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The role of olfactory cues in the sequential radiation of a gall-boring beetle

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Bucknell University

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**THE ROLE OF OLFACTORY CUES IN THE SEQUENTIAL
RADIATION OF A GALL-BORING BEETLE**

By

Bradley C. Rhodes

A Thesis

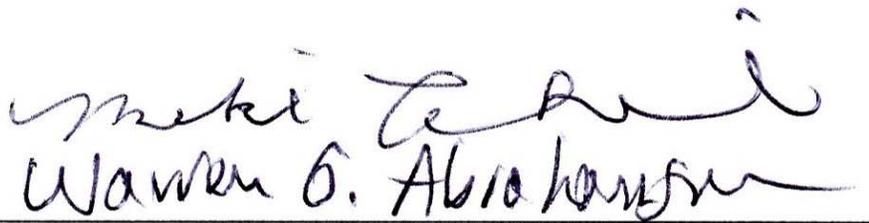
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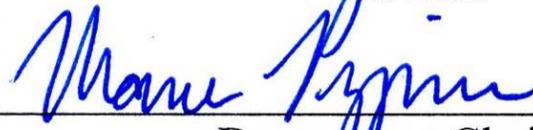
In Partial Fulfillment of the Requirements for the Degree of

Master of Science in Biology

Approved:


Warren O. Abrahamson

Adviser


Mame Pyzdek

Department Chairperson

Date:

5/2/11

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Abstract

Species diversity itself may cause additional species diversity. According to recent findings, some species modify their environment in such a way that they facilitate the creation of new niches for other species to evolve to fill. Given the vast species diversity of insects, the occurrence of such sequential radiation of species is likely common among herbivorous insects and the species that depend on them, many of them being insects as well. Herbivorous insects often have close associations with specific host plants and their preferences for mating and ovipositing on a specific host-plant species can reproductively isolate host-specific populations, facilitating speciation. Previous research by our laboratory has established that there are two distinct populations of the gall fly, *Eurosta solidaginis* (Tephritidae), which attack different species of goldenrods, *Solidago altissima* (Asteraceae) and *S. gigantea*. The gall fly's host-associated differentiation is facilitating the divergence and potential speciation of two subpopulations of the gall-boring beetle *Mordellistena convicta* (Mordellidae) by providing new resources (galls on stems of the goldenrods) for the gall-boring beetles. These beetles exist as two host-plant associated populations of inquilines that inhabit the galls induced by the gall fly. While our previous research has provided genetic and behavioral evidence for host-race formation, little is known about the role of their host plants in assortative mating and oviposition-site selection of the gall-boring beetles' host-associated populations. Volatile emissions from host plants can play a major role in assisting herbivores to locate their natal host plants and thus facilitate assortative mating and host-specific oviposition. The present study investigated the role of host-plant

volatiles in host fidelity (mating on the host plant) and oviposition preference of *M. convicta* by measuring its behavioral responses to the host-plant volatile emissions using Y-tube olfactometers. In total, we tested behavioral responses of 615 beetles. Our results show that *M. convicta* adults are attracted to their natal host galls (67% of *S. altissima*-emerging beetles and 70% of *S. gigantea*-emerging beetles) and avoid the alternate host galls (75% of *S. altissima*-emerging beetles and 66% of *S. gigantea*-emerging beetles), while showing no preference for, or avoidance of, ungalled plants from either species. This suggests that the gall beetles can orient to the volatile chemicals emitted by the galls and can potentially use them to identify suitable sites for mating and/or oviposition. Thus, host-associated mating and oviposition may play a role in the sequential speciation of the gall-boring beetle.

Introduction

The topic of biodiversity and how it forms holds keen interest in evolutionary ecology. One suggestion generated by this interest is the idea that species diversity itself may drive the creation of more species (Emerson and Kolm 2005). One reason for this is that organisms often modify their environment in such a way that they create new environments or new sources of food for other organisms to exploit (i.e., ecosystem engineering, Jones et al. 1994, Odling-Smee 2003, Wright and Jones 2006). Ecosystem engineering encompasses all of the changes that an organism makes to its environment (Wright and Jones 2006) and many of these changes have the potential to trigger speciation events which in turn cause more positive effects on species diversity and abundance (Jones et al. 1997).

Plant-insect interactions make excellent model systems for studying evolutionary phenomena, including diversification and the effects of ecosystem engineering, because of their abundance, remarkable species diversity, and rapid generation times. Many herbivorous insects are specialists that form intimate associations with a specific host plant or a specific host-plant organ (Gullan and Cranston 2005). Furthermore, the diversification of herbivorous insects with new host plants can lead to the subsequent diversification of other organisms that depend on the resources the herbivores provide, following the herbivore to novel hosts in a process known as sequential radiation (Abrahamson et al. 2001, 2003, Abrahamson and Blair 2008). When a population of an herbivorous insect shifts to a novel host plant, such a shift can result in the formation of insect host races, which are at least partly sympatric populations that are adapted to

different hosts. As a result, host races have limited inter-host gene flow at rates typically between one and 6% per generation (Feder et al. 1994, Drés and Mallet 2002). Genetic differences between the groups can then build up over time, which can lead to the formation of new species (Kelleher and Barbash 2010, Michel et al. 2010). Thus, host races exist on the continuum between a single polymorphic population and sister species (Drés and Mallet 2002). This phenomenon is likely widespread among herbivorous insects (Feder et al. 1994, Stireman et al. 2005) and other insects that depend on resources they provide (Stireman et al. 2006, Abrahamson and Blair 2008, Kolaczan et al. 2009).

In order for host races to develop in sympatry, some reproductive barriers must exist to facilitate host-associated genetic divergence (Abrahamson et al. 2001, Drés and Mallet 2002). One such reproductive barrier is host fidelity, which is the tendency to mate on an individual's natal host-plant species (Feder et al. 1994). Host fidelity reduces the chance of mixed matings between groups because their reproduction is segregated spatially. A simple preference for their host plant may be enough to facilitate differentiation of the host-associated populations. The effect is stronger if host fidelity is paired with habitat avoidance, which is the tendency to avoid the natal host-plant of closely related populations (Forbes et al. 2005). A second potential reproductive barrier is the tendency of females to oviposit on their natal host-plant species (Diehl and Bush 1984). Host fidelity and host-specific oviposition are the two barriers that will be investigated in the present study.

Several examples of sequential radiation have been described (Abrahamson and Blair 2008). One of the most extensively studied examples is that involving the sequential radiation of the parasitoid wasp, *Diachasma alloeum*, which has diversified into at least three host races following the shift of its host tephritid fly, *Rhagoletis pomonella*, onto novel fruit hosts (Glas and Vet 1983, Stelinski et al. 2004, Forbes et al. 2009). Another example, which is the model system of the present study, involves the gall-boring beetle, *Mordellistena convicta*, which is an inquiline of galls formed on two species of goldenrods produced by two host races of the tephritid fly, *Eurosta solidaginis* (Abrahamson and Weis 1997, Abrahamson et al. 2001). *Eurosta solidaginis* acts as an ecosystem engineer by inducing a sizeable gall on its host's stem. Although the production of the gall provides benefits for the fly larva, it also provides a resource that can be exploited by other insects, including *M. convicta* (Ping 1915, Uhler 1951, Abrahamson et al. 2001). Both behavioral evidence (Eubanks et al. 2003) and genetic and ecological evidence (Blair et al. 2005) suggest that *M. convicta* has formed host races that specialize on the galls of one of the two species of goldenrod subsequent to the host-race formation of the gall-inducer *E. solidaginis* (Abrahamson et al. 2001, 2003, Blair et al. 2005). The genetic evidence is supported by differences in the size of beetles associated with the two gall species and differences in parasitoid attack rates (Blair et al. 2005), either strong oviposition preference or differential larval survival on the two gall species, allochronic emergence of gall beetles, and limited evidence of assortative mating (Eubanks et al. 2003). Although Eubanks et al. (2003) found evidence of assortative mating, the levels at which mixed matings occurred in their experiments are considered

too high to be an effective barrier to gene flow. However, these experiments were conducted in the absence of host plants; hence host fidelity was not investigated. In order for organisms to display host fidelity and oviposition preference, they need to be able to recognize their natal host plant and to respond differently to stimuli provided by an alternate host plant. The present study seeks to explore the gall beetles' responses to potential volatile cues from their host plants, and based on how they respond to them, to infer the role of the host plants in the sequential speciation of the gall-boring beetle.

