A Geographic Mosaic of Speciation in *Eurosta solidaginis*

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Dedication

This thesis/project is dedicated to my mother who earned her master's in Physical Therapy while raising three children.

Abstract

Ecological speciation occurs when divergent selection in different habitats creates strong selection for reproductive isolation which counteracts the homogenizing effects of gene flow between populations using those different habitats. Multiple divergent selection pressures can affect isolation and the strength of these selection pressures can vary geographically. Sympatric pairs of populations using different habitats in separate geographic areas may experience varying degrees of diversifying selection. Differing degrees of reproduction isolation between the populations result in what may be called a "Geographic Mosaic of Speciation". A Geographic Mosaic of Speciation provides an opportunity to study the process of speciation in various degrees of completion. *Eurosta solidaginis* (Diptera: Tephritidae) is a fly that induces galls on tall goldenrod (*Solidago altissima*; Compositae) in North America with populations in the forest and prairie biomes of Minnesota, which are under divergent ecologically-based selection pressures. Previous research indicates that the goldenrod, the gallfly, and the natural enemies of the gallfly in the prairie and the forest biomes are genetically differentiated. I measured characteristics of each member of this three-trophic-level interaction across the prairie-forest biome border in order to characterize divergent selection on members of this interaction. I found that differences in the host plants, flies, and their natural enemies were distributed in a geographic mosaic at the prairie-forest biome border. Some neighboring populations were highly differentiated in a range of characteristics while others showed less differentiation. I then tested two pairs of neighboring fly populations to measure their degree of reproductive isolation and their adaptation to host-plants. The goldenrods from these paired sites were morphologically differentiated

from each other. Flies from three out of the four sites tested had higher survival on plants from their own site than plants from the neighboring site, which shows that differences in plant morphology are correlated with differential selection of host-plants on *E. solidaginis* flies. Reproductive isolating mechanisms of the flies across the boundary are also distributed in a geographic mosaic which supports our hypothesis that these flies show a Geographic Mosaic of Speciation.

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Introduction

Green plants and the herbivorous insects that feed upon them account for much of the earth's biodiversity. Understanding their diversification is fundamental to understanding the biodiversity on the planet today (Berlocher & Feder, 2002; Bush & Butlin, 2004; Thompson, 2005). Because the genetic diversity of communities can have important impacts on ecological interactions in the communities (Elton, 1958; Tilman, 1996), it is important to understand how genetic diversity arises and is maintained. Plant and insect diversity may also be responsible for diversification in higher trophic levels. Specialized herbivorous insects may differentiate into new species when they shift to utilizing a new host plant (Bush, 1969, 1994; Via, 2001). I tested the hypothesis that a geographic mosaic distribution of differential environmental selection pressures, including selection pressures from host-plants, has resulted in a Geographic Mosaic of Divergence of *Eurosta solidaginis* (Diptera: Tephritidae) fly populations which may lead to a Geographic Mosaic of Speciation, and that this mosaic may represent an unrecognized source of biodiversity.

It is useful to think about the process of speciation as a continuum between populations that freely exchange genes to populations that do not exchange any genes (Via, 2009). While populations that fall in the middle of that continuum may not necessarily be considered reproductively isolated species, they still represent distinct entities that are considerably different than populations that freely exchange genes. These partially reproductively isolated populations may still function in an ecosystem like 'good' species and therefore contribute to biodiversity in other trophic levels (Harrison, 1998; Via, 2009).

Speciation can occur in a sexually reproducing species without effective geographical isolation (Maynard Smith, 1966) if there are ecological or temporal barriers to reproduction for populations which are geographically coincident. Support for sympatric speciation has accumulated in the latter half of the 20th century (Coyne & Orr, 2004). Sympatric speciation may be fairly common in phytophagous insects that switch host plants (Berlocher & Feder, 2002; Bush, 1969, 1994; Via, 2001; Walsh, 1867).

Reproductive isolating mechanisms are factors that keep two populations from freely exchanging genes (Dobzhansky & Dobzhansky, 1937; Mayr, 1963). Oftentimes, behavioral reproductive isolating mechanisms can evolve before genetic incompatibilities accumulate (Via, 2009). However, once two species are completely separated it may be impossible to tell which reproductive isolating mechanisms acted initially to produce divergence (Via, 2009). If two taxa are in the process of speciation, and if they are distributed in a fragmented mosaic of populations, some of those populations may be at different stages along the speciation continuum, i.e., they may be distributed in a Geographic Mosaic of Speciation (Via, 2009; West, 2008). This type of spatial variance may provide a window into the reproductive isolating mechanisms that initiate the process of speciation (Howard, Waring, Tibbets, & Gregory, 1993; Via, 2009).

Ecological speciation, which can happen in all geographic scenarios, results from environmental selection pressures that either act as, or select for, reproductive isolating mechanisms (Schluter, 2001). Local adaptation to different habitats can cause differentiation within species (Darwin, 1859; Funk, Filchak, & Feder, 2002; Douglas J.

Futuyma & Moreno, 1988; Schluter, 2001). If diversifying selection pressures between different habitats are strong enough they can select for additional reproductive isolating mechanisms to conserve adaptations to the different habitats in the face of gene flow (Rundle & Nosil, 2005; Schluter, 2000). Parapatric and sympatric populations are more conducive to the production of reproductive isolating mechanisms because there is potential for selection against maladapted hybrids (Cain et al., 1999; Rundle & Nosil, 2005). Local adaptation can lead to ecological speciation most easily via assortative mating when mate choice is determined by habitat choice (Rice, 1987; Rundle & Nosil, 2005; Schluter, 2001; Turelli, Barton, & Coyne, 2001; Via, 2001).

Phytophagous insects are a model system for studying how local adaptation can lead to ecological speciation via host shifts are combined with assortative mating (Funk et al., 2002). Fitness of some insects is tightly linked to their ability to adapt to their host plant (Funk et al., 2002). Internal feeders, including galling insects, are under especially strong selection to adapt to host plant defenses (Cornell & Hawkins, 1995; Gaston, Reavey, & Valladares, 1992). If a host shift occurs, selection is strong for assortative mating or other reproductive isolating mechanisms in order to preserve any adaptations to the new host plant (Funk et al., 2002).

Often times, ecological speciation results from several partial reproductive isolating mechanisms that work together in concert (Nosil, Harmon, & Seehausen, 2009; Rice & Hostert, 1993). In addition to assortative mating, diversifying selection pressures that do not involve mate choice can also affect reproductive isolation through immigrant inviability (Nosil, Vines, & Funk, 2005). If immigrants from other populations that have evolved under different selection pressures have reduced survival

rates in neighboring sites, there will be a reduction of gene flow between those populations (Nosil et al., 2005). Divergent ecological selection pressures that vary geographically in composition and strength may result in variable amounts of reproductive isolation (Itami, Craig, & Horner, 1998; Thompson, 2005; West, 2008).

The degree of reproductive isolation among partially differentiated populations is affected by at least three interacting factors (Itami et al., 1998; Thompson, 2005; West, 2008). These factors include: 1) Differing amounts of genetic diversity within populations that affects potential for adaptation. For example, some small populations may not have the genetic potential to adapt to certain selection pressures. 2) Multifarious divergent selection pressures that are distributed in a geographic mosaic. 3) Differences in the amount of gene flow between differentiated populations. All these factors interact to produce a Geographic Mosaic of Divergence (West, 2008). If the Geographic Mosaic of Divergence ultimately results in a complete speciation event, this distribution could also be called the Geographic Mosaic of Speciation (West, 2008).

