloem (phloem sap).

of the model of speciation by host shifts (review in Bush 1994). Recently, models of the evolution of specialization (Fry 1996; Kawecki 1996, 1997; Kawecki et al. 1997), sympatric speciation (Rice 1987; Rice and Hostert 1993), and ecological speciation (Schluter 1996, 1998) have also suggested that characters differ in the roles they play in these evolutionary processes. However, for these authors, the crucial issue is the extent to which a character under divergent selection also promotes assortative mating. When traits that lead to assortative mating are either direct targets of divergent selection or are genetically correlated with divergently selected traits, they influence the genetic structure of populations by causing assortative mating. This process may lead to extremely rapid evolutionary divergence of populations in different habitats and may be an important mechanism leading to rapid “ecological speciation” (speciation that occurs because of selection and adaptation to different environments, e.g., Schluter 1996, 1998; Nagel and Schluter 1998) or to sympatric speciation (e.g., Rice 1987; Rice and Hostert 1993; Doebeli 1996; Kondrashov and Kondrashov 1999).

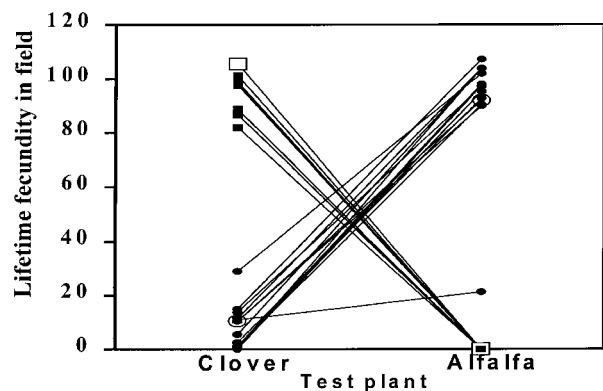
In sum, characters that directly influence both specialized resource use in the different environments and the extent of assortative mating are predicted to be of particular importance in the evolution of ecological specialization and its potential to lead to speciation. Is this dual effect of characters commonly seen in specialized populations and species, or are there just as many cases in which the traits that cause specialization differ from those that influence mate choice?

As pointed out by Schluter (1996, 1998), there are few empirical examples of natural populations in which both the characters under divergent selection and those that contribute to assortative mating are known. However, available examples show remarkable parallels in widely diverse systems. For example, stickleback fish in recently

formed postglacial lakes have diverged into benthic and limnetic species with distinct morphologies (review in Schluter 1996). The difference in morphology is highly correlated with both feeding efficiency in the two environments (Schluter 1995) and with mate choice (Schluter and Nagel 1995), which leads to a high degree of assortative mating. A similar pattern of divergent morphology (beak depth) and assortative mating based on the ecologically divergent trait is also seen in different species of Darwin’s finches (Ratcliffe and Grant 1983; review in Schluter 1996).

Herbivorous insects are one of the most diverse and specialized groups on Earth. Might a link between specialization and speciation contribute to this diversity (e.g., Thompson 1994)? In two well-known examples of host-associated insect races, habitat choice plays a role in reproductive isolation of the races, but it does not appear to have a functional link to the mechanism of specialization. In the apple maggot fly *Rhagoletis pomonella*, races on apple and hawthorn are thought to be divergently selected by differing host phenology (Filchak et al. 1999), which leads directly to some assortative mating. However, population divergence is augmented by an additional preference for ovipositing on the “home” plant (review in Feder et al. 1997). In the ball-gallmaker *Eurosta solidaginis*, races on different species of goldenrod are divergently selected because of differences in gall size, and oviposition preference is thought to be important in maintaining race identity (review in Itami et al. 1997). In both of these cases, habitat choice appears to be somewhat independent of the characters under direct selection within the habitat (phenology and gall characteristics, respectively), although the proximate cause of habitat choice is not known in either case.

Populations of the pea aphid *Acyrtosiphon pisum* Harris in closely adjacent fields of clover and alfalfa show striking genetic divergence in demographic performance



**Figure 2:** Demography of pea aphids on clover and alfalfa in a field trial following the protocol described in Via (1991a). Lifetime fecundity on each plant of 21 aphid genotypes collected from clover and alfalfa fields in Tompkins County, New York. Closed circles are clones collected in alfalfa fields; closed squares are clones collected in clover fields. The focal clone *A1* is represented with an open circle; *C1* is represented with an open square.

on the two hosts (Via 1989, 1991a, 1991b, 1994, 1999). Gene flow between races on the two crops is low, and these aphid populations appear to be incipient species that almost certainly continue to exchange genes that are neutral or beneficial in both environments (Via 1999). As such, the pea aphid races provide an excellent opportunity to identify the factors involved in the joint evolution of ecological specialization, genetic divergence, and reproductive isolation without the confounding effects of extensive postspeciation evolution in each group. Pea aphids are introduced from Europe, and it is possible that the local adaptation may have originated there. However, a preliminary phylogeographic analysis suggests evidence for some independent evolution of specialization in geographically separated populations in North America (D. J. Hawthorne and S. Via, unpublished data). The data presented here thus bears directly on the contemporary maintenance of divergence between sympatric populations on the two hosts and may also allow us to formulate testable hypotheses about the origin of that divergence.

Pronounced host plant preference by pea aphids has been suggested in field studies and verified with laboratory observations (Via 1999). Given that mating in pea aphids occurs on the host plant with no evidence of choice among mates on a given host (S. Via, unpublished data), this host choice behavior and the assortative mating that it causes may be responsible for much of the gene flow restriction between races (Via 1999).

Here, we identify the proximate determinants of plant specialization and habitat choice in pea aphids through a detailed analysis of feeding behavior and its role in spe-

cialized resource use. In this study, we test two hypotheses: the first is that the same character(s) that determine host choice (and thus assortative mating) also affect survival or fecundity on the host; the second, that specialization involves plant toxicity, which would imply that trade-offs in detoxification are important in specialization in this system (e.g., Futuyma and Moreno 1988; Fry 1996). We show that specialized feeding behavior may underlie both the habitat choice that leads to assortative mating and the poor demographic performance on nonhost plants. Moreover, we illustrate that feeding is not required for host choice, obviating the role of phloem-based toxins in the host choice of these aphids.

### The Mechanisms of Host Choice

“Host plant acceptance” is a complex behavioral trait. Feeding behavior in aphids consists of penetration of leaf or stem with the stylets and a search for vascular tissues. Previous work suggests that chemical or physical attributes of various plant tissues may deter aphid acceptance of a host plant (Srivastava and Auclair 1975; Campbell and Dreyer 1985; Dreyer et al. 1985, 1987; Klingauf 1988; Caillaud and Niemeyer 1996). Thus, acceptance involves interaction not only with the phloem sap, which is the source of food, but also with other plant tissues encountered during the search for the phloem.