Importance of Olfaction and Y-tube Olfactometer Bioassay

The olfactory apparatuses of insects are typically very sensitive to minor differences in volatile chemicals produced by plants and hence many herbivorous animals that depend on plants are able to readily distinguish among plant species (Visser 1986, Bernays and Chapman 1994, Bruce et al. 2005). Some insects also have the ability to distinguish developmental stages of their host plants (Bengtsson et al. 2001) or can detect plant tissues that have been wounded by herbivory (Pare and Tumlinson 1997, De Moraes et al. 1998, Delphia et al. 2007).

Using a Y-tube apparatus, a Y-shaped tube of glass with air flowing into both of the arms of the tube and out the stem, it is possible to test if certain volatile chemicals are discernable by an organism and how exposure to the volatiles affects its behavior (Sabelis and Van de Baan 1983). The importance of olfactory cues from natal host plants has been well established for the sequential radiation of the parasitic wasp, *D. alloem* (Forbes et al. 2009), as well as among several species of beetles. In three species of longhorn beetles (*Megacyllene caryae*, *Neoclytus m. mucronatus*, and *Xylotrechus colonus*), presumably

unmated individuals of both sexes are attracted to volatiles of their natal plants, supporting the hypothesis that the beetles meet on distressed plants to mate, (Ginzel and Hanks 2005). Field-collected cranberry weevils, *Anthonomus musculus*, were also shown to be attracted to the volatile emissions from their host plants for mating, suggesting host fidelity (Szendrei et al. 2009). Although the ability of females to orient to these volatiles may be attributed to mate-seeking behavior and/or oviposition-site seeking behavior (depending on if they have mated or not), the attraction of males to the same volatiles is a sign that the orientation to the plant is a mate-seeking behavior (Linn et al. 2003).

In addition to being attracted to the volatile emissions of the natal host, there is evidence to suggest that the volatile emissions of alternative hosts may be avoided. One host race of *Rhagoletis pomonella* avoids odors of non-natal plants that are used by another host race of *R. pomonella* (Forbes et al. 2005). A tendency to move away from alternative hosts was also observed in *D. alloeum*, a parasitoid wasp of *R. pomonella* (Forbes et al. 2009). Specific chemicals or chemical cocktails associated with an inappropriate host may act as repellents, decreasing the likelihood of backcrossing and hybrids. Hybrids would receive conflicting attraction and avoidance signals from both hosts which could cancel one another (Forbes et al. 2005, Feder and Forbes 2010). The lack of response to the chemical emissions of either potential host plant by hybrids decreases their chances of finding a mate (Linn et al. 2004). Because of the decreased fitness of hybrids, active avoidance of alternative host plants may be under strong selection (Forbes et al. 2005).

Questions and Hypotheses

If host fidelity plays a role in the divergence of the host races of *M. convicta*, then gall beetles should orient to the volatile chemical emissions of their host-plant species or to galls of their natal host plant. Olfactory cues may be important in detecting host plants especially in dense sympatric fields. If unmated males and females and mated males orient themselves to the volatiles of their natal host plants or galls, this suggests that host plants or galls may be used as rendezvous sites at which beetles meet potential mates. Mated males will continue to show a similar preference, since they mate multiple times (Blair et al., unpublished data). If mated females orient themselves to the volatiles of their natal host galls, this suggests that olfaction is important to identification of appropriate oviposition sites. The motivation to eat the plants can be ruled out because adult gall-boring beetles do not feed on the host-plant goldenrods or their pollens when most of the mating is going on. Finally, if an avoidance behavior of alternative hosts occurs, such behavior would suggest that mechanisms are in place to prevent backcross mating between hybrids and parental host races. This would occur because hybrids would inherit conflicting attractance/avoidance signals from their parents (Forbes et al. 2005). Using both mated and unmated gall-boring beetles of both sexes from both hosts, the following three hypotheses were examined:

- (1) Male and unmated female beetles can sense volatile chemical emissions presumably to locate their natal host galls to use them as rendezvous sites for mating (host fidelity).

- (2) Mated female beetles can sense volatile chemical emissions presumably to locate their host galls for oviposition (oviposition-site preference).
- (3) Mated and unmated male and female beetles avoid alternate hosts (alternative-host avoidance).

Methods

Study System

The focal organism in the present study is the gall-boring beetle, *Mordellistena convicta* (Coleoptera: Mordellidae). It is a tumbling flower beetle. Gall-boring *M. convicta* exist as at least two host races (Abrahamson et al. 2003, Eubanks et al. 2003, Blair et al. 2005, Abrahamson and Blair 2008) that attack the galls created on species of goldenrod plants, including *Solidago altissima* (Asteraceae) and *S. gigantea*, by the host races of the gall-inducing fly, *Eurosta solidaginis* (Abrahamson and Weis 1997, Abrahamson et al. 2003). Larvae of gall-boring *M. convicta* tunnel through and feed on the gall tissues and often facultatively prey on the gall-fly larva if they tunnel into it (Uhler 1951, Blair et al. 2005).

Solidago altissima and *S. gigantea* are closely related plant species in the *S. canadensis* species complex (Abrahamson and Weis 1997, Abrahamson et al. 2001, 2003). The two species are widely distributed throughout much the continental United States and southern Canada, with the exceptions of the west coast and the southwest. These species are sympatric over much of their ranges, and clones of both species are often found living side-by-side, although the plants have slight differences in microsite

preferences (Abrahamson 2005). They tend to live in open spaces such as in old fields and along roadsides (Abrahamson et al. 2003, 2005).

Eurosta solidaginis oviposits in the apical bud of its goldenrod host (Uhler, 1951). Upon hatching, the larva tunnels to the base of the apical meristem where it often induces the development of a gall. *Eurosta solidaginis* larvae feed on the nutritious gall tissues that surround them and remain in their galls as diapausing third-instar larvae throughout the winter. In the spring, the larvae quickly pupate, and adult gall flies emerge from their galls to mate and oviposit (Abrahamson and Weis 1997, Weis and Abrahamson 1998). *Eurosta solidaginis* exists as at least two host races, specializing on *S. altissima* and *S. gigantea* (Waring et al. 1990, Craig et al. 1993, 1997, Brown et al. 1996). The two host races are behaviorally isolated via host fidelity, which leads to assortative mating, and oviposition-site preference (Craig et al. 1993). The two host races are also partially temporally isolated via allochronic emergence, although the tail end of the breeding season of the *S. gigantea* host race overlaps with the beginning of the breeding season of the *S. altissima* host race (Craig et al. 1993, Craig et al. 2000). Also, *E. solidaginis* larvae that had been oviposited in the galls of the alternate host plant during a forced no-choice oviposition experiment showed reduced survival and lower emergence rates compared to those that were raised in their natal host plant (Craig et al. 2007). Furthermore, natural selection acts against hybridization given that hybrid and backcross offspring suffer low gall-induction rates and hence, low survival rates (Craig et al. 1997).