The theory of the Geographic Mosaic of Coevolution explores how distributions of interacting species vary geographically and how that can affect the degree of coevolution (Thompson, 2005). Research on mosaic hybrid zones investigates the geographic distribution of populations and how this mosaic distribution can contribute to production of reproductive isolating mechanisms (Cain et al., 1999). The 'multifarious selection hypothesis' states that speciation is more likely to occur when there are multiple divergent selection pressures working in concert (Nosil et al., 2009). The combination of these ideas creates the Geographic Mosaic of Divergence, or a Geographic Mosaic of Speciation as proposed by West (2008).

Eurosta solidaginis induce galls on tall goldenrod, *Solidago altissima* (see Abrahamson & Weis, 1997; Uhler, 1951 for a full reveiw). Gall size and shape is a result of the interaction among fly genes, plant genes, and abiotic conditions (Weis & Abrahamson, 1986). These flies are univoltine and emerge in the spring from galls, mate on host plants, then females oviposit into host plants (Abrahamson & Weis, 1997; Uhler, 1951).

These flies are subject to different selection pressures in the forest and prairie biomes of the Midwest USA (Craig & Itami, 2011). Strong diversifying selection pressures include differences in their *S. altissima* host plants and the natural enemies in the two biomes (Craig & Itami, 2011). *Eurosta solidaginis* flies must be able to overcome specific defenses of their host plant, *S. altissima*, and their host plants are differentiated at the subspecies level (*S. altissima altissima* in the forest and *S. altissima gilvocanescens* in the forest) between the biomes (Craig & Itami, 2011; R.E. Cook & Semple, 2006). In addition, there are different selection pressures from natural enemies in the prairie and the forest. Birds in the forest, including downy woodpeckers, *Piscoides pubesens* (Piciformes: Picidae) and black-capped chickadees, *Poecil atricapillus* (Passeriformes: Paridae), prefer to eat larvae from large galls and therefore select for flies that induce smaller galls (Craig & Itami, 2011; Craig, Itami, & Horner, 2007; Weis, Abrahamson, & Andersen, 1992). The parasitoid wasp, *Eurytoma gigantea* (Hymenoptera: Eurytomidae) causes higher mortality in small galls in both the prairie and the forest which selects for flies with round, large galls (Craig & Itami, 2011; Weis, McCrea, & Abrahamson, 1989). The parasitoid's ovipositor is too short to reach larvae at the center of round, large galls and this exerts selection for larger galls (Craig et al.,

2007; Weis et al., 1989). Predation by a beetle, *Mordellistina convicta* (Coleoptera: Mordellidae), in the prairie also selects for round, large galls because the mordellids are more likely to run into, and subsequently eat, fly larvae in smaller galls (Craig et al., 2007). These selection pressures create large round galls in the prairie and smaller, elliptical galls in the forest (Craig et al., 2007)

Due to host-plant adaptation and assortative mating, *E. solidaginis* populations distant from the prairie-forest border are partially reproductively isolated even when geographic isolation is removed in a common garden setting (Craig & Itami, 2011). Flies from the different biomes assortatively mate and oviposit on plants from their own biome and also survive better on plants from their own biome (Craig & Itami, 2011). Hybrids between the prairie and forest populations have lower survival than the pure host races (Craig & Itami, 2011). Therefore, flies far from the biome border are partially reproductively isolated due to a combination of habitat preference, local adaptation, and geographic isolation which limits gene flow. The populations therefore may be in an intermediate stage of ecological speciation, and have been designated as host races (Craig & Itami, 2011).

Previous to this study, little was known about how these host races interacted at the border between the prairie and forest biomes where there is diversifying selection combined with a strong potential for gene flow due to the lack of geographic isolation. Here I discuss how the populations of prairie and forest *E. solidaginis* flies, and selection pressures affecting them, are distributed across the biome border in southern Minnesota in order to determine if there is a Geographic Mosaic of Speciation. First, I determined whether there was gene flow occurring between the host races at the biome

border. To answer that question, I sampled fly populations along transects across the biome border and analyzed the morphological differentiation and host-plant adaptation to the host plants among those sites. Second, I analyzed the geographic distribution of selection pressures that have previously been found to differ between the prairie and the forest by measuring plant morphology and natural enemies from the same sites where I collected flies. Third, I tested the strength of prezygotic reproductive isolating mechanisms between populations including host plant preference as a proxy for assortative mating, and temporal isolation due to differences in emergence times.

Methods

Geographic distribution of morphological fly characteristics

1. Collection sites

To determine where prairie and forest flies came into contact, I measured previously collected galls from an extensive north-south transect running through eastern Minnesota across the prairie biome border, which I term the "long interval transect". I measured at least one hundred galls from sites that were separated from each other by a mean of 88 kilometers. I selected a region where there were large changes in gall shape (diameter/length), indicating a shift between prairie and forest populations. In the fall of 2009, I collected galls containing diapausing flies from a "short interval transect" in this area to obtain a smaller-scale picture of the distribution of the forest and prairie host races. I collected approximately 350 galls from each site, which were separated by an average of 26 kilometers (Figure 1). When a gall was found, all the galls within a meter of that gall were also collected to avoid non-random sampling.

A total of 21 sites were sampled along a north-south transect running approximately at 93˚ longitude between 42˚ and 44˚ latitude. Site numbers from south to north are: 14, 15, 16, 17, 19, 18, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 20, CA3. Galls from all fall transect sites (1-13) were stored at ambient Duluth temperatures in plastic garbage bags until temperatures rose above freezing, after which I transferred the galls to mesh bags to avoid mold growth. All additional galls collected in the spring were also stored at ambient Duluth temperatures until the beginning of May when all galls

were transferred to the lab and kept at approximately 20˚C with natural light cycles throughout emergence.

I also collected galls with diapausing flies in the fall of 2010. I re-collected galls from sites 3, 4, 8, and 10 for use in a common garden experiment that was performed during the spring and summer of 2011. In addition, I collected galls from 3 sites in between sites 8 and 10 (the 'Micro transect') to see how flies were changing on an even smaller geographical scale between these two morphologically different fly sites. I also collected galls from an additional transect west of our original transect (the 'Western short interval transect') to determine if the prairie and forest host races exhibit a mosaic distribution across the biome border in other areas (Figure 1). Galls were stored at ambient Duluth temperatures throughout the winter.

2. *Geographic distribution of galls*

I analyzed approximately 250 galls from each site after flies had emerged. Using electronic calipers, I measured the length and diameter of each gall to the nearest tenth of a millimeter. Next, I cut open the gall using garden shears and determined cause of death. If I determined that the larva died before complete formation of the gall, I excluded that gall from shape and size analysis. I also excluded fused galls, which are galls occurring in adjacent internodes that have grown together, from analysis because I could not measure gall length accurately.

3. Geographic distribution of wing patterns

Each day in 2010, shortly after the daily flies emerged, I took pictures of each fly with a Nikon E995 digital camera mounted to a dissecting scope. Then, using a

metric ruler, I measured the digital images of the wings while they were displayed on a computer screen. The Hyaline ratio is a measure of the ratio of non-pigmented area to pigmented area in the R^{4+5}cell and is a morphological characteristic that distinguishes prairie from forest populations (Ming, 1989). Hyaline ratios were measured for all flies from each site unless the cell was not visible due to breakage or lack of scleritization. Because the wing pattern data is proportional data, I used an arcsine square root transformation to account for non-normal distribution. I then analyzed hyaline ratios using a single factor analysis of variance with a Tukey's multiple comparison test.

4. Wing patterns from individual rearings

In February of 2011, galls from the 2010 cohort from sites 30-40 (Micro transect and Western transect) were reared in the laboratory. I put each gall into an individual, labeled 4 oz. PLA plastic portion cup. Cups were flipped over onto moist paper towels. The paper towels were then moistened daily until fly emergence had ceased. As flies emerged, I took pictures of their right wings, and measured wings using the same technique used above. Again, I transformed proportional wing data using an arcsine square root transformation and preformed regression analysis of wing data with gall shape and gall size of all individual flies from each site to determine correlation between wing pattern and gall size/shape in order to determine the amount of trait remixing that occurs at the boundary area.