In order to determine the mechanisms of host acceptance and resource specialization, we need to identify the plant tissues where the host or nonhost nature of the plant is first perceived. For example, if a plant is rejected before stylet penetration or before feeding begins, then plant toxicity and nutritional quality are not involved in the proximate discrimination among hosts. In such a case, host specialization may be due more to behavioral rejection of some plants as hosts than to physiological adaptation to plant defenses. Alternatively, if a plant is rejected only after the phloem is reached by the aphid stylets or after a period of feeding, then the chemical composition of the phloem sap or its nutritional quality must play a role in host acceptance.

We dissect host acceptance into four successive steps (fig. 1): prelighting behavior (step 1), exploration of the plant surface and probing of subepidermic tissues (step 2), deep probing in the plant tissues and searching for the nutritional tissues (step 3), and evaluation of the phloem sap as suitable for ingestion (step 4). This study extends a preliminary analysis that focused on the exploration of the surface tissues of the two hosts (step 2; Caillaud 1999). Our identification of the characters leading to host plant choice and specialization paves the way for an analysis of the genetic architecture of these important traits (M. C. Caillaud and S. Via, unpublished manuscript; S. Via, M. C. Caillaud, and D. J. Hawthorne, unpublished data.).

**Table 1:** Variables measured to characterize behavioral specialization in pea aphids

Step	Variable
Prealighting behavior (1)	Percentage of aphids reaching either clover or alfalfa (%) Time needed to reach either plant (s)
Exploration of the plant surface and subepidermis (2; 30-min observation)	Time to first penetration (s) Duration of first penetration (s) Total number of penetrations Total time spent penetrating (s) Mean duration of a penetration (s) Number of nymphs produced Percentage of adults remaining on plant after 30 min (%) Time before plant was left (s)
Search for the phloem vessels and ingestion (3, 4; EPG)	Time to first penetration (s) Total number of E1 Total number of E2 Number of R-PDs Duration of a R-PD (min) Duration of NP (min) Duration of C (min) Duration of E1 (min) Duration of E2 (min)

Note: Abbreviations are as follows: EPG = electropenetrography; E1 = waveform that corresponds to aphid's injection (in preparation for ingestion) of saliva inside the sieve elements of the phloem; E2 = waveform that corresponds to a sustained ingestion of plant sap by aphid; R-PD = repetitive potential drop; NP = nonpenetration activities (resting or walking); C = search through extracellular spaces for nutritional tissues.

## Methods

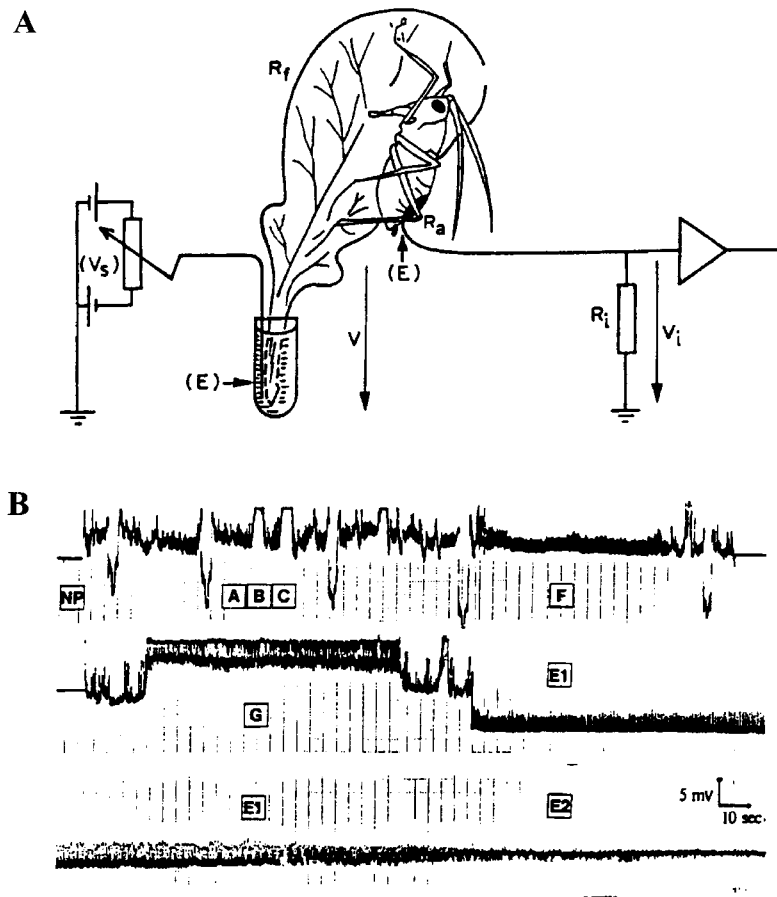
### *Study Organism*

Pea aphids are specialists on legumes (Eastop 1973). In dairy-farming areas, such as our study site in upstate New York, they can be found in abundance on their two major perennial hosts, alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*). They may also be found to a much lesser extent on a variety of wild annual and perennial legumes (Eastop 1973; M. C. Caillaud and S. Via, personal observation). Pea aphids are cyclical parthenogens with a single sexual generation in the fall. Sexual morphs are induced in the fall by a combination of cold temperatures and decreasing photoperiods. Fertilized eggs overwinter on the dead herbaceous hosts, and they hatch in the spring into fundatrices that establish the first parthenogenetic lineages. Most opportunities for migration occur during the parthenogenetic generations at the beginning of the summer or immediately preceding sexual reproduction, but some gene flow through dispersal of winged males is also possible. Thus, it is important to test the host acceptance behavior both of the parthenogenetic forms that give rise to the sexuals and of the sexuals themselves.

### *Feeding Behavior of the Specialized Races*

Because the behavioral experiments we performed are very time consuming to analyze and require considerable replication, we focused our work on two pea aphid genotypes. They were specifically chosen because they typify the racial specialization of the populations on alfalfa and red clover in upstate New York that we have studied for 10 yr (see fig. 2 for phenotypes of clones from our study area). Genotype *A1* was collected from an alfalfa field, and genotype *C1* was collected from a nearby clover field in Tompkins County, New York. These two genotypes have been maintained parthenogenetically on the crop from which each was collected ("native host"; for *A1*, alfalfa [cv. "Oneida VR"]; for *C1*, clover ["Medium Red"], 14°C, 16L:8D). Aphids in each of these clonal lineages were reared on their native host so that we could evaluate the behavior of dispersing aphids from each race that had grown up in the field on their home host. This is the comparison that is most relevant for issues of gene flow and assortative mating.