Although the gall inducer and the causes of the divergence of its host races are well understood, the reproductive barriers between the host races of *M. convicta* have not

been as well investigated. Eubanks et al. (2003) tested assortative mating in the absence of host-plant cues, oviposition choice and no-choice experiments with natal and alternate host-species galls, and allochronic emergence under different temperature conditions. The beetles showed a preference for mating assortatively, but the percentage of mixed matings that occurred in the mating experiments was too high for this to be an effective barrier by itself if mating occurs in the absence of the host plants. For beetles reared from *S. altissima* galls, over 21% of their matings occurred with beetles reared from *S. gigantea* galls. For beetles reared from *S. gigantea* galls, nearly 15% mated with beetles reared from *S. altissima* galls. Because these mating experiments were not carried out in the presence of host plants, any effect that cues from their host plants may have on host fidelity were not investigated. This means that an appreciable but insufficient amount of assortment occurs in the absence of host plants for the host-race formation of the gall-boring beetle. However, plant cues may strengthen these assortative behaviors. In choice oviposition experiments, upwards of 80% of emerging beetles came from the natal host species of the parent, indicating that the same trend holds when the beetles were provided with multiple plants they could oviposit on. The difference in the timing of the emergence of the two host races of *M. convicta* (approximately 2 to 10 days depending on temperature) is small compared to the lifespan of the beetle (one month or more). Hence, it is unlikely that allochronic emergence is an important barrier to reproduction. This suggests that gall beetles have display host fidelity, a preference for ovipositing on their natal host, decreased larval survival rates on alternative hosts, or some combination of these.

Sampling

To obtain *E. solidaginis* and *M. convicta*, ball galls stimulated by *E. solidaginis* on *S. altissima* and *S. gigantea* stems were for this experiment and another one collected between 17 and 20 December 2009 and on 20 March 2010 from six sites in VT and northern NY, a region where the two host races of gall-boring *M. convicta* beetles occur in sympatry (Table 1). Beetles from a sympatric area were used because beetles from these populations must be actively segregating themselves, otherwise genetic differences between the two populations would be swamped out due to hybridization (Blair et al. 2005). The galls were collected after the goldenrod stems had senesced and the insects inside the galls had entered winter diapause. Enough galls were collected to rear out approximately 500 adult beetles from each host species of goldenrod.

Table 1. Collection dates, sites, and numbers of galls.

Date Collected	Site	Coordinates	<i>S. altissima</i> Galls	<i>S. gigantea</i> Galls
12/17/2009	Philadelphia, NY	N44°10' W75°39'	1500	100
12/18/2009	Upper and Lower Lakes Wildlife Management Area, Canton, NY	N44°37' W75°15'	520	3900
12/19/2009	11 km northeast of Potsdam, NY	N44°44' W74°52'	0	2500
12/19/2009	5 km north of Altoona, NY	N44°55' W73°40'	0	1750
12/19/2009	Northwest Corner of Vermont	N44°58' W73°13'	0	750
12/20/2009	Georgia Center, VT	N44°42' W73°61'	7000	0
3/20/2010	Georgia Center, VT	N44°42' W73°61'	4000	0

Preparation of Plant Specimens

Rhizomes of *S. altissima* and *S. gigantea*, which are both clonal, were collected from Missisquoi National Wildlife Refuge, VT (44°57'14.96"N, 73°12'22.31"W) on 18 October 2009 and from Green Mountain Audubon, VT (44°20'51.17"N, 72°59'33.53"W) on 22 October 2009. Both sites contained galled plants of both species. The rhizomes

were overwintered in ProMix BXTM with Mycorise Pro (Premier Horticulture, Quakertown, PA) in a cool greenhouse. During the last week of March 2010, the rhizomes were dug up, cut into 5-cm long pieces, and planted in 53 x 26 cm trays containing ProMix BXTM with Mycorise Pro with up to 60 rhizome fragments of a single genet and allowed to grow in a warm greenhouse. After new ramets sprouted, they were transplanted into 20-cm standard pots. Pots were watered as needed and treated with a solution of Peters ProTM 15-16-17 (N-P₂O₅-K₂O, 3.71 g/l, 119 ml per pot) fertilizer once every two weeks. Greenhouse pest populations (mostly thrips) were controlled as needed by hand and with the predatory mite (*Neoseiulus cucumeris*) from IPM Laboratories (Locke, NY).

Galls were induced on 72 *S. altissima* and 14 *S. gigantea* ramets using *E. solidaginis* flies that emerged from the same galls the gall beetles emerged from. Six of each of these were used in this experiment and the rest were used in another. The gall flies were released into 3.2 x 1.0 m mesh-covered oviposition cages within the greenhouse to freely mate and oviposit on their host plants in May and June 2010. Since the gall flies live for only days after emergence, they were reared sequentially in two cohorts. The second cohort was held in the -20°C freezer for three more weeks than the first cohort. Each host race of gall flies was caged separately so that goldenrod ramets could be oviposited on by flies that emerged from the same plant species. None of the goldenrod ramets used in the ungalled stem conditions of the experiment were placed in oviposition cages or observed to have oviposition scars on their apical bud, which indicates that these were not attacked by the female flies.

Insect Care

The collected galls were stored at -20° C until they were removed from storage for rearing. To rear the insects, galls were placed in screen-covered emergence cages in growth chambers at 23° C, 80% relative humidity, and 15:9 hrs of light:dark daily, which mimics the photoperiod at their normal spring emergence time. Cages were monitored multiple times daily for emerged insects. During periods of emergence, the beetles were removed from the cages five times a day, with more frequent checks corresponding to time of day when most of the beetles emerged (mid-day). Beetle removal was carried out so frequently in order to catch the beetles as they emerged, before they had a chance to mate. Captured beetles were housed individually in 30-ml plastic containers containing a disk of filter paper and a small bit of crushed bee pollen from Y.S. Organic Bee Farms for food. The filter paper was moistened twice a week to provide water to the gall beetles. The beetles were housed for at least 2 days before attempting to mate them and use them in the experiment. Previous research has indicated that there is a lag between emergence and oviposition (Weis and Abrahamson 1985). During the waiting period, the containers were stored in growth chambers at 10°C at 80% relative humidity and with a 15:9 hour light:dark cycle. The beetles were removed from the growth chambers for one hour every day, during the light period to allow them to warm up and eat or drink. This also served to reduce the possibility that beetles would search for food while being used in the experiment.

Mating Procedure

Beetles emerging from the galls of a single species of goldenrod were placed in Petri dishes in groups of three, 2-3 days after emergence. Groups of three were used because it is not possible to determine the sex of *M. convicta* by external observation. If only pairs were used, about half of the beetles would have been paired with members of the same sex. Gall beetles were observed for at least an hour or until they mated, and then they were returned to their 30-ml containers. Beetles that were observed to mate were used in the experiments with mated beetles and those observed not to mate were used in the unmated experiments.