5. *Geographic distribution of local adaptation to host plants*

To test whether flies at neighboring, differentiated sites, were locally adapted to plants from their sites I compared survival rates of flies on plants from their own sites

and plants from the neighboring site. In the spring of 2010, I measured fly preference for plants from the prairie and forest in an experiment at the University of Minnesota Duluth Research Farm. I grew plants from three prairie sites and three forest sites from far within the prairie and forest biomes in Pro-Mix BX potting soil in 3 gallon pots and watered as needed. Forty 1.5 m^3 cages were constructed with rebar, irrigation tubing, and Remay row cover cloth. The largest plants were selected from each site and randomly distributed to all 40 cages. Each cage contained one pot from each site for a total of six pots arranged in two rows of three near the center of the cage.

When a female *E. solidaginis* fly inserts her ovipositor into the apical meristem of the goldenrod, an oviposition scar is produced which is termed an ovipuncture (Abrahamson & Weis, 1997). The number of ovipunctures are highly correlated with number of eggs laid by the female (Craig, Abrahamson, Itami, & Horner, 1999; Craig, Horner, & Itami, 1997). Once the oviposition preference of flies in each cage had been measured (see methods below), I continued to add mated females to each cage in order to increase the number of stems with eggs in the cage. If a stem was ovipunctured more than 20 times, that pot was removed from the cage and stored in an overflow cage to increase the probability that flies would ovipuncture the remaining stems within the cage. I continued to add mated females and/or unmated males and females evenly among cages until all females were distributed. Plants were watered as needed throughout the summer. In the fall, I collected galls from the stems and compared that with the number of original ovipunctures in the cage to determine rate of survival. I used a general linear model with plant site, fly site, cage, and plant*fly site interaction as factors affecting survival.

Geographic distribution of morphological plant characteristics

I collected *S. altissima* seed heads from several plants at each site in the fall of 2009. Seeds were kept at ambient Duluth temperatures until they were planted on February 27th, 2010. For each site, I planted 3 flats (6 x 4") of seeds in Pro-Mix PGX soilless seed starting medium. Sites that did not germinate well initially were replanted from the same stock of seeds and some from new seed heads collected on April 9th, 2010. Site 5 and Site 1 did not germinate. I transplanted seedlings into 4 inch pots with Pro-Mix BX on April 28th, 2010. Plants continued to grow in the greenhouse setting until after the last frost at which time I transferred the plants to our common garden and transplanted them into 1 gallon pots with the same soil. Plants were then maintained for the remainder of the summer. I watered the plants as needed and applied one application of 20-20-20 soluble fertilizer and one application of 20-20-20 slow release fertilizer pellets. Plant measurements were taken for the tallest ramet in the pot and included the total height, stem diameter at 5cm from soil level, leaf width and length of the 10th leaf below the apical meristem, and total number of leaves. In September of 2010 I measured the tallest ramet within each pot from all sites.

Rhizomes from many of the pots from sites 3, 4, 8, and 10 were split and planted in new pots to produce multiple replicates in the fall of 2010. Additional rhizomes from those sites were also collected in March of 2011 and planted into 3 gallon pots with the same potting mix. All plant morphology measurements were repeated in September of 2011.

Previously, the same measurements were taken from plants collected from far within the prairie and the forest (Craig & Itami, 2011), and I used these data to conduct

a discriminate function analysis, which correctly classified 77% of the original plants used to make the model as prairie or forest plants. I then applied that discriminate function to the plant morphology measurements from our boundary sites. Each plant was given a probability of being a prairie or forest plant based on the discriminate function analysis. A one-way ANOVA among boundary sites of 'probability prairie' was preformed along with a Tukey's multiple comparison test. I also used a MANOVA to test for differences among sites using all morphological characteristics measured.

Geographic distribution of natural enemies

When dissecting galls, I used evidence of predation to determine cause of fly death if the fly did not successfully emerge. I used data from all galls collected to determine percentage mortality explained by the three major natural enemies in this system: Parasitoid wasps, *Eurytoma gigantea*, stem–boring beetles *Mordellastina convicta*, and bird predation by Downy woodpeckers, *Picoides pubescens*, and Chickadees, *Poecile atricapillus*.

Testing for reproductive isolating mechanisms

1. *Allochronic isolation*

I recorded the emergence date and sex of the fly. Only flies from the original 13 sites were assessed for emergence because they were collected at the same time and kept under the same winter conditions. Emergence day was the number of days after the first fly from any one of the sites emerged. Allochronic isolation was tested with a one-way ANOVA of emergence day by site with Tukey's multiple comparison test.

Sexes were analyzed separately at each site because male *E. solidaginis* flies always emerge before female flies (Itami et al., 1998).

2. Oviposition preference

I measured oviposition preference for each fly site by placing flies into cages with prairie and forest plants and subsequently counting the number of ovipunctures on all plants within the cage. Oviposition preference has been shown to strongly correlate with assortative mating in *E. solidaginis* flies in previous studies (Craig et al., 1997, 1999), and I therefore used it as a proxy for assortative mating. For some flies, I placed males and females together in a mating cup-cage to increase mating success and thus the likelihood of oviposition, while other flies were released unmated into the cages. I tested the oviposition preference of flies from 13 sites using five replicate cages for each site that were placed in a fully random design within a 40 cage grid. Because the galls collected in the spring were not held at the same temperature as the galls from the October transect, the flies from spring-collected galls began to emerge first. Whenever five cages were finished and the plants replaced, a new fly site was tested for oviposition preference. The first set of sites tested for preference included sites 14, 16, 17, 18, 5, 8, 20, and CA3 for a total of 8 sites. Flies were added to cages as soon as possible after emergence. After flies were added to cages, ovipunctures were counted between 24 to 48 hours later. I measured the number of stems in each pot, the height of each stem, and the number of ovipunctures on each stem. Initially, stem heights were not measured for finished replicates and were instead measured up to two weeks later. Ovipunctures were also recounted when stem heights were taken. Analysis of the correlation between ovipunctures and stem height used only the second set of

ovipuncture counts. When a cage replicate was finished and the data collected I removed any plants with ovipunctures from the cage and replaced them with unused plants from the same site. The second set of sites tested for preference included 18, 6, 7, 9, 10, and 12. I ran a general linear model looking at the effects of plant biome, fly site origin, cage replicate, and plant biome*fly site origin interaction. I originally ran the general linear model with stem height as a covariate because flies tend to prefer tall plants in general but our results did not change.

In the spring of 2011, I performed another preference experiment. Based on gall morphology and wing pattern of the flies, I chose two pairs of sites that were geographically close, yet had morphologically differentiated flies. These two pairs of sites were 3 & 4 and 8 & 10. One hundred pots of plants from each of the above sites were randomly assigned to cages. Each cage contained 5 replicate pots from each of the paired sites. There were 20 cages containing plants from sites 3 & 4 and 20 cages containing plants from sites 8 & 10. Those 20 cages were split between the flies from each of those sites. Mated females from a site were placed in a cage with plants from their own site and the neighboring site. For example, mated females from site 4 were placed in cages with plants from sites 3 & 4. If I was not able to mate females before adding to cages, I added males and females to cages simultaneously.

Cages were checked for oviposition marks within 48 hours after mated females were added to the cages. If a cage contained more than 10 oviposition marks, that replicate was considered finished and a total number of ovipositions on each stem were tallied along with the height of each stem within the cage. I ran a general linear model

looking at the effects of plant origin site, fly origin site, cage, and plant site*fly site interaction on oviposition.