To verify that the behavior of these two clones reflects that of the races that they represent, a limited behavioral analysis of additional parthenogenetic clones was conducted in a separate set of experiments, with *A1* and *C1* retested for comparison. In addition, sexual forms were



**Figure 3:** DC-electropenetrography (EPG) technique. *A*, Schematic representation of the electrical circuit (reprinted with permission from Tjallingii 1988). *B*, Electrical waveforms recorded by DC-EPG. In *B*, *C* = aphid search for nutritional tissues (xylem or phloem); *E1* = an entrance in the sieve elements of the phloem and injection of saliva; *E2* = a sustained ingestion of plant sap; *G* = an ingestion of the xylem sap (water and minerals); *NP* = nonpenetration activities (resting or walking); and *F* = a stress response.

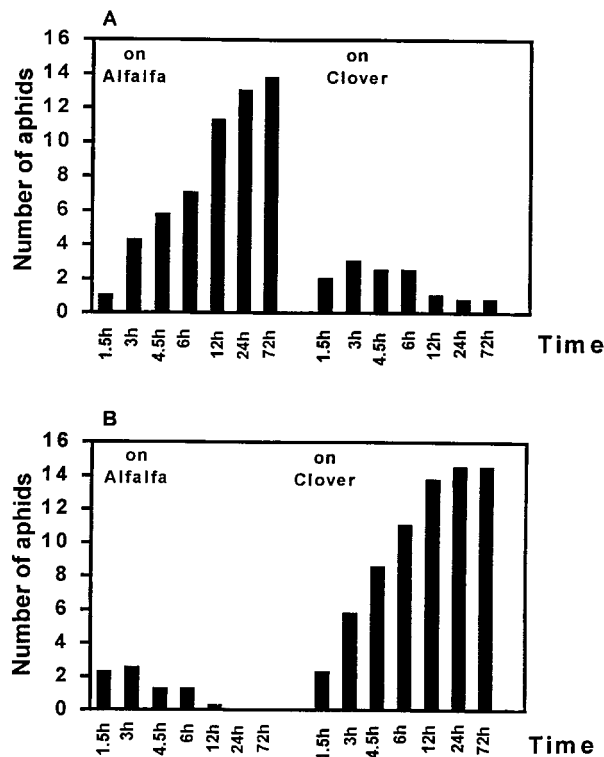
induced for several genotypes, including *A1* and *C1*, to check whether or not the sexuals also exhibited the same degree of specialized behavior seen in the parthenogenetic forms of our two focal clones.

Three types of bioassays were used to study the successive steps of host acceptance in pea aphids (fig. 1). Except for the examination of prealighting behavior (step 1), we studied "host acceptance" rather than "host preference" because aphids were not given a choice between alfalfa and clover. This no-choice situation is a good representation of what aphids encounter when searching in agricultural fields, most of which are planted in monoculture.

*Step 1: Prealighting Behavior (Fig. 1).* We conducted two experiments to evaluate whether or not pea aphids could distinguish between hosts before landing. In the first ex-

periment, 20–25 winged aphids of a given genotype were released from an aerial platform into a  $1 \times 0.7 \times 0.7$ -m cage containing two pots of each host, as described in Via (1999). Within each cage, the number of aphids on each plant was scored after 1.5, 3, 4.5, 6, 24, and 72 h. Four to five trials were performed for each genotype. If aphids distinguish between plants on the basis of prealighting cues, we would expect a clumped distribution to be seen soon after aphids were released into the cage. In contrast, if aphids need interaction with the host in order to discriminate, we would expect aphids to aggregate gradually on the preferred host.

In a second test of prealighting behavior, winged aphids were released onto the floor of a  $0.9 \times 0.6$ -m rectangular arena. A pot of alfalfa and a pot of clover were located at



**Figure 4:** Number of individuals of two aphid genotypes scored on alfalfa and clover following their release in a large cage containing pots of alfalfa and clover. Observations were made at 1.5, 3, 4.5, 6, 12, 24, and 72 h. Each trial involved 20 winged individuals. A, Alfalfa specialist (genotype A1), mean of four trials. B, Clover specialist (genotype C1), mean of five trials.

opposite edges of the arena. Ten aphids deposited in the center of the arena were followed individually for 7 min (10–15 trials/clone/plant). The percentage of aphids that reached either plant and the time needed to reach it were recorded. If aphids can identify the preferred plant before probing the plant surface, we expect that aphids would rapidly reach their preferred host and ignore the alternate host.

*Step 2: Exploration of Plant Surface and Immediate Sub-epidermic Tissues (30-min Observations).* To test the behavior of freely moving aphids immediately after contacting a plant, we placed four aphids directly on either a clover or an alfalfa plant in the 0.9 × 0.6-m arena described above. Aphids were observed for 30 min, and each behavior was recorded (table 1; nine to 11 replicates/genotype/host) with a computer-based event recorder called “The Observer” (Noldus, Wageningen). For each replicate, the observation was the mean of the behavior of the four aphids. We know from our electropetrography (EPG) recordings (steps 3 and 4) that the phloem tissue is generally not reached within 30

m of penetration of either clover or alfalfa. Thus, if we observe rejection of one of the plants by A1 or C1 within a 30-min observation, we can conclude that information obtained before the phloem is reached is used to evaluate potential hosts. In this and all of the following experiments, the wingless aphids were 4–7-d-old adults when observed. Aphids were starved for 3 h before experiments to increase their reactivity to the plant and to standardize their physiological condition. Experiments were performed at the same time each day in a room held at  $21^{\circ} \pm 1^{\circ}\text{C}$ . Plants were grown from commercially available seed of these cultivars and were 5 wk old at the time of the experiments. Individual plants within hosts were randomized over all trials so that variation among plants could not contribute to differences among genotypes.

