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Ecological Speciation in a Multi-trophic Complex: Gall Midges, Goldenrods, and Parasitoids

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ECOLOGICAL SPECIATION IN A MULTI-TROPHIC COMPLEX:
GALL MIDGES, GOLDENRODS, AND PARASITOIDS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

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2010

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ABSTRACT

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Ecological Speciation in a Multi-trophic Context: Goldenrods, Gall Midges, and Parasitoids

The importance of ecological interactions in the origin and maintenance of species diversity remains unclear. The current study assesses how ecological interactions shape the process of evolutionary diversification using a gall midge-host plant system in Ohio involving the gall midge, Asteromyia carbonifera (Diptera: Cecidomyiidae), and its goldenrod (Solidago) host-plants. A. carbonifera form four morphologically distinctive gall morphs and differ genetically. I studied phenology, host-plant specialization, and parasitism at three field sites in Southwestern Ohio. Phenology was assessed for twelve weeks while host-plant distribution and pressure from parasitoids were measured by monthly plot and rearing gall collections. Relative gall frequencies and eclosions were used to evaluate if temporal barriers exist (phenology) while host plant distributions were evaluated to observe if spatial barriers were present. Parasitism differences among morphs were also measured. Although phenology and host-plant preference were not significant, parasitism results revealed significantly distinctive patterns of parasitism among gall morphs.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Ecological Speciation in a Multi-trophic Context: Goldenrods, Gall Midges, and Parasitoids</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Speciation</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Adaptive Radiation</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A Classic Example: Adaptive Radiation in Cichlid Fishes</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cases of Adaptive Radiation</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ecological Speciation</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Cases of Ecological Speciation and Phytophagy</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Research Focus</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Literature Cited</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Chapter 2: Phenology and Distribution of a gall-making midge, Asteromyia carbonifera</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Adaptive Radiation</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Ecological Speciation and Phytophagy</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Phenology</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Diversification in Asteromyia Gall Mides</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Phenological Isolation of Asteromyia gall morphs has allowed their genetic and morphological</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>divergence</td>
<td></td>
</tr>
</tbody>
</table>
Genetic Divergence among Gall Morphs has been driven by differences in preference for and performance among host plant genotypes .................................................................20

Study System........................................................................................................21

The Host Plant.....................................................................................................23

Fungal Mutualism and Asteromyia .....................................................................24

II. Methods.............................................................................................................25

Study Sites..........................................................................................................25

Transects...............................................................................................................26

Phenological Isolation of Gall Morphs...............................................................27

Host-plant Distribution.......................................................................................28

Statistical Analyses.............................................................................................29

III. Results.............................................................................................................32

Gall Morph Frequencies over Space and Time..................................................32

Phenological Isolation of Asteromyia Gall Morphs...........................................35

Distribution of Gall Morphs among Plants.........................................................45

Among Ramets....................................................................................................45

Pair-wise General Linear Models and AIC at the Ramet Level .......................46

Among Plots.........................................................................................................52

Principal Component Analyses.........................................................................58

IV. Discussion.......................................................................................................64

Phenology.............................................................................................................64

Gall Morph Distribution......................................................................................68
V. Chapter 3: Divergence and Coexistence of a gall-making midge, Asteromyia carbonifera