Results

Fly morphology

Gall size and shape of the 2009 cohort were significantly different among sites, thereby forming a geographic mosaic distribution (Figure 2, Table 1). I use the term geographic mosaic to describe fluctuation in the amount of differentiation between neighboring sites. I.e., some neighboring sites are significantly different while others are not and those relationships between sites repeat across a geographic transect. Site pairs 3 & 4 and 8 &10 were significantly different from their neighbors in both size and shape (Figure 2, Table 1). Wing patterns also showed a significant, mosaic distribution among sites (Figure 2, Table 1). Again, the wing patterns at site pairs 3 & 4 and 8 & 10 were significantly different from their neighbors (Figure 2, Table 1). At site 4, there was a bimodal distribution of wing patterns indicating that there may be two differentiated populations of flies mixed together at the site (Figure 3).

Gall shape and gall size of the 2010 cohort from site pairs 3 & 4 as well as 8 & 10 were again significantly different between the neighboring sites (Table 1). Gall size, gall shape, and wing patterns from the micro transect sites 30, 31, and 32, between sites 8 and 10, also differed significantly from each other (Table 1). Gall size, gall shape, and wing pattern also differed significantly among sites along the western transect with a geographic mosaic pattern (Table 1). Gall size and gall shape were also positively correlated with wing pattern (gall size=17.8 + 4.24 arcsin of ratio, df= 156, r= 0.27, $p< 0.0001$; gall shape=0.714 + 0.0780 arcsin of ratio, df=156, r = 0.15, $p< 0.0001$).

Host plant adaptation

Flies from sites 8 and 10 did not form any galls on plants from the neighboring site but formed galls on plants from their own site (Table 2, Figure 4). Flies from site 3 formed significantly more galls on plants from their own site compared to plants from site 4 (ANOVA $F_{1, 98}$=7.02, p<0.009). However, site 4 flies formed the same number of galls formed on plants from their own site and plants from site 3 (ANOVA $F_{1, 98}$=0.07, p<0.793). More fly larvae from sites 8 and 10 survived on plants from their own site compared to plants from the neighboring site (Table 3) but there was no difference in fly larvae survival from sites 3 and 4 on plants from the neighboring site (Table 3). Site 3 flies had no surviving larvae on site 4 plants, but the number of surviving larvae on site 3 plants was so low it was not significantly different from zero (ANOVA $F_{1, 98}$=2.82, p<0.096). This low survival was due to high bird predation rates on site 3 flies.

Plant morphology

There were alternating differences among sites for all plant morphological characteristics (Table 4), indicating a geographic mosaic distribution of plant populations. All morphological traits of plants from experimental sites 3 and 4 were significantly different from each other (Table 4). Plants from experimental sites 8 and 10 were significantly different in plant height, stem diameter, leaf length, and leaf width (Table 4). A one-way ANOVA of the probability of a prairie score (from discriminate function analysis) showed significant differences among sites (ANOVA $F_{10, 267}$=6.66, p<0.0001, Figure 5). Experimental site pairs 3 & 4 and 8 & 10 were significantly different from their neighbors in their mean probability prairie scores (Sites 8 & 10: ANOVA $F_{1, 28}$=9.54, p < 0.005; Sites 3 & 4: ANOVA $F_{1, 17}$=14.69, p< 0.001). Means

for some sites were intermediate between the two extremes, indicating that there may be two different populations of plants or intermediate, hybrid plants at some sites. Scores for the probability of being a prairie plant displayed a bimodal distribution in site 4 indicating that there may be two differentiated populations at that site (Figure 6).

Natural enemy distribution

Percentage mortality differed significantly among sites for the three types of natural enemies measured (χ^2 (38, N=5104) = 2052.055, p<0.0001, Figure 7). There was similar distribution of natural enemy mortality among the western transect sites (χ^2 (14, N=1863) = 540.1434, p<0.0001, Figure 8).

Reproductive isolating mechanisms

1. Allochronic isolation

Time of fly emergence differed significantly among sites (ANOVA $F_{12, 1666}$=14.23, p< 0.0001). There was significant difference between emergence time for site 8 males and site 10 females, site 10 males and site 8 females, and site 4 males and site 3 females (ANOVA $F_{7, 469}$=13.36, p< 0.0001, Figure 9). There was no significant difference between emergence times for site 3 males and 4 females.

2. Host plant preference

Host plant preference is strongly correlated with assortative mating in *E. solidaginis* flies, and it was used as a proxy for assortative mating in this experiment (Craig et al., 1999, 1997). For the preference experiment preformed in the spring of 2010, I found that overall flies did not prefer to oviposit on plants from one biome over the other in general (Table 5). I also found that flies from the different sites did not

significantly differ in their number of ovipositions (Table 5). Cage replicates did differ significantly because in some cages flies tended to oviposit more than in others (Table 5). The plant biome by fly site origin interaction was significant, indicating that flies from some sites preferred plants from one biome while flies from other sites preferred plants from the other biome (Table 5, Figure 10).

For the preference experiment performed in the spring of 2011, I used only flies from sites 3 & 4 and 8 & 10. Flies from sites 8 and 10 significantly preferred to oviposit on plants from their own site versus plants from the neighboring site (Table 5, Figure 10). Flies from site 3 preferred to oviposit on plants from their own site (ANOVA $F_{1, 402}$=22.50, $p<0.0001$, Figure 10). Flies from site 4 preferred to oviposit on plants from site 3 instead of plants from their own site (ANOVA $F_{1, 372}$=10.87, $p<0.001$, Figure 10).

Discussion

Geographic Mosaics of Divergence can lead to Geographic Mosaics of Speciation when several diverging populations under similar divergent selection pressures occur over a large geographic range (West, 2008). Varying levels of genetic variation, amount of gene flow, and composition of divergent selection pressures among different geographic populations contribute to a Geographic Mosaic of Speciation (Itami et al., 1998; Via, 1999; West, 2008). These Geographic Mosaics of Speciation are made up of populations that are at different points along the continuum from undifferentiated populations to fully reproductively isolated species and are therefore useful for the study of reproductive isolating mechanisms that initiate and complete the process of speciation (Via, 2009; West, 2008).

I found that the prairie and forest host races of *Eurosta solidaginis* flies are distributed in a geographic mosaic across the prairie-forest boundary in the southern Minnesota. I also found that their host plants, *S. a. altissima* and *S. a. gilvocanescens,* and their natural enemies are also distributed in a geographic mosaic across the prairie-forest boundary, potentially creating a mosaic of diversifying selection that could select for genetic differences seen in the host races. Finally, I demonstrated that there are varying degrees of reproductive isolation due to host-plant preference (a proxy for assortative mating) among these boundary sites suggesting that these flies show a Geographic Mosaic of Speciation.

I found significant differences among flies in their wing patterns, the gall morphology they induced, and in their ability to survive on the subspecies of *S. altissima*. The geographic mosaic pattern of neighboring sites with strong differences

between fly populations indicates the existence of two partially reproductive isolated host races with little gene flow between sites. The sites where flies were intermediate in the characters that Craig and Itami (2011) used to differentiate between the host races indicates that there has been gene flow between the populations. Together these indicate that there is geographic variation in either prezygotic or postzygotic isolation, or both. The correlation of wing pattern and gall shape was significant indicating that there is some linkage of these traits, but there are also intermediates indicating that there is gene flow resulting in trait remixing.

In order for host-associated assortative mating to evolve as a reproductive isolating mechanism, there must be some sites where there are parapatric or sympatric distribution of plant populations for the flies to utilize so that there can be selection for the flies to evolve the ability to differentiate among host plants. I measured a suite of morphological plant characteristics from each site and found that some neighboring sites are morphologically distinct from each other. This indicates that there may be reproductive isolation at the biome border between these prairie and forest *S. altissima* populations. Probability prairie scores from the discriminate function showed bimodal distribution at site 4, indicating that differentiated plant populations may be truly sympatric at that site. These differentiated plant populations could exert diversifying selection pressures on the flies for the evolution of reproductive isolating mechanisms which could result in ecological speciation. Because these significantly differentiated and intermediate plants populations are distributed in a geographic mosaic, there is a geographic mosaic of selection pressure for flies to adapt to their local plant populations.