*Steps 3 and 4: Search for the Nutritional Tissues and Ingestion of Nutrients (EPG Recordings).* To study the activity of the aphid stylets inside the plant tissues during the search for the vascular tissues, we used an electronic technique (DC-EPG) to monitor feeding (fig. 3). In this technique, an aphid is attached to a fine wire, and the aphid and plant are made part of an electrical circuit. The penetration of the aphid stylets in the plant modifies the voltage of the signal, and different waveforms (fig. 3) reveal information about aphid behavior as well as the location of the stylet tips during penetration. Thus, this method can be used to distinguish between events that occur during the search for phloem (step 3; fig. 1) and events that occur inside the phloem vessels (step 4; fig. 1). The biological significance of each waveform has been carefully calibrated (Tjallingii 1978, 1988, 1990), and this technique is commonly used among aphid and whitefly biologists (Caillaud et al. 1995; Lei et al. 1999). For example, waveform “NP” (fig. 3B) represents “nonpenetration” activities (resting or walking). The waveform “C” represents the search through extracellular spaces for the nutritional tissues (xylem or phloem). During their progression toward the feeding sites, the aphid stylets probe briefly (a few milliseconds) into plant cells located along their route, producing a potential drop (PD). Many plant allelochemicals are stored in cell vacuoles, and aphids might be able to perceive the presence of these secondary compounds when they pierce cells during PDs. Another waveform called repetitive potential drop (R-PD) is longer than a single PD and corresponds to brief intracellular punctures of sieve elements of the phloem (Tjallingii and Gabrys 1999). Waveform “E1” is scored when aphids, in preparation for ingestion, inject saliva inside the sieve elements of the phloem. Waveform “E2” corresponds to a sustained ingestion of plant sap (nutrients, mostly amino acids), while “G” is an ingestion of xylem sap (water and minerals). Waveform “F” is often observed when aphids appear to be under stress (M. C. Caillaud, personal observation).

**Table 2:** Means and standard deviation for variables characterizing prealighting behavior

	Genotype <i>A1</i> (alfalfa specialist)	Genotype <i>C1</i> (clover specialist)
Percentage of aphids that:		
Reached clover	59	48
Reached alfalfa	41 <sup>a</sup>	52 <sup>a</sup>
Time (s) needed to:		
Reach clover	97.6 (56.1)	93.6 (60.6)
Reach alfalfa	98.7 (53.3) <sup>a</sup>	110 (50.1) <sup>a</sup>

Note: Standard deviations in parentheses. Significance levels refer to differences between behavior on clover and alfalfa for each genotype and were determined by one-way ANOVA with plant as a fixed factor.

For the EPG, aphids were attached to a 25- $\mu$ m gold wire by means of a droplet of conductive silver paint, then transferred to a leaf of a potted alfalfa or clover and monitored according to techniques described in Caillaud et al. (1995). Because aphids are tethered during EPG recording, they are not able to leave the test plant even if it is rejected. Eight plant/aphid combinations were tested simultaneously, and stylet activities during plant penetration of individual aphids were recorded for 7.2 h. Two replicates per specialized genotype were tested on each host per day (block) and were randomized on the eight-unit apparatus (10–16 replicates/genotype/host).

EPG waveforms were digitized with an analog to digital converter (MacAdios 8ain; GW Instruments, Somerville, Mass.) and the computer program Acq. MacAdios (G. Febvay and Y. Rahbe, unpublished program). Analysis of EPG files (waveform recognition and timing) was achieved with MacStylet (Febvay et al. 1996). After eliminating some highly correlated variables, a total of nine behavioral variables were analyzed from each EPG recording (table 1). Collectively, these variables can be used to determine the mechanisms of feeding specialization. For example, if the recording of *A1* feeding on clover contains the EPG waveform R-PD or E1 but not E2, it indicates that this genotype reached the phloem on the alternate host but did not succeed at ingesting sap. This would suggest that the composition of the phloem sap influences rejection of clover as a host by alfalfa specialists. Alternatively, if no R-PD, E1, or E2 are seen in a given recording, it indicates an earlier rejection of the plant mainly on the basis of cues in surface, subepidermic, or mesophyll tissues, without influence of phloem ingestion.

#### *Tests of Additional Clones and Sexual Forms from Each Race*

To check the generality of the observations made on the two genotypes, *A1* and *C1*, we performed two additional

sets of experiments. First, 30-min observations and EPG analyses were performed on nine other genotypes (four from alfalfa and five from clover; six to nine replicates/genotype/host), all collected in Tompkins County, New York, in 1998. Genotypes *A1* and *C1* were tested at the same time for comparison. Second, EPG was also performed on oviparae (sexual females) and males of three clover genotypes and five alfalfa genotypes (including *A1* and *C1*, five to six replicates per genotype/host). This permits evaluation of the extent of host choice in the mating forms themselves as well as in the more typical asexuals, which perform most of the migration.

#### *Statistical Analyses*

For all analyses, “genotype” and “plant” were considered to be fixed factors. Block and all interactions with block were considered as random factors (PROC MIXED; Littell et al. 1996). When the main effect of block and its interactions were not significant, these factors were dropped and the model was recomputed.

*Step 1: Prealighting Behavior.* For each genotype, the mean number of aphids in the large cage on each plant type at a given time, the proportion of aphids that reached either plant in the small arena, and the time they needed to reach each plant were log transformed and compared by ANOVA (PROC GLM; SAS 1990).

*Step 2: Exploration of the Plant Surface.* To evaluate differences between the specialized genotypes in their overall behavior during 30 min of free movement, a MANOVA was performed on the variables in table 1 (PROC GLM; SAS 1990). We first considered the full model using the whole data set to test for host specialization by means of a genotype  $\times$  host interaction. Then, the data set was partitioned in order to test for behavioral differences between genotypes on each host separately. The total canonical correlation for each factor permits evaluation of the extent to which the set of measured behaviors explain differences between genotypes or hosts. Univariate ANOVAs were also performed on several variables to aid in interpreting the differences between the genotypes.

*Steps 3 and 4: Search for the Nutritional Tissues and Ingestion.* MANOVAs and univariate ANOVAs were performed on these behaviors as described for step 2.

*Tests of Additional Genotypes.* The 30-min observations and the EPGs on the additional genotypes were analyzed as described above.

#### **Results**

*Step 1: Prealighting Behavior.* For both genotypes, equal numbers of individuals were found on alfalfa and clover 1.5 h after they were each released into a large cage con-

**Table 3:** MANOVA of behavior of freely moving aphids on potted plants (30-min observations) and aphids monitored by EPG during plant penetration

Source of variation	Wilks's $\lambda$	$F$	Pr > $F$	Total canonical correlation
Freely moving aphids (30-min observations):				
Complete model:				
Genotype	.47	4.74	.001	.72
Host	.48	4.67	.012	.72
Genotype $\times$ host	.036	114.6	.0001	.98
Alfalfa habitat:				
Genotype	.0057	30.3	.0001	.97
Clover habitat:				
Genotype	.007	203.7	.0001	.99
Aphids monitored by EPG (7.2-h recordings):				
Complete model:				
Genotype	.61	1.7	.061	.55
Host	.51	2.8	.032	.61
Genotype $\times$ host	.02	55.6	.0001	.97
Alfalfa habitat:				
Genotype	.01	73.5	.0001	.99
Clover habitat:				
Genotype	.03	25.5	.0001	.98

Note: EPG = electropenetrography.

taining pots of both hosts (*A1*:  $F_{\text{host}} = 3$ ,  $P = .13$ ; fig. 4A; *CI*:  $F_{\text{host}} = 1.1$ ,  $P = .33$ ; fig. 4B). This indicates little discrimination between the plant types upon the initial approach of the aphids. However, the proportion of aphids on either plant soon started to diverge. Significant differences in the number of *A1* individuals on alfalfa and clover were seen by 4.5 h ( $F_{\text{host}} = 16.3$ ,  $P = .007$ ; fig. 4A), and differences between hosts were seen by 3 h for *CI* ( $F_{\text{host}} = 12.9$ ,  $P = .005$ ; fig. 4B). Over time, an increasing number of *A1* aphids settled on alfalfa, while *CI* aphids accumulated on clover (fig. 4). During the entire first day of the experiment, we observed that aphids were flying, landing on plants, and taking off again. By 72 h, most individuals had accumulated on one of the hosts.