Apparatus

Y-tube

olfactometers were used to examine the gall beetles' ability to detect volatile cues, and their preferences for them. The design of our olfactometers followed that of Sabelis and Van de Baan (1983) and Tooker et al. (2005) (Fig. 1). A 2-cm inner-diameter glass

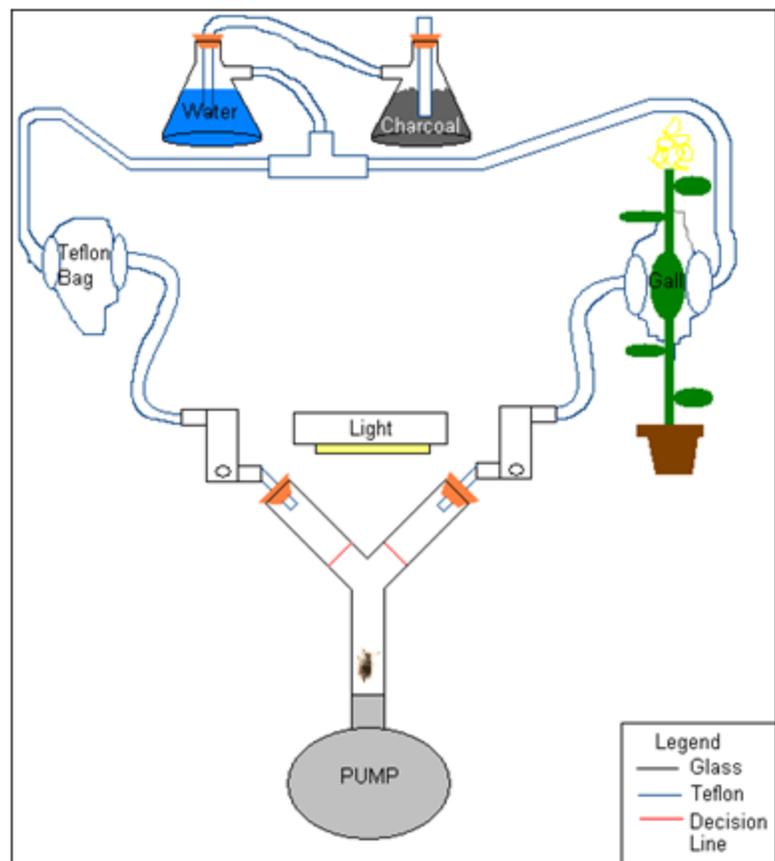


Figure 1. Y-tube olfactometer.

Y-shaped tube was connected at its stem to a vacuum pump, which pulled air through the tube. Each Y-tube arm was connected to an acrylic-tube rotameter with stainless-steel end fittings (Muis Control Ltd., model FR-2A12-SVVT) to facilitate fine adjustment of the volume of air flow through each side of the apparatus at a rate of 100 ml per minute (200 ml per minute through the stem tube and into the pump). Teflon PDA tubing (McMaster-Carr, catalog numbers 51805K73 and 51805K55) connected each of the rotameters to a 2-mm thick, 15.24 x 17.78-cm Teflon bag with 6.35-mm Kynar fittings in the middle of each of the two faces (Welch Fluorocarbon Inc.). The bags were used to contain either the volatile-emitting sample or an odorless control (empty glass vial). Each bag was connected to Teflon tubing that joined at a Teflon PFA tube-fitting tee (McMaster-Carr, catalog number 52195K43) which connected to a Büchner flask containing water and finally to another Büchner flask containing activated charcoal (Kalyx, catalog number 210058-51).

All volatile-emitting samples were intact plants rather than cut stems to ensure that none of the volatile emissions were associated with plant-wound responses. The bags were not sealed across the top or bottom so they could be slipped on over the top of the plant and moved down to the height where galls develop or to similar-age non-galled stems. Once positioned at the right height, the bags were tied shut using twist ties firmly secured around the stems of the plants. The twist ties were secured as tightly as possible without damaging the stems of the plants. They were positioned above and below a length of the plant that contains the gall and as little stem and leaf material as possible or

that contains a bare stretch of the stem (depending on the experimental stimulus). This procedure separated the response to volatiles emitted by the gall or stem of the plant.

During a trial, the vacuum pump pulled air through the activated charcoal to filter out any ambient organic volatiles. Then, the air passed through the flask with water to rehydrate the air inside the Y-tube apparatus. After passing through the water, the air stream was split into two paths which met again in the Y-shaped glass tube carrying air that had been exposed to either the experimental treatment or a blank control and past the beetle subject before being pulled into the vacuum pump. All materials used in the construction of the olfactometer are inert to prevent confounding effects of volatile emissions from the apparatus itself.

Gall beetles, like many insects, show preferences for climbing and moving toward sources of light (B.C. Rhodes, personal observation). Because of this, elevation and light were used to entice the beetles to make a decision in the experiment, as has been done in similar studies (Ginzel and Hanks 2005, Voss et al. 2009). The Y-tube was angled at 15° so that the arms of the Y-tube were higher than the stem. A 15-watt T-8 fluorescent 4100 K white light was centered in front of the Y-shaped tube. Light intensity emanating from the light fixture at the distance where gall beetles were loaded was 3.0 $\mu\text{E}/\text{m}^2/\text{s}$ and the light intensity at this distance inside the Y-tube apparatus was 2.3 $\mu\text{E}/\text{m}^2/\text{s}$ – the difference being due to the filtering effect of the glass.

Experimental Procedure

The gall beetles' ability to sense, and their responses to, volatile emissions from their natal and alternate host plants and galls were tested using the Y-tube olfactometer.

All trials were carried out in a photographic dark room and the only source of light during testing was the fluorescent light in front of the Y tube. The plants were not visible to the beetles so that orientation to visual cues can be ruled out. Beetles were loaded into the stem of the Y-tube and then the system was sealed and the vacuum pump turned on. Beetles were observed for 10 min or until they moved more than 2 cm up one of the arms of the Y-tube. Ten min was chosen as the observation time because conventional times used by Y-tube olfactometer-assay studies span 5 to 15 min (Sabelis and van de Baan 1983, Takabayashi and Dicke 1992, Ginzl and Hanks 2005, Szendrei et al. 2009, Voss et al. 2009) and in preliminary trials, beetles that did not make a decision in 10 min were not active and did not make a decision within 15 minutes (B.C. Rhodes, personal observation). Any trial where the beetle failed to move past either of the Y-tube arm decision lines after 10 min was recorded as a no-decision. The experimental stimulus side of the Y-tube was alternated after every five trials of the experiment and the plant was changed every ten trials. These two measures were taken to control for any directional preferences of the beetles in the Y-tube and to overpower the variation between individual galls.

A number of experimental conditions were carried out to test the beetles' preferences when exposed to the galls and ungalled stems of their natal and alternative host species. Unmated and mated male beetles and unmated female beetles were used to test Hypothesis 1 that they use olfactory stimuli to orient to their natal hosts' galls as a mate-seeking behavior. Mated females were used to test Hypothesis 2 that they use olfactory stimuli to orient to their natal hosts' galls for oviposition. Only galls were used

as stimuli for the mated beetles because oviposition necessarily occurs on galls and it is unlikely that a sufficient number of mated beetles were obtained to test them using both galls and ungalled stems. No-choice experiments with alternate host's galls and ungalled stems were also carried out to test Hypothesis 3 that beetles avoid alternate host plants or galls to prevent mixed matings. The experimental conditions tested are summarized in Table 2. All of the experiments were carried out with beetles that were 3 to 25 days old. The mean age of gall beetles tested was 13.4 days with 0.226 standard error.

Table 2. Expected results for each no-choice experimental condition.