I also found that natural enemies of *E. solidaginis* flies are distributed in a geographic mosaic across the prairie-forest boundary. Depending on how these additional differential selection pressures match up with the differential selection pressure of the host plant, they may increase or decrease the selection for the evolution of reproductive isolating mechanisms among populations of *E. solidaginis* flies. For example, in a typical prairie site with prairie plants, low bird predation, abundant mordellid beetles, and *E. gigantea* with long ovipositors would produce selection for adaptation to prairie plants and production of large round galls. An adjacent forest site with forest plants, high bird predation, low mordellid densities, and *E. gigantea* with short ovipositors would exert selection for adaptation to forest plants and the production of small galls. The combination of these selection pressures at each site creates more selection pressure for reproductive isolating mechanisms to avoid the production of hybrid offspring with low fitness in either site. A site with prairie plants bordering a forest area could have high bird predation resulting in selection of flies adapted to prairie plants, but with small galls.

I also found that prezygotic reproductive isolating mechanisms are present in this geographic mosaic. Difference in emergence times among populations could produce significant if incomplete reproductive isolation. I found differences in emergence times of males and females from neighboring populations of about two days. Since flies live 10-14 days in cage environments, this would not by itself produce complete reproductive isolation (Itami et al., 1998). However, females usually mate the first day after emergence and male mating success decreases with age (Itami et al., 1998) so small differences in emergence times could have a large impact on

reproductive isolation. Also, flies will wait to emerge from their galls and/or will refuse to mate if weather conditions are not favorable (Itami et al., 1998). If males from one population emerge, and if there was poor weather for several days in a row, these small differences in emergence times could produce strong reproductive isolation.

I also found indications of prezygotic isolation due to assortative mating. Plant preference for oviposition site is strongly correlated with assortative mating in this system (Craig et al., 1999, 1997; Craig & Itami, 2011). I found that there was a geographic mosaic of plant preference across the biome border when I tested the preference of flies from sites near the prairie-forest boundary for plants from far within the prairie or the forest. In the second field season I used pairs of fly sites that were morphologically different yet geographically adjacent to test for preference of the flies using plants grown from their own sites and from their neighboring site. I found that flies from three out of four of the sites significantly preferred to oviposit on plants from their own site versus plants from the neighboring site. This indicates that host-plant preference (a proxy for assortative mating) is functioning as a prezygotic reproductive isolating mechanism in this system at some sites. Site 4 flies did not prefer to oviposit on plants from their own site but the bimodal distribution of wing patterns at this site suggests there may be two, partially reproductively isolated populations of flies present. Because I did not test preference of individual flies, I do not know if the flies I used to test preference had forest or prairie type wing patterns which may be linked to differences in preference. Plants from site 4 also show a bimodal distribution of probability prairie scores, suggesting there may be two populations of plants present at this site which further complicates the preference results from this site. If these

differentiated populations are truly sympatric at site 4 then further investigation of plant and fly populations present at site 4 is warranted.

Funk et al. (2006) have shown that a combination of number of relatively weak reproductive isolating mechanisms can produce strong reproductive isolation. I found that there are several reproductive isolating mechanisms. Differences in emergence times, assortative mating, and spatial isolation combined with low hybrid survival (Craig & Itami, 2011), though relatively small in their individual impact, could in combination create strong reproductive isolation and potentially result in ecological speciation. Conversely, a combination of weak reproductive isolation due to each of these factors at some sites could result in significant gene flow and the genetic homogenization of populations. I conclude that sites with different combinations of plant populations and natural enemies could create a geographic mosaic of diversifying selection resulting in a geographic mosaic of reproductive isolating mechanisms. Reproductive isolating mechanisms that vary in strength and composition may produce varying levels of gene flow which produces a range of degrees of reproductive isolation among populations creating a Geographic Mosaic of Speciation.

There have been a limited number of other integrative studies that have looked at an organism's distribution in relation to habitat use and selection and how that might affect reproductive isolating mechanisms (Itami et al., 1998; Nosil, Crespi, & Sandoval, 2002; Nosil, Sandoval, & Crespi, 2006; West, 2008). Results described here provide the first indication that the differentiation of *Eurosta solidaginis* system across the prairie-forest boundary occurs in a geographic mosaic which may produce varying degrees of reproductive isolation resulting in a Geographic Mosaic of Speciation.

Our understanding of this system would benefit from further investigations into interactions between prairie and forest populations of *E. solidaginis*. A large scale experiment with grid sampling of sites along the boundary area could be used to answer questions about the extent of the Geographic Mosaic of Speciation present in this system. An increase in the number of sites measured for preference could be analyzed for correlation with the presence of certain divergent selection pressures. This would give us a better idea of what selection pressures are influencing the development of reproductive isolating mechanisms.

Further development of genetic markers identifying prairie and forest plants and flies would aid in determining which plants and flies are present in each site. Using information about population genetics at these sites, we could also determine the amount of genetic variance at each site which is one of the three factors laid out by West (2008) that may affect the Geographic Mosaic of Speciation.

Another factor discussed by West (2008) is the of variation in the amount of gene flow between divergent populations. I have shown that there is variation in the amount of prezygotic reproductive isolation among sites which affects the amount of gene flow. Other factors that might affect the amount of gene flow include the geographic isolation between populations and the size of population patches. Larger populations will produce more migrants into smaller populations and may therefore swamp local adaptation and development of reproductive isolating mechanisms (Kareiva, 1983; McCauley, 1991; West, 2008).

Geographic distribution of differential selection pressures affects the amount of reproductive isolation between closely related taxa in different populations (Itami et al.,

1998; West, 2008). This geographic variation may explain the level of divergence between differentiated taxa and may shed light on which selection pressures drive speciation (Via, 2001). Biodiversity in part depends on these types of speciation mechanisms. With a greater understanding of the impact of different selection pressures and how they function at different geographic scales we can make informed choices about the development of conservation plans to conserve biodiversity.

Figures and Tables

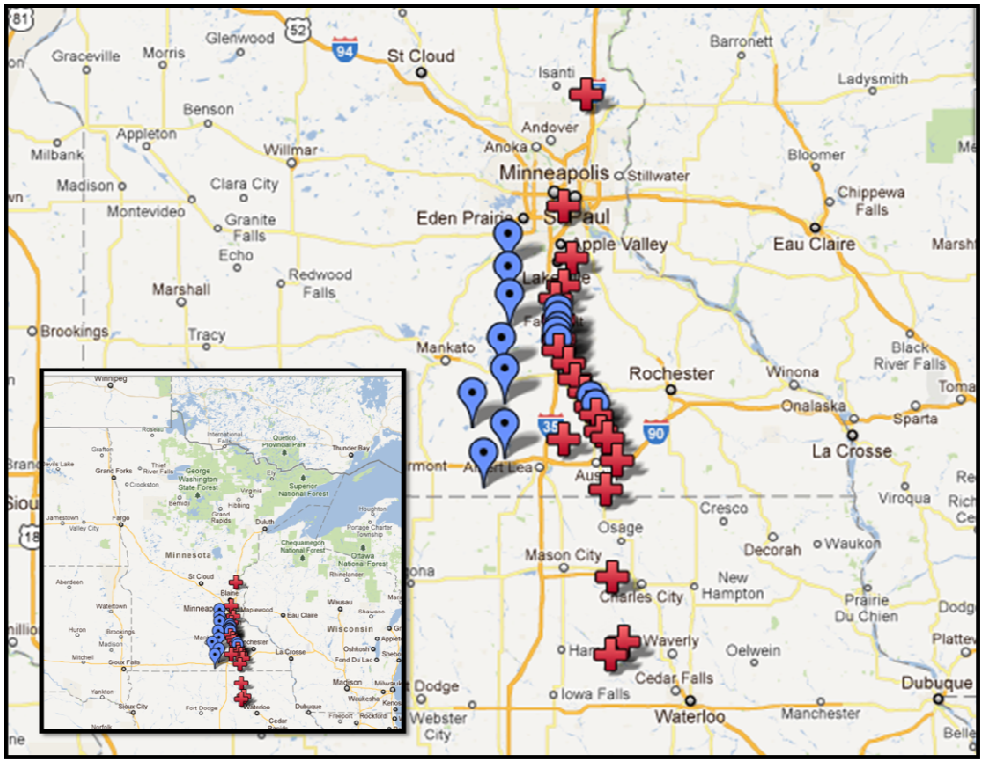

Figure 1. Gall Collection Sites. Flies from the 2009 cohort are marked with red crosses. Flies from 2010 cohort are marked with blue pointers. Sites 3, 4, 8 & 10 were collected both years.