When aphids were released in the center of an arena and given the choice between clover and alfalfa, both genotypes again approached each of the two hosts roughly equally (table 2). Thus, whether approaching plants from the air or from the ground, specialized genotypes *A1* and *CI* do not appear to discriminate between clover and alfalfa as potential hosts on the basis of visual or olfactory cues, at least in an enclosed arena setting.

*Step 2: Exploration of the Plant Surface and Subepidermis.* When freely moving aphids were placed directly upon a plant and observed for 30 min, we found a significant multivariate genotype  $\times$  environment interaction in overall behavior (genotype  $\times$  host; table 3). Analysis of the two genotypes on each plant separately also revealed that *A1* and *CI*

differ dramatically in multivariate behavior on both potential hosts (table 3). The variables that we measured explained nearly 100% of the total difference in behavior between aphid genotypes (total canonical correlations for factors genotype  $\times$  host and genotype; table 3).

There was again no indication that the plants were distinguished as host or nonhost before stylet penetration (fig. 5A). However, for both genotypes, the first penetration on the alternate host was significantly shorter than on the native host (fig. 5B). This first probe was followed by numerous and increasingly shorter penetrations (fig. 5C), such that the total time spent penetrating the plant tissues (i.e., attempting to feed) on the alternate host was much shorter than on the native host (fig. 5D). By the end of the 30-min observation, 47% of *A1* had left clover and 56% of *CI* individuals had left alfalfa. No reproduction was seen for any aphid placed on the alternate host. In contrast, all aphids placed on their native host settled into a long penetration within 10 min, and most of them started reproducing within 30 min. Based on this bioassay, the specialized genotypes *A1* and *CI* appear to distinguish clover from alfalfa soon after their stylets have penetrated the plant, possibly during the first probe, but before enough time had elapsed to find the phloem.

*Steps 3 and 4: Search for the Phloem Vessels and Ingestion.* The specialized genotypes also showed highly significant differences in multivariate behavior while searching within the internal tissues of alfalfa and clover (table 3). As before,

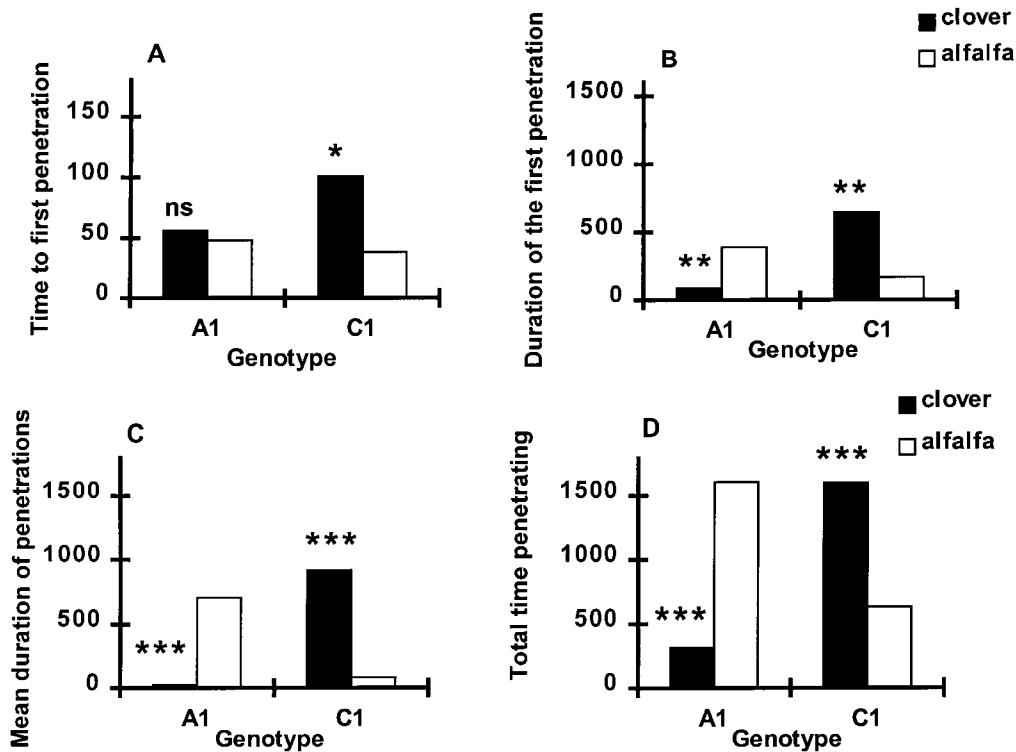


Figure 5: Means for behaviors of freely moving aphids on plants (30-min observations). A, Time (s) to first penetration. B, Duration (s) of first penetration. C, Mean duration (s) of a penetration. D, Total time (s) spent penetrating. Significance levels obtained from one-way ANOVAs within each genotype (factor = test plant) are shown. *Single asterisk*,  $P < .05$ . *Double asterisk*,  $P < .01$ . *Triple asterisk*,  $P < .001$ .

neither genotype showed a difference in the time to first penetration of the two plants (table 4), but pronounced host-associated differences were seen soon after stylet penetration. Both clones spent little time attempting to feed on the alternate plant (duration of NP; table 4). In contrast, most of the time on the native plant was spent searching for food (duration of C), attempting to feed (number of R-PD; duration of E1), and feeding (duration of E2; table 4). A few individuals of each specialized genotype sampled phloem vessel cells of the alternate host (number of R-PD; table 4), but none stayed long enough to inject saliva inside the phloem vessels (duration of E1, preparation of the ingestion phase) or to ingest phloem sap (duration of E2; table 4). Aphids on the alternate host seemed to be strongly inhibited shortly after their first penetration of the plant tissues. It is important to stress that this first penetration always included a PD, indicating that aphid stylets sampled a cell, either of the mesophyll or of the epidermis, before aborting plant penetration. In sum, specialist aphids prevented from leaving the alternate host during EPG recording hardly ever reached the phloem

vessels and never ingested nutrients on the alternate host during observations of  $>7$  h.