Stimulus	Natal Host of Beetle	Mating Status	Predicted Outcome	Hypothesis Tested*
Natal host gall	<i>S. altissima</i>	Unmated	Attraction	1
Natal host gall	<i>S. gigantea</i>	Unmated	Attraction	1
Natal host gall	<i>S. altissima</i>	Mated	Attraction/Indifference**	1, 2
Natal host gall	<i>S. gigantea</i>	Mated	Attraction/Indifference**	1, 2
Alternative host gall	<i>S. altissima</i>	Unmated	Avoidance	3
Alternative host gall	<i>S. gigantea</i>	Unmated	Avoidance	3
Alternative host gall	<i>S. altissima</i>	Mated	Avoidance/Indifference***	3
Alternative host gall	<i>S. gigantea</i>	Mated	Avoidance/Indifference***	3
Natal host stem	<i>S. gigantea</i>	Unmated	Attraction or Indifference	1
Alternative host stem	<i>S. gigantea</i>	Unmated	Avoidance or Indifference	1
Both Galls	<i>S. gigantea</i>	Unmated	Toward Natal Host	1, 3

*Hypothesis 1 is host-fidelity; hypothesis 2 is oviposition-site preference; hypothesis 3 is alternative-host avoidance.

**Females should be attracted; males may be attracted or indifferent.

***Females should avoid; males may avoid or be indifferent

Sexing of Beetles

The sex of *M. convicta* cannot be determined in life unless they are observed to mate so they were sexed post mortem. Gall beetles were placed under a Wild dissecting scope at 75X to 625X and pressure was applied to their abdomen using a probe or a pin

while the beetle lay on its side in order to evert either the female ovipositor or the male aedeagus. The structures were identified on sight.

Statistical Analysis

A binary logistic regression was carried out using Predictive Analytics Software (PASW), version 18.0 (SPSS Inc.) to test for effects of stimuli on the gall beetles' decision because there are only two choices that can be made in the olfactometer. Furthermore, the binary logistic regression model allows for potential predictive variables to be considered (Ramsey and Schafer 2002). If there is no effect of volatile chemicals on the preference of the beetle, it would have an equal chance of choosing either arm so any deviations were compared to the null hypothesis of 0.5. Instances where the gall beetle did not cross either of the decision lines during the experiment were not used in this analysis, but are reported.

A number of potential predictive variables were considered in each of the statistical models run on the data (Table 2). For each of the no-choice experiments in which the stimulus was a gall, the following variables were included in the model: genotype of the stimulus plant, age of the gall beetle (i.e., days since emergence), mating status of the beetle (mated or unmated), gall collection site from which the beetle came from, sex of the beetle, and every possible interaction between the age, mating status, and sex. For the two no-choice experiments in which unmated beetles emerging from *S. gigantea* galls were presented with ungalled stems, the following variables were considered in the original model: genotype of the stimulus plant, age of the gall beetle, field site from which the gall which produced the beetle came from, sex of the beetle, and

the interaction between the age and sex of the beetle. For the choice experiment, the following variables were considered: genotype of the stimulus *S. altissima* plant, age of the gall beetle, genotype of the stimulus *S. gigantea* plant, origin of the gall which produced the beetle being used, sex of the beetle, the interaction between the age and sex of the beetle, and interaction between the two plants used as stimuli. A manual backwards elimination was carried out for each of the statistical analyses, removing variables one by one until the best model was determined for each. The variables tested in each condition are summarized in Table 3. The quality of each model was evaluated using the Akaike information criterion (AIC) and the Bayesian information criterion (BIC) to determine the goodness of fit of the respective statistical models. In every case, the two information criteria were in agreement concerning the best model.

Table 3. Predictive variables considered in each experimental condition. The list of predictive variables is provided below the table.

Stimulus	Predictive Variables Considered
One gall	1, 2, 3, 6, 7, 8, 9, 10, 11
One ungalled stem	1, 2, 3, 7, 9
Both galls	1, 2, 4, 5, 7, 9, 12

1. Age of gall beetle
2. Gall collection site from which the gall beetle came from
3. Genotype of plant used
4. Genotype of *S. altissima* plant used
5. Genotype of *S. gigantea* plant used
6. Mating status of gall beetle
7. Sex of gall beetle
8. Interaction between age and mating status of gall beetle
9. Interaction between age and sex of gall beetle
10. Interaction between mating status and sex of gall beetle
11. 3-way interaction among age, mating status, and sex of gall beetle
12. Interaction between the two plants used

Results

Experimental Validity and Sample Sizes

A chi-square test for an intrinsic Y-tube arm bias of the beetles showed that the gall beetles displayed no significant preference for one arm or the other (52.0% right vs. 48.0% left), $\chi^2(1, 552) = 1.13, p = 0.228$. A total of 615 gall beetles were tested across all experiments and of these, 578 made decisions. The number of non-deciding beetles was 37, accounting for 6.0% of the total. The 25 beetles that made a decision, but could not be sexed were also excluded from the analyses. The total number of excluded observations accounted for a total of 10.1% of the data. The numbers of beetles in each condition that were excluded from the analysis are reported in Table 3.

Table 3. Number of trials and hypotheses tested in each of the experimental conditions.

Stimulus	Natal Host of Beetle	Mated Status	Number of Trials (Number Excluded)	Hypothesis Tested
Natal host gall	<i>S. altissima</i>	Mated	31 (5)	1**, 2**
Natal host gall	<i>S. altissima</i>	Unmated	41 (9)	1**
Natal host gall	<i>S. gigantea</i>	Mated	56 (5)	1**, 2**
Natal host gall	<i>S. gigantea</i>	Unmated	56 (7)	1**
Alternative host gall	<i>S. altissima</i>	Mated	28 (2)	3**
Alternative host gall	<i>S. altissima</i>	Unmated	45 (5)	3**
Alternative host gall	<i>S. gigantea</i>	Mated	59 (3)	3**
Alternative host gall	<i>S. gigantea</i>	Unmated	64 (6)	3**
<i>S. altissima</i> stem	<i>S. gigantea</i>	Unmated	59 (8)	1
<i>S. gigantea</i> stem	<i>S. gigantea</i>	Unmated	57 (7)	1
Both galls	<i>S. gigantea</i>	Unmated	57 (5)	1**, 3**

**Hypothesis 1 is host-fidelity; hypothesis 2 is oviposition-site preference; hypothesis 3 is alternative-host avoidance.

**Hypothesis supported.

All of the experimental conditions were carried out for the beetles emerging from the *S. gigantea* galls. There were not enough beetles emerging from the *S. altissima* galls to carry out all possible tests. Beetles from *S. altissima* were not tested in either

experiment involving ungalled stems. Additionally, a choice experiment was carried out with unmated beetles that emerged from *S. gigantea* galls to test their response when exposed to volatiles from the galls of both hosts simultaneously, mimicking conditions frequently experienced in the beetles' natural environment. The goal was to have 60 replicates of each treatment condition, but low emergence of beetles from *S. altissima* galls, non-deciduers and beetles that could not be sexed altered the number of beetles used in each treatment. The actual numbers of trials of each condition carried out are reported in "number of trials" column in Table 3.