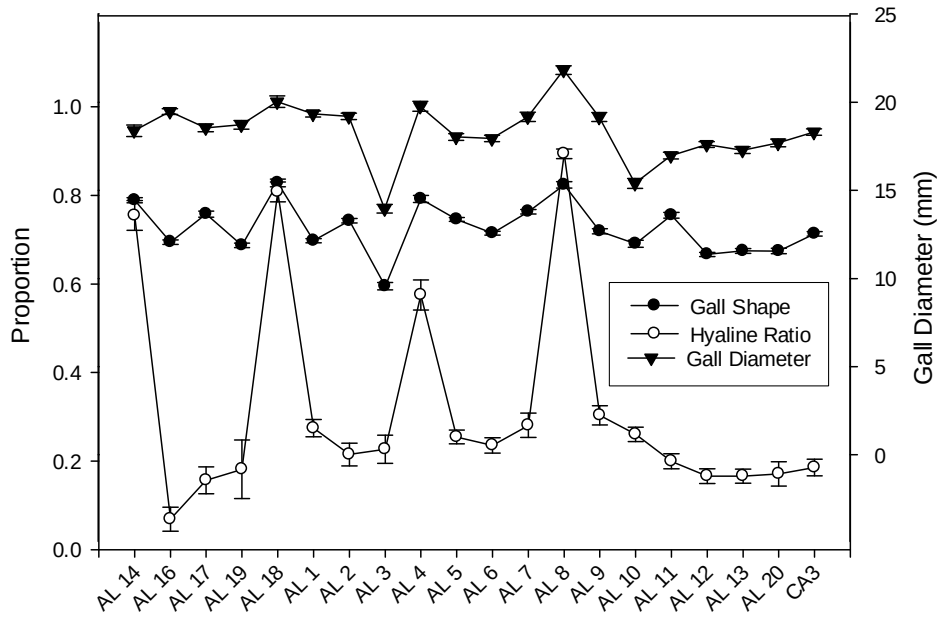

Figure 2. Geographic distribution of morphological fly characteristics. All characteristics show a geographic mosaic distribution with some neighboring sites that are significantly different from each other (P<0.05).

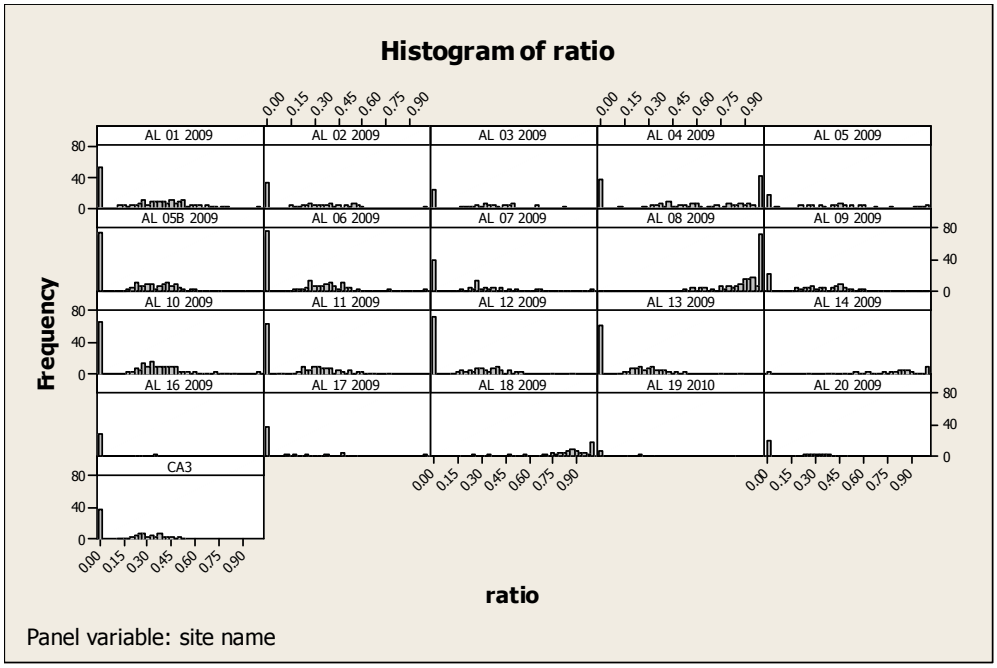

Figure 3. Distribution of wing ratios within sites. Site 4 shows bimodal distribution indicating there may be two, differentiated, populations of flies present.

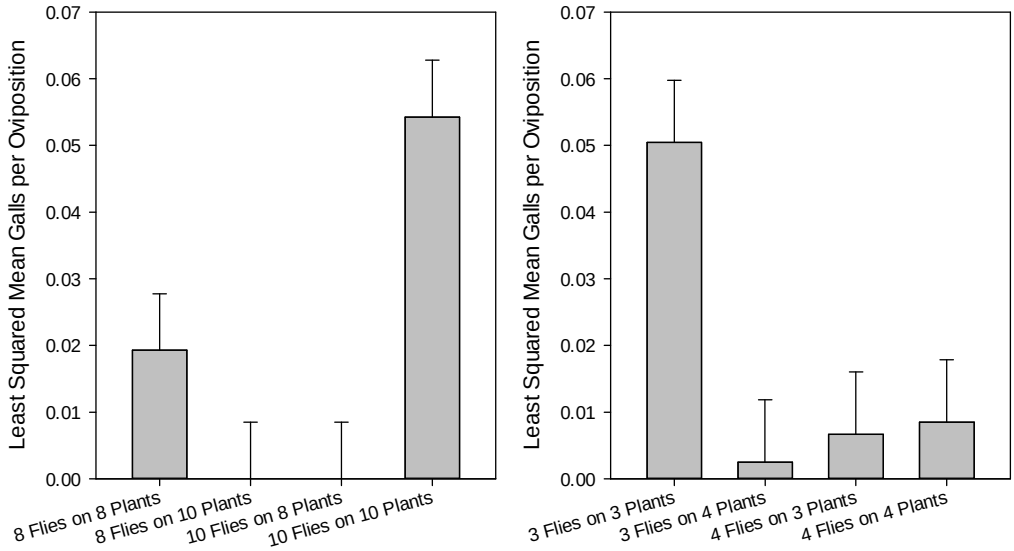

Figure 4. General linear model of number of galls formed per oviposition. Combinations are labeled by site (#) flies (F) on site (#) plants (P).