*Feeding Behavior of Other Specialist Clones.* In a separate set of 30-min observations on freely moving aphids and EPG experiments on wired aphids, we tested the behavior of five additional clover genotypes and four other alfalfa genotypes collected from the same study area 10 yr after A1 and C1 were collected. Our two focal clones were re-tested for comparison. Both experiments verify the results seen above in our detailed analyses of A1 and C1. In the observations of freely moving individuals, all genotypes from each race performed a shorter first penetration on the alternate host than on their native host (fig. 6A), suggesting that the alternate host was recognized and rejection began during this first penetration. Furthermore, no genotype of either specialist race ingested any phloem from the host of the other race in the 7.2-h EPG trial (fig. 6B). Therefore, the picture of behavior drawn for the two specialized genotypes A1 and C1 appears to apply in general to these two specialist races, at least for our study populations in New York.

**Table 4:** Means for behaviors (monitored by EPG) comparing host and alternate plant for each genotype

	<i>A1</i> on alfalfa		<i>C1</i> on clover	
	(host)	<i>A1</i> on clover	(host)	<i>C1</i> on alfalfa
Time to first penetration (min)	2.9	10.1	5.97	4.9
Total number of E1	5.1 <sup>***</sup>	0	4.1 <sup>***</sup>	0
Total number of E2	3.5 <sup>***</sup>	0	2.5 <sup>***</sup>	0
Number of R-PDs	71.9 <sup>***</sup>	1.6	72.6 <sup>***</sup>	1.5
Duration of a R-PD (min)	.22 <sup>***</sup>	.07	.24 <sup>***</sup>	.08
Duration of NP (min)	47 <sup>***</sup>	340	110 <sup>***</sup>	326
Duration of C (min)	138 <sup>***</sup>	32	154 <sup>***</sup>	55
Duration of E1 (min)	16 <sup>**</sup>	0	13 <sup>*</sup>	0
Duration of E2 (min)	191 <sup>***</sup>	0	122 <sup>***</sup>	0

Note: Significance levels refer to the differences between the host and alternate plant for each genotype and were determined by one-way ANOVA with plant as a fixed factor. Abbreviations: E1 = waveform that corresponds to aphid's injection (in preparation for ingestion) of saliva inside the sieve elements of the phloem; E2 = waveform that corresponds to a sustained ingestion of plant sap by aphid; R-PD = repetitive potential drop; NP = nonpenetration activities (resting or walking); C = search through extracellular spaces for nutritional tissues.

\*  $P < .05$ .

\*\*  $P < .01$ .

\*\*\*  $P < .001$ .

*Feeding Behavior of Sexual Forms.* Oviparae (sexual females) and males also show the same specialized feeding behavior as seen in the parthenogenetic forms of the two focal genotypes *A1* and *C1*. The EPG analyses reveal that, as before, no ingestion of phloem on the alternate host was attempted by oviparae or males from any of the eight genotypes tested (fig. 7).

### Discussion

These experiments demonstrate that each of the specialized pea aphid races from populations in upstate New York can quickly distinguish between their preferred host and the alternate host used by the other race. Discrimination appears to require only a brief penetration by the aphid's stylets, and the detection of the alternate host results in abandoning the plant within just a few minutes without any feeding. In contrast, aphids on the preferred host settle rapidly into feeding and show little tendency to move. The outcome of this behavior is that specialized genotypes accumulate on their favored host. Given that pea aphids mate on the host, this will produce de facto assortative mating among specialists. Moreover, unwillingness to feed on the alternate host leads directly to reduced individual fitness and could explain the local adaptation among populations on the two hosts that is seen in demographic characters (e.g., Via 1991a, 1994). Our results thus suggest that the same complex character, host acceptance, appears to contribute not only to assortative mating but also to fitness differences in the two environments. This is expected to

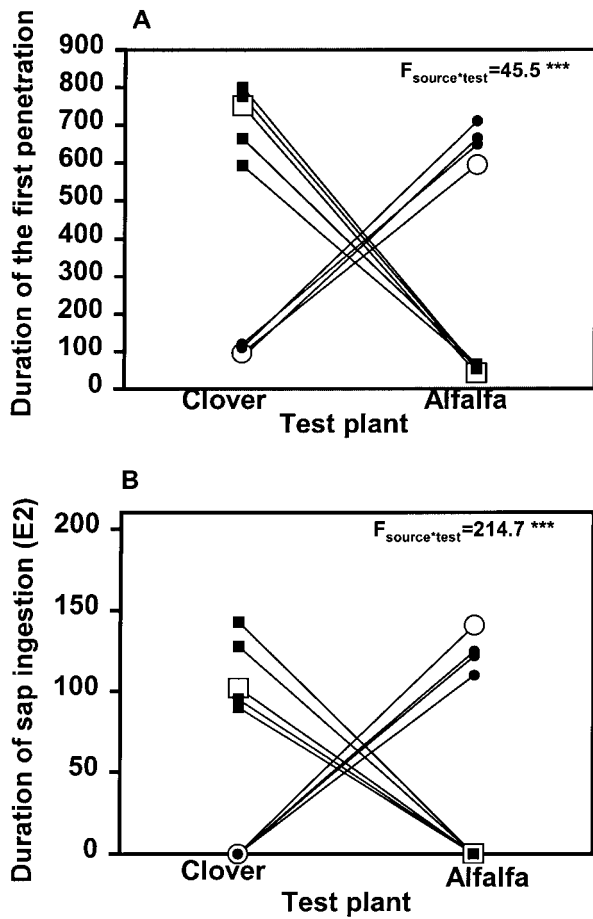
greatly facilitate genetic divergence and reproductive isolation in these sympatric races.

### Host Acceptance and Assortative Mating

This study shows that although aphids appear unable to distinguish between plants before alighting, freely moving pea aphids are likely to leave the alternate plant within a few minutes. Half of those tested here had left the alternate plant within 30 min. None produced any offspring before leaving. When aphids were tethered to the plant in the EPG trials, none of the specialists fed on the alternate plant, but a few offspring were deposited. When confined on the alternate host for their entire life, however, some feeding must eventually occur because a few offspring are typically produced, especially by some of the less specialized alfalfa clones placed on clover (see fig. 2 and Via 1999).

The impact that host choice and the scattering of offspring that may be produced on the alternate host have on assortative mating must be understood within the context of the fact that pea aphids are cyclically parthenogenetic. There are 10–12 asexual generations from May to September and a single sexual generation in the fall. Most movement involves winged asexuals migrating between fields or colonizing new fields. Peaks of alate production occur in early summer and again in August.