Attraction to Natal Host Galls

Both host races of beetles displayed a preference for moving toward the chemical emissions from their natal host galls. *Solidago altissima* beetles significantly preferred the Y-tube arm from which the volatiles were coming, $Wald \chi^2 (1, 71) = 8.95, p < 0.001$ (Figure 2 [top]). *Solidago gigantea* beetles also displayed a significant preference for their natal host gall, $Wald \chi^2 (1, 111) = 17.74, p < 0.001$ (Figure 2 [bottom]). An analysis of the goodness of fit using AIC and BIC showed that the best model took no predictive variables into effect, including the sex and mating status of the beetles. The finding that there was no effect of mating status or sex means that the first and second hypotheses, concerning host fidelity and oviposition preference, can be considered together since the preferences of mated females are not significantly different from those of unmated males or females or mated males. The findings support the first two hypotheses.

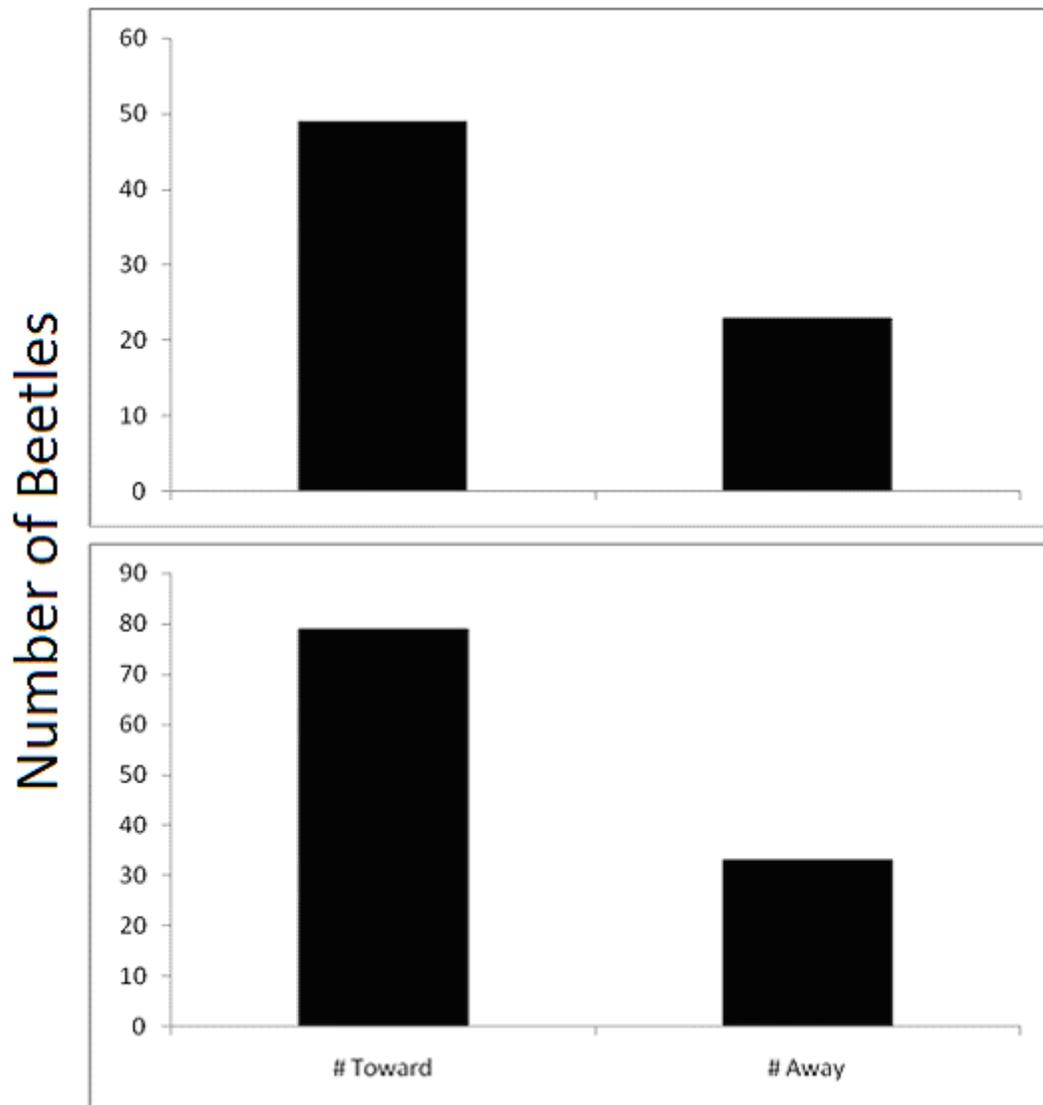


Figure 2. Decisions of gall beetles when exposed to their natal host galls. The bars show the number of gall beetles that made each decision from the (top) *S. altissima* ($p < 0.001$) and (bottom) *S. gigantea* ($p < 0.001$) host races.

Avoidance of Alternate Host Galls

The beetles displayed a significant preference to move away from the volatile chemical emissions of the alternate host-race's gall, supporting the third hypothesis. This was true both for beetles emerging from *S. altissima* galls, $Wald \chi^2 (1, 72) = 13.00, p < 0.001$ (Figure 3 [top]), and *S. gigantea* galls, $Wald \chi^2 (1, 122) = 10.78, p < 0.001$ (Figure

3 [bottom]). The best model for the beetles emerging from *S. gigantea* galls included no predictive variables. This is consistent with the third hypothesis, which predicted that the beetles would avoid the volatile chemical emissions of the galls of the alternate host race.

Two predictive variables remained during the analysis of beetles emerging from *S. altissima* galls. The sex of the beetle was significant ($Wald \chi^2 [1, 72] = 6.901, p = 0.009$), with males being more likely to avoid the alternate host gall than females (Figure 3 [top]). The interaction between age and sex was also significant, with older females being more likely to avoid the alternate host gall than younger females ($Wald \chi^2 [1, 42] = 6.524, p = 0.011$). There was no significant trend among males with regards to age ($Wald \chi^2 [1, 29] = 0.184, p = 0.184$).