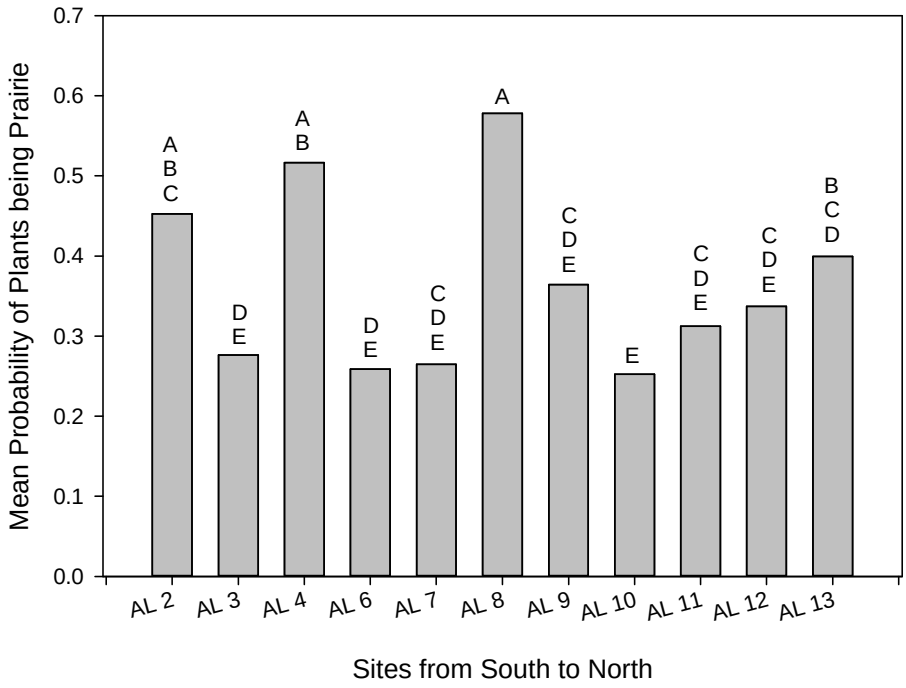

Figure 5. Using discriminate function probability scores for each plant, I took the mean 'probability prairie' score and compared among sites. There was statistical differences among sites with the experiment sites (3, 4, 8 & 10) being statistically different from their neighboring site.

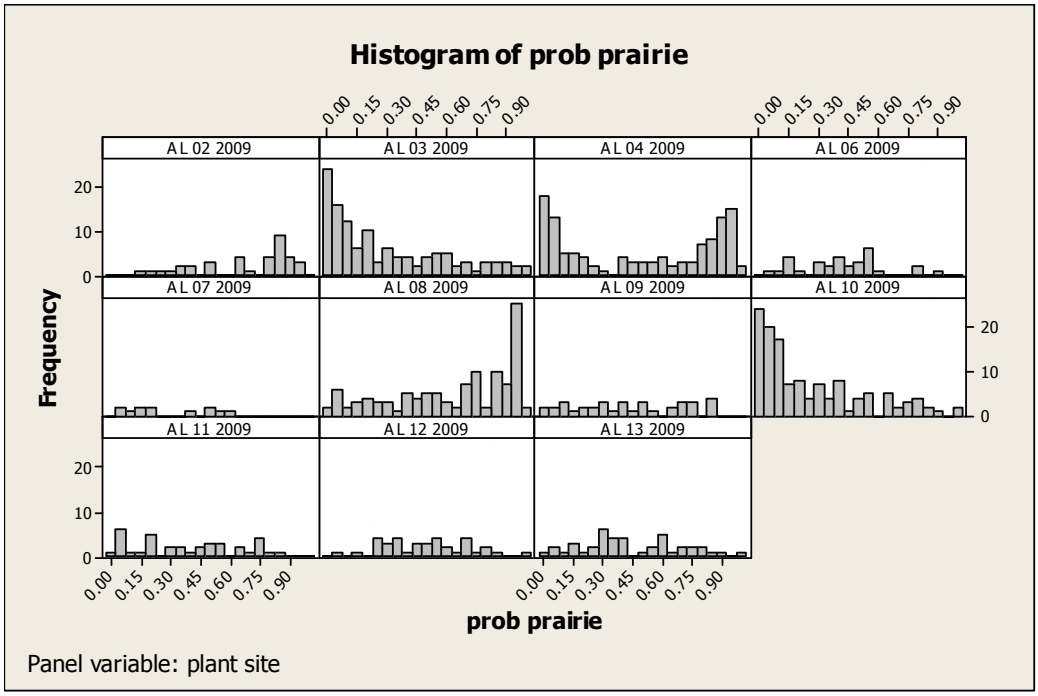

Figure 6. Distribution of probability scores for plants being prairie. Site 4 shows bimodal distribution indicating they may be two populations of differentiated plants present.

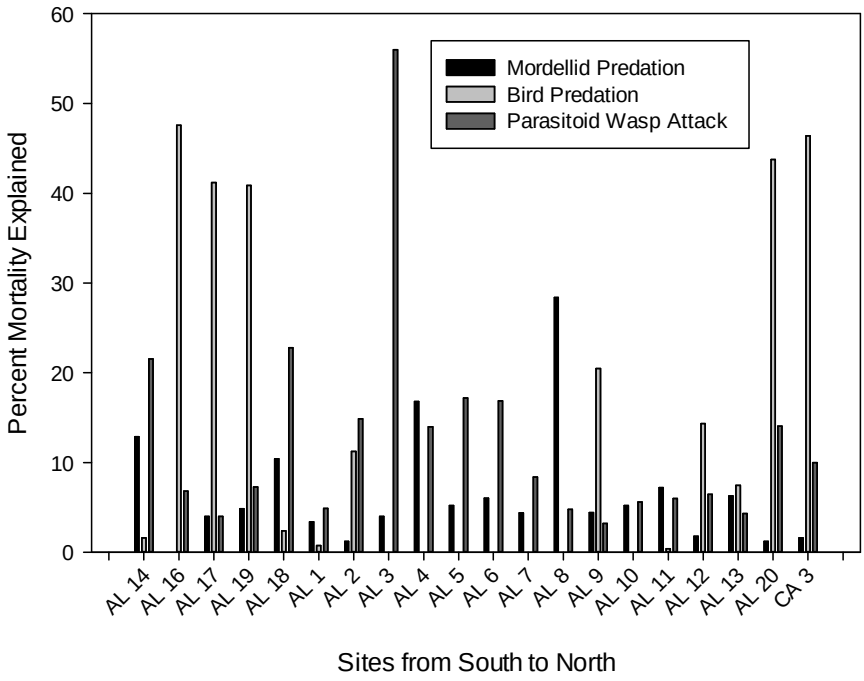

Figure 7. Percent mortality of original transect flies explained by three natural enemies. Type of natural enemy and/or abundance of natural enemies varies among sites.

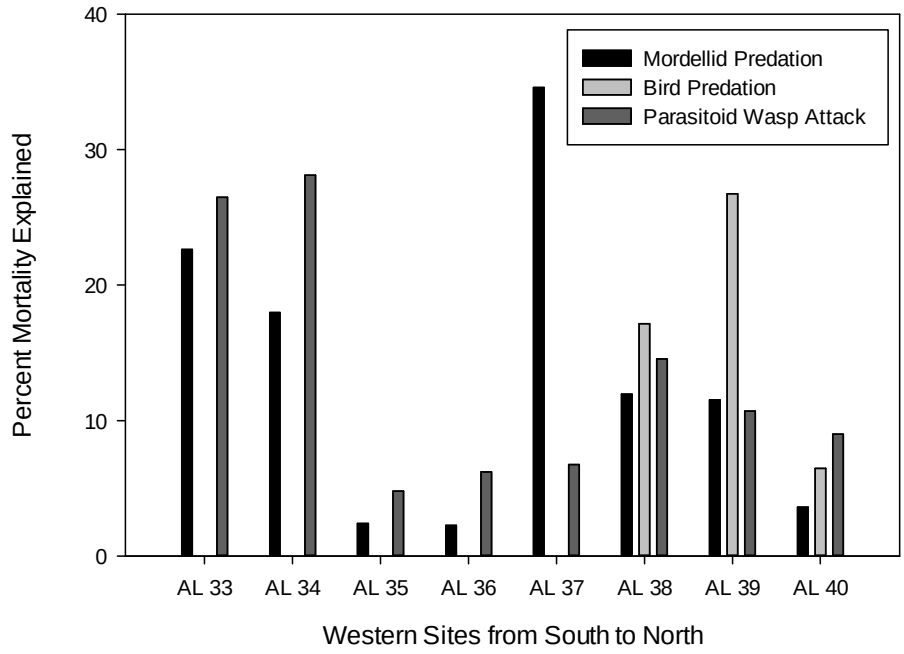

Figure 8. Percent mortality explained by three natural enemies of *E. solidaginis* in the western transect. Type of natural enemy and/or percentage mortality explained varies among sites.