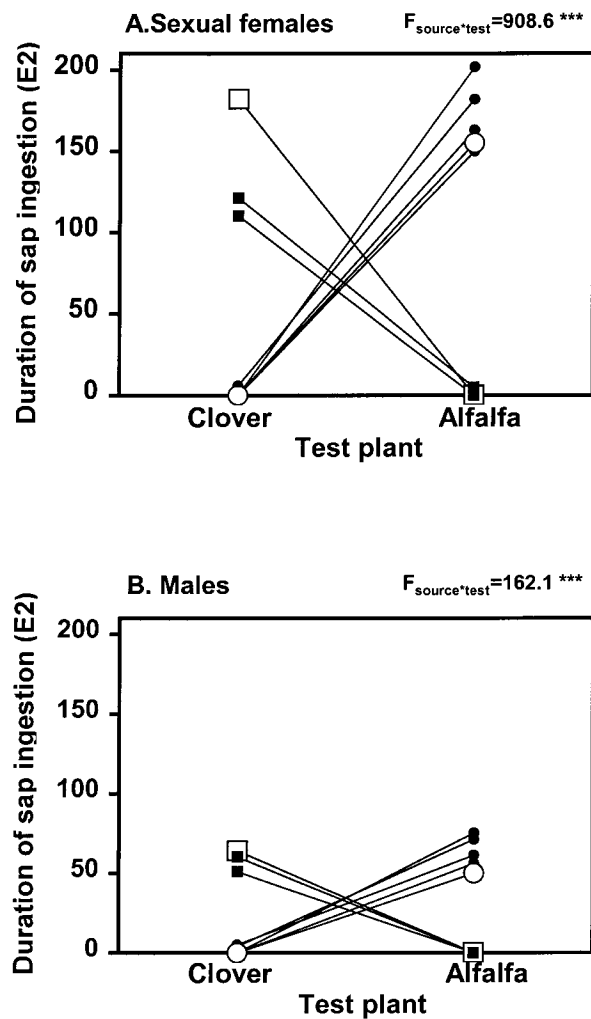
Only those migrants between crops (or their offspring) that survive until the formation of sexuals occurs can participate in mating. Early season migration may have little effect on gene flow because performance by migrants be-



**Figure 6:** Characterization of the feeding behavior on clover and alfalfa of four parthenogenetic clones collected in alfalfa fields (*closed circles; open circles, A1*) and five parthenogenetic clones collected in clover fields (*closed squares; open squares, C1*). **A**, Duration (s) of the first penetration for freely moving aphids onto the leaf surface (30-min observations). **B**, Duration (min) of sap ingestion as shown by electropenetography monitoring. Each data point is the mean of six to nine replicates. Triple asterisks,  $P < .001$ .

tween crops is so low relative to residents (Via 1991a; Via et al. 2000) that they may be lost during the asexual generations that pass before mating. Parthenogenetic forms migrating later in the season could possibly produce a few offspring on the alternate host that become induced to produce sexuals. However, the production of sexuals takes three clonal generations, and again, if these migrants have low fitness, they may not complete the process of sexual production. Sexual females are always wingless, and so they are unlikely to be migrants. In males, there is a one locus wing polymorphism (Smith and MacKay 1989), so not all males are likely to be able to migrate to the other crop. However, for those that are winged, our EPG data (fig.

7B) suggests that they are unlikely to remain on the alternate host long enough to obtain many (if any) matings with native females. Thus, the typical specialized genotype seems unlikely to be a source of much gene exchange between races. Previous studies suggest that some gene flow does occur (e.g., Via 1999), and at present, we believe that it results primarily from the occasional production and dispersal of less specialized genotypes. There is a range of specialization in field populations (Via 1991a, 1994; S. Via, unpublished data), and the least specialized individuals will accept the alternate plant for feeding and production of offspring, although they do not perform as well



**Figure 7:** Characterization of the feeding behavior (duration [min] of sap ingestion as shown by electropenetography monitoring) of sexual forms for five genotypes collected in alfalfa fields (*filled circles; open circles, A1*) and three genotypes collected in clover fields (*filled squares; open squares, C1*). Each data point is the mean of five to six replicates. **A**, Oviparae (sexual females). **B**, Males. Triple asterisk,  $P < .001$ .

on it as on their home host (Via 1991b, 1999). Future studies of hybridization in field populations should illuminate the extent of hybridization and inter-race introgression that actually occurs.

*Proximate Mechanisms of Plant Discrimination by Specialized Aphid Genotypes*

We found no evidence that pea aphids distinguish among plants before contacting them. However, during the 30-min observations of unrestrained aphids, we found that all of the clones tested from these two specialized races rejected the alternate plant shortly after a brief first penetration of the plant tissues (figs. 5B, 6A). This suggests that information is quickly gathered that dissuades the aphid from continuing to probe. Freely moving aphids on the alternate host then performed a series of additional short penetrations at different locations on the plant before running off the plant. This very active rejection is in contrast to the behavior on the typical host, which consisted of a few penetrations that were each quite long (fig. 5C, 5D). No aphids left their home plant during the 30-min observation.

The EPG experiments showed that first aphid penetration on any plant always involved the voltage drop that corresponds to an intracellular puncture (PD). Thus, we suspect that rapid rejection of the alternate host is triggered by chemicals located inside the epidermal cells or in the first row of mesophyll cells. The EPG experiments also showed that, for both races, there were many fewer penetrations of phloem cells on the alternate plant than on the usual host (R-PD; table 4) and that there were no attempts to prepare the phloem vessels for ingestion and no feeding from phloem tissue on the alternate plant (E1 and E2; table 4). These internal activities of the stylets are entirely consistent with the behavioral outcomes observed in freely moving animals. Even though aphids were tethered on the plants during the EPG, most of the time on the alternate host was spent in nonpenetration (waveform NP; table 4), while most of the time on the native host was spent in activities associated with feeding (waveforms C, R-PD, E1, and E2). Because rejection usually occurred well before location of the phloem tissue and always before phloem sap was ingested, direct nutritional deficiencies or ingested toxins cannot be involved in host rejection.

These specialized pea aphid races differ in their behavioral responses to alfalfa and clover. Each race accepts only one of these plants and rejects the other. This could either be due to genetically based chemosensory differences or to variation between clones in the central integration of responses to one or more plant allelochemicals (deterrents or stimulants). In other insects, evidence of genetic variation in the behavioral responses to specific plant extracts or particular allelochemicals has been reported between

species (Bierbaum and Bush 1990; Frey and Bush 1990) and strains (Wieczorek 1976; Harrison and Mitchell 1988; Du et al. 1995). Genetic variation in chemoreception or integration of chemosensory information is clearly the raw material required for host choice behavior to play a key role in population divergence, reproductive isolation, and speciation. Thus, the study of intraspecific genetic variation in chemical ecology, as seen between these specialized aphid genotypes, will provide a good opportunity to evaluate the role of chemically mediated behavior in the early stages of speciation.