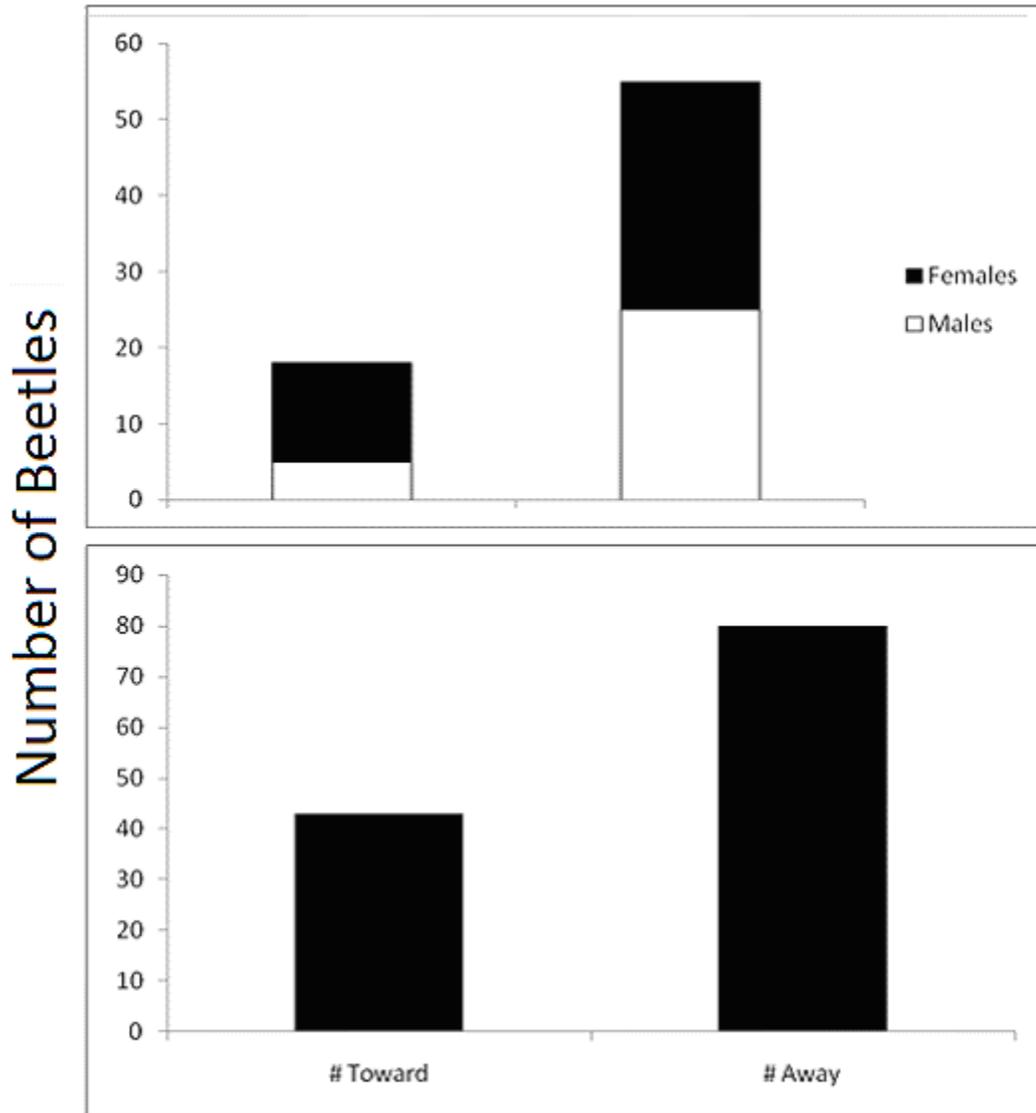


Figure 3. Decisions of gall beetles when exposed to the alternate host galls. The bars show the number of beetles that made each decision from the (a) *S. altissima* ($p < 0.001$) and (b) *S. gigantea* ($p < 0.001$) host races.

Lack of Preference for Ungalled Stems

Gall beetles displayed no significant attraction to their ungalled host plant, *S. gigantea*, $Wald \chi^2 (1, 56) = 0.437, p = 0.508$. The best model took no predictive variables into account. Similarly to the results of the condition in which they were exposed to the

volatile chemical emissions of *S. altissima*, no significant trends were found, $Wald \chi^2 (1, 58) = 0.827, p = 0.363$. The best model excluded all predictive variables. The gall beetles' behavior when confronted with the volatile emissions from the ungalled stems of its host plant and the alternate host were investigated to test if the beetles were specifically responding to the volatiles emitted by the galled plants or if they had a general preferences regarding the host plants. Only beetles emerging from *S. gigantea* were used in these conditions.

Choice Experiment

A choice experiment was carried out in which *S. gigantea* beetles were exposed to the volatile chemical emissions of a natal *S. gigantea* gall and the gall of *S. altissima*. The *gigantea* beetles showed a preference for moving toward the volatile cues of their natal host and away from the cues of the alternate host, $Wald \chi^2 (1, 56) = 7.363, p = 0.007$ (Figure 4). There were no significant predictive variables in the best-fitting model. These findings are consistent with what would be predicted by the hypotheses. Hypotheses 1 and 2 predicted that gall beetles would show a preference for their natal host gall while hypothesis 3 predicted that they avoid the alternate host gall. The experiment was set up to be more like what the beetle would experience in the field, with olfactory cues coming to the beetle from more than one plant species.

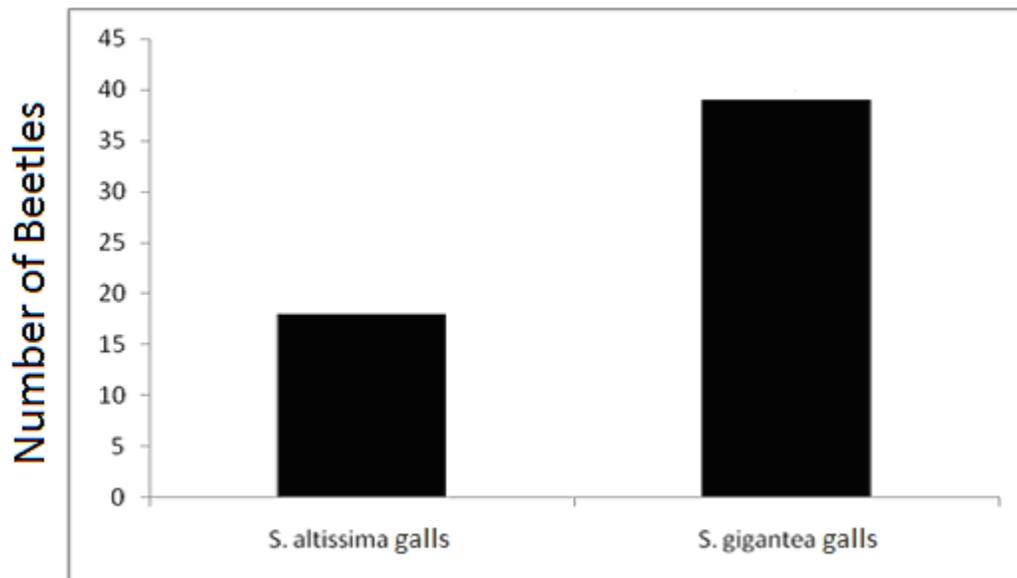


Figure 4. Decisions of gall beetles from *S. gigantea* galls when simultaneously exposed to the galls of both host plants ($p = 0.007$).

Discussion

Attraction to Natal Host Galls

The results of the experiments show that gall beetles can sense and react to the volatile chemical emissions from their own natal host gall and an alternate host gall. Gall beetles had clear preferences when exposed to each of these stimuli. This is consistent with findings in other insect-host plant interactions (Visser 1986, Bernays and Chapin 1994, Bruce et al. 2005). Gall beetles are likely using the volatile emissions from host plants to distinguish among these two plants, as well as other closely related species they encounter in the field. This opens up the possibility of the host plant acting as a spatial barrier to reproduction between the two host-associated populations of gall beetles.

Since there were no differences between mated and unmated beetles in any of the experimental conditions, and males and females display the same basic patterns across conditions. This suggests that these insects are probably using the same mechanisms to locate their host plant for finding mates and finding oviposition sites. This makes the model more parsimonious because it appears that only one change in preference has occurred, instead of two.

The gall beetles were attracted to the volatile chemical emissions of their natal host plants regardless of their sex or mating status. They have more opportunity to mate with members of the same host race if males and females of the same host race come to the same type of site when seeking mates (Linn et al. 2003, Szendrei et al. 2009) and oviposit on the correct host if they are naturally drawn to it. These findings support Hypotheses 1 and 2, which proposed that the beetles are attracted to the volatile chemical cues of their host plants for pairing up mates and ovipositing, respectively. The findings suggest that host fidelity and host-specific oviposition are two barriers to gene flow that exist between the two host races. Taken together with the findings of previous research including oviposition/differential survival experiments (Eubanks et al. 2003), and gall beetle transplant experiments (Blair et al. 2010), there is strong evidence for oviposition-site preference, and relatedly host fidelity since the two seem to be operating via the same mechanism.