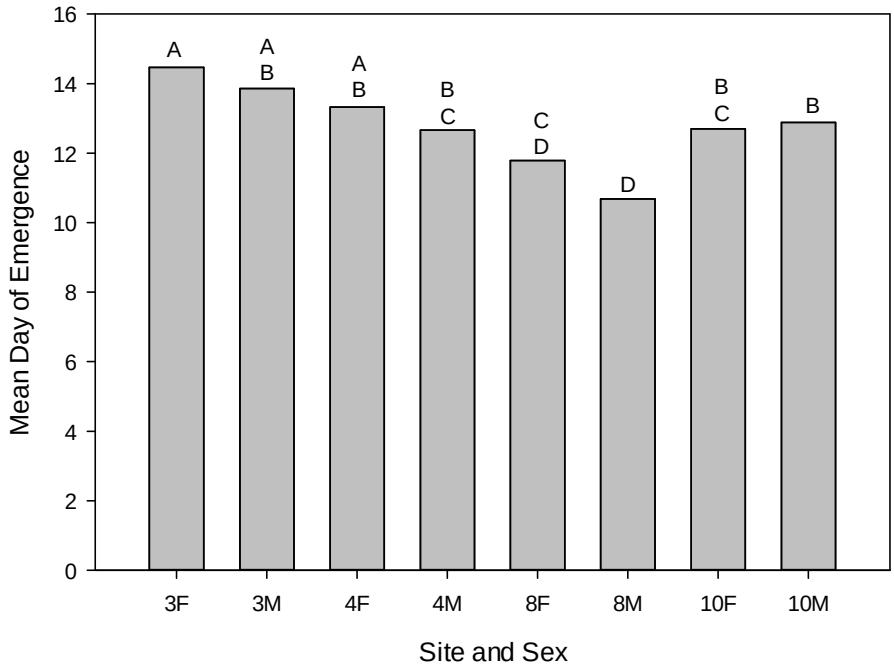

Figure 9. Mean day of emergence after the first fly emerged. Letters indicate groups from Tukey's multiple comparison test. Bars that do not share a letter are significantly different.

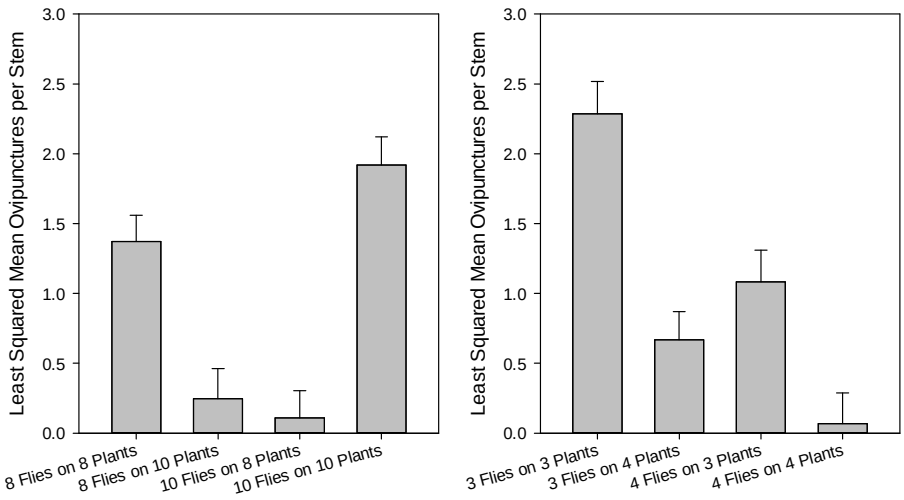

Figure 10. General linear model of fly preference measured in least squared mean ovipunctures per stem. Combinations are labeled by site(#) flies (F) on site(#) plants (P).

Table 1. Analysis of variance of gall diameter, gall shape, and wing pattern in 2010 and 2011.

Source	df	gall diameter F	P	gall shape F	P	df	wing patterns F	P
2010	19	53.21	0.000	84.52	0.000	20	86.15	0.000
2010 (sites 3 & 4)	1	231.72	0.000	289.04	0.000	1	29.91	0.000
2010 (sites 8 & 10)	1	287.84	0.000	159.73	0.000	1	746.34	0.000
2011 (sites 3 & 4)	1	73.13	0.000	0.63	NS	NA	NA	NA
2011 (sites 8 & 10)	1	55.12	0.000	29.13	0.000	NA	NA	NA
2011 (microtransect)	2	3.07	0.048	15.98	0.000	4	195.45	0.000
2011 (western transect)	7	66.12	0.000	73.09	0.000	7	25.70	0.000

Table 2. Analysis of variance of gall formation in the 2011 common garden experiment.

Factor	Sites 3 & 4 Df	F	P	Sites 8 & 10 Df	F	P
Fly site	1	2.20	NS	1	4.16	0.056
Plant site	1	6.10	0.014	1	4.24	0.041
Cage (fly site)	18	1.86	0.021	18	1.02	NS
Fly site*Plant site	1	7.11	0.008	1	18.75	0.000
Error	178			177		

Table 3. Analysis of variance of larval survival in the 2011 common garden experiment. There was significantly higher larval survival in sites 8 & 10 on plants from their own site versus plants from the neighboring site. There was no significant difference for sites 3 & 4 between survival on their own plants and survival on neighboring plants.

Factor	Sites 3 & 4 Df	F	P	Sites 8 & 10 Df	F	P
Fly site	1	2.11	NS	1	.62	NS
Plant site	1	2.35	NS	1	.57	NS
Cage (Fly Site)	18	0.83	NS	18	.93	NS
Fly site*Plant site	1	2.94	NS	1	5.99	0.015
Error	178			177		

Table 4. Multivariate analysis of variance of plant morphological characteristics from 2011 field season.

Plant trait	All Sites $F_{10,1034}$	P	Sites 3 & 4 $F_{1,297}$	P	Sites 8 & 10 $F_{1,308}$	P
Plant height	17.20	0.000	25.36	0.000	36.73	0.000
Stem diameter	26.02	0.000	58.83	0.000	90.55	0.000
Total leaves	7.29	0.000	4.83	0.029	0.52	0.473 (NS)
Leaf length	4.27	0.000	5.06	0.025	16.96	0.000
Leaf width	10.10	0.000	17.82	0.000	51.93	0.000

Table 5. Analysis of variance of oviposition preference of flies in 2010 and 2011.

Factor	All sites 2010 Df	F	P	Sites 3 & 4 2011 Df	F	P	Sites 8 & 10 2011 Df	F	P
Fly site	11	1.14	0.362 (NS)	1	3.32	0.085 (NS)	1	0.15	0.707 (NS)
Plant site	1	0.44	0.506 (NS)	1	36.20	0.000	1	2.93	0.087 (NS)
Cage (Fly Site)	35	2.18	0.000	18	5.25	0.000	18	7.38	0.000
Fly site*Plant site	11	4.00	0.000	1	1.90	0.169 (NS)	1	54.24	0.000
Error	1496			756			906		

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