*The Link between Feeding Specialization and Performance*

Rejection of the alternate host by these specialized clones was rapid and of long duration. In >7 h of observation during the EPG trials, aphids on the alternate host did not even attempt to ingest plant sap, even though they were starved for 3 h before the beginning of the experiment and could not leave. Rejection of the alternate plant by these specialized clones occurs even in the face of starvation. Similar behavior has been witnessed in both field and laboratory experiments in which adult pea aphids that are confined on the alternate host often die before any offspring are produced (e.g., Via 1991a). The unwillingness of these highly specialized aphid genotypes to feed on the alternate host thus provides a clear mechanism for the low average fitness of host-associated genotypes on the alternate host (e.g., Via 1991a; fig. 2).

*Ecological Specialization: Trade-offs Not Essential?*

One of the most long-standing hypotheses for the causes of ecological specialization is that high performance in one environment comes at a cost of performance in another environment (the "trade-off" hypothesis). Despite an intense search for such trade-offs in performance traits, measured as negative genetic correlations in performance in different environments, few have been found (review in Fry 1996), leading to speculation that performance trade-offs are not essential for the evolution of ecological specialization (Fry 1996; Kawecki 1996; Whitlock 1996; Kawecki et al. 1997).

The results presented here also suggest that trade-offs in physiological characters determining performance may not always drive specialization. The simple unwillingness of specialist genotypes of pea aphid to feed on the alternate host could produce the large reductions in fitness on the alternate host that we have observed (e.g., Via 1989, 1991a) without involving any nutritional deficiency or toxicity. In fact, we found that the alternate plant is rejected before the phloem is even located, supporting the hypothesis that ecological specialization in this system is not driven in a

proximal sense by nutritional deficiencies of the alternate hosts or trade-offs associated with an inability to detoxify secondary chemicals in the alternate host. Clearly, however, we cannot eliminate the possibility that toxic effects of the phloem could have served as a selective force in the past to favor the evolution of responsiveness to a chemical cue in the plant periphery that can be detected more rapidly.

It is ironic that the pea aphid system is one in which fitness trade-offs are seen at the population level in reciprocal transplants to different hosts, and this is sometimes cited as an exception to the general absence of trade-offs in empirical studies (Via 1990; Kawecki 1997). However, significant negative genetic correlations in performance on the two hosts have been seen only among populations, not within populations (Via 1991*a*). It is, thus, entirely possible that the reduced fitness experienced by pea aphids on the alternate host does not stem from a fundamental genetic inability to perform well on both hosts. Instead, races may have initially diverged because of genetic change in host acceptance. If acceptance behavior is not determined by opposite alleles at the same loci, there may be no fundamental genetic trade-off, and it may be that an aphid population could evolve that could use both hosts successfully. The intermediate demographic performance of hybrids on both hosts is consistent with this hypothesis (Via et al. 2000).

#### *Ecological Specialization and Speciation*

Models of sympatric speciation have long suggested that it occurs most readily when assortative mating of individuals in different environments is produced by a trait that is either a direct target of disruptive selection (such as habitat choice) or is correlated with a divergently selected character (Rice 1987; Rice and Hostert 1993). This has recently been affirmed as the most direct and least problematic route to sympatric speciation (Kondrashov and Kondrashov 1999).

The key to speciation as a result of specialization appears to lie in an evolutionary synergism between ecological specialization and reproductive isolation that occurs when the same trait causes both specialization and assortative mating. In such a case, initial colonization of multiple habitats and exposure to divergent selection may not only cause adaptive specialization but also ever-increasing assortative mating. This effect on mating would then feed back to facilitate the evolution of specialization by limiting gene flow between individuals exposed to selection in the alternate environment (and the consequent import of genes with maladaptive effects). Thus, the joint determination of specialization and assortative mating by a single trait or a pair of highly correlated traits initiates an evolutionary

positive feedback. As reproductive isolation evolves, the accumulation of deleterious mutations expressed in the alternate environment(s) adds further fuel to the evolution of habitat-associated population divergence and local adaptation (e.g., Kawecki 1997). When the same trait influences assortative mating and specialization, the first stage of speciation (divergence: *sensu* Kondrashov et al. 1998) is facilitated because it does not require extra selection (or genetic drift: Dieckmann and Doebeli 1999) to produce linkage disequilibrium between loci for assortative mating and specialization. However, speciation is not assured because, even when a genetic correlation is established between specialization and assortative mating (e.g., Dieckmann and Doebeli 1999; Kondrashov and Kondrashov 1999), other factors, such as the fitness of hybrids, may influence the final stages of speciation (e.g., Kondrashov and Mina 1986; Kondrashov et al. 1998).

In pea aphids, we have shown that specialized clones are unwilling to search for nutritional tissues on the alternate host. Feeding behavior, therefore, not only determines host choice (and thus the pool of potential mates), it also strongly influences performance on the alternate plant. This is precisely the type of association between assortative mating and performance in different habitats that is thought to favor the evolution of ecological specialization and, potentially, to lead to ecological speciation (e.g., Schluter 1998). Given genetic variation in host choice behavior, population divergence and ecological specialization by subpopulations on different hosts could proceed quite rapidly by the mechanisms outlined above.

The results presented here suggest that specialization in herbivorous insects may sometimes be based in variability in sensory systems or neural integration that produces differences among genotypes in feeding behavior and host preference. A genetic trade-off requires that at some of the loci influencing host use, alternate alleles produce high fitness in different environments. It is possible that instead of involving loci that affect detoxification or digestion, alternate alleles at loci that affect host preference cause the use of one or the other host. In other words, perhaps there is a preference trade-off. Are all combinations of host acceptance possible, or does accepting one host reduce the likelihood that others will be accepted? Quantitative trait locus mapping analyses can be used to evaluate this hypothesis at the genetic level (Via and Hawthorne 1998). Does preference for the two possible hosts map to the same or different places in the genome? If acceptance of alfalfa and acceptance of clover map to different chromosomal segments, they are not influenced by the same loci and thus cannot be involved in any fundamental trade-off. This work is currently in progress.

Pea aphids offer a unique opportunity to study both the genetic divergence between natural populations in the

field (e.g., Via 1991a, 1999) and the chemical and physiological basis of ecological specialization and assortative mating through habitat choice. Such studies can potentially open the door for the eventual identification of the underlying molecular and genetic mechanisms that give rise to local adaptation, genetic divergence, and even speciation in the absence of physical barriers to gene flow.

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