Avoidance of Alternate Host Galls

The gall beetles tended to avoid the emissions of the alternative host galls. The avoidance behaviors observed in this experiment are similar to findings from two other

species with multiple host races, *R. pomonella* (Forbes et al. 2005) and *D. alloeum* (Forbes et al. 2009), and are consistent with Hypothesis 3. Avoidance behaviors for non-natal hosts can be an isolating mechanism that operates to prevent backcrossing between hybrids and parental host races. This would be the case if the insects have the potential to inherit the attraction preference for each of the host plants as well as the avoidance preferences for each of the host plants, resulting in non-preference for either plant species. This makes it hard for them to find potential mates or oviposition sites. This mechanism allows for increased specialization and decreased production of hybrids with lower fitness (Linn et al. 2004, Forbes et al. 2005, Feder and Forbes 2010).

In addition to decreasing the fitness of hybrids, avoidance of the alternate host galls may help to reduce mixed matings and oviposition mistakes. It may lead to the spatial segregation of beetles, on a microhabitat level, because they are repelled by the volatiles emitted from the alternate host galls and attracted to the natal host galls. Since the two species of goldenrods tend to grow in patches that may be interspersed in a field, the attraction behavior may draw the beetles into a patch of the correct species and the avoidance behavior may keep them confined to that patch once they find it. If this is the case, then the beetles looking to mate will mostly segregate by host plants. Within such environments, and given choices of mates that would be expected in the field, the rate of assortative mating that occurs between host races may be smaller than the roughly 20% chance of mixed matings observed by Eubanks et al. (2003). Assortative mating probably evolved after the change in host preference as an additional barrier to mixed matings.

The only treatment in which any of the predictive variables were included was that in which gall beetles emerging from *S. altissima* plants were exposed to volatiles from *S. gigantea* galls. Here, male *altissima* beetles were more likely to move away from the volatile chemical emissions of *gigantea* galls than were female *S. altissima* beetles. In addition, older females were more likely to move away from the galls than younger females. This result seems puzzling since females presumably invest more energy into reproduction than the males do via egg production versus sperm production.

A reason for why males may respond to mating cues earlier in their lives than females may be that they are employing some tactic to gain reproductive advantages over other males (Emlen and Oring 1977). One possibility is that they are trying to make themselves available to females for a longer length of time. Males may produce viable sperm earlier in their lives than females produce mature eggs. Displaying host preferences earlier in their lives may grant them an increased chance to mate. It may even be the case that males claim territories. Why this tendency occurs in only one population of the beetles is unclear without further investigation.

Reactions to Ungalled Stems

Gall beetles emerging from *S. gigantea* displayed no attraction for, or avoidance of, ungalld stems of either host-plant species. This suggests that the beetles are only responding to the galls, rather than the plant itself. This makes sense since the larvae of this host race of beetles is adapted to feed on galls, not stems (Blair et al. 2010). A lack of preference for stems could help to keep them separate from other species within the *M. convicta* species complex that attack the stems of these plants (Blair et al. 2005). The

volatiles emitted from the galls and ungalled stems must be different, especially if the beetles are cuing in on a wound response emitted by the plants in response to gall formation (Takabayashi et al. 2006) since the beetles emerge as the first galls become discernible (Weis and Abrahamson 1985, Abrahamson and Weis 1997). There is evidence that the volatiles emitted by galled and ungalled *S. altissima* plants have different concentrations of salicylic acid (Tooker et al. 2008), a hormone frequently emitted by plants that have been wounded that appears to provide them protection against some pathogens and herbivores (Bennett and Wallsgrave 1994, Rani 2006, Jahangir et al. 2009).

Sequential Radiation and Ecosystem Engineers

To date, most of the research on sequential radiation has focused on parasitoids of herbivorous insects (Abrahamson et al. 2003, Abrahamson and Blair 2008, Feder and Forbes 2010), but niche exploiters of ecosystem engineers seem to follow the same basic ecological and evolutionary trends as organisms that directly parasitize. Both groups use volatile chemicals emitted by plants to locate their hosts and both seem to be strongly associated with the host plant, though the beetles investigated in this study specifically respond to plants being attacked by the gall fly. Like parasites and other natural enemies, niche exploiters such as in the goldenrod-insect system, have the potential to affect their niche constructor and may pose a threat to the organisms they are evolutionarily tracking (Crespi and Abbot 1999, Abrahamson et al 2003, Feder and Forbes 2010).

Although gall-boring *M. convicta* do not always kill the niche constructor (Uhler 1961, Blair et al. 2005), other organisms exploiting niches opened up by herbivorous

insects do. Members of the genus *Kladothrips* (Thysanoptera: Phlaeothripidae) stimulate gall production on plants of the genus *Acacia* (Fabaceae) (Crespi 1992). The galls created are invaded by members of the genus *Koptothrips* (Thysanoptera: Phlaeothripidae) which kill the gall inducers and use the conquered galls for mating and ovipositing (Crespi and Abbot 1999). *Kladothrips* attack many *Acacia* species and are tracked evolutionarily by members of *Koptothrips* that seek to take over their galls (Crespi and Abbot 1999). Although it is not known how the *Koptothrips* find their respective hosts, at least oviposition preference for their natal hosts is apparent. Although the *Koptothrips* are more interested in the engineered ecosystem than their prey, they act functionally as a parasitoid because the killing of the gall-inducer is obligate.

In both of the plant-insect systems (i.e., *Eurosta* and *Rhagoletis*) in which sequential radiation has been well-studied (Abrahamson et al 2003, Feder and Forbes 2010), the niche exploiter poses an appreciable threat to the ecosystem engineer, but this does not necessarily need to be the case. Since niche exploiters do not need to feed on the organisms they are evolutionarily tracking, there is no reason that the other organism always must be killed. In fact, it would be beneficial to the niche exploiter to leave its ecosystem engineer alone to relieve the evolutionary pressure being applied and enable the potential for more suitable habitats to open up for subsequent generations. Morris et al. (2000) discovered an example of an organism like this that exploits the habitat of the *Acacia* galls. *Advenathrips inquilinus* (Thysanoptera: Phlaeothripidae) is a species of thrips that is a true inquiline, leaving its ecosystem engineer unmolested. Therefore, these organisms do not need to be natural enemies. It may be necessary to broaden the

definition of sequential radiation (Abrahamson et al. 2003) from “systems in which the differentiation of an herbivore influences the differentiation of a second unrelated natural enemy, with the result of increasing biodiversity up the trophic system” to systems in which the differentiation of an herbivore influences the differentiation of a second unrelated organism that depends on specific resources provided by the herbivore on the host plant, with the result of increasing biodiversity in the trophic system.

Following the line of reasoning that the ecosystem engineer does not need to be harmed in order for the organism dependent upon it (not necessarily a natural enemy) to flourish, a single ecosystem engineer may support multiple commensal organisms. In addition, the organisms supported may also be herbivores meaning that more resources will be available than if the supported organism were at a higher trophic level. This means that instead of biodiversity increasing solely up the trophic ladder as previously proposed (Abrahamson et al. 2003), sequential radiation may be able to account for increasing biodiversity at a single trophic level.

Although cascading diversity up the trophic ladder explains a lot of the diversity observed among insects, cascading diversity sideways across a single rung of the trophic ladder has the potential to explain even more. Each horizontal step also opens up room for specialist natural enemies as well. Just as *M. convicta* has different parasitoids than *E. solidaginis*, each niche exploiter across the trophic ladder will open up additional niches for natural enemies to fill as well. The result is increasing biodiversity throughout the entire trophic system. Biodiversity seems to have a huge potential to open up new niches and allow for the creation of more biodiversity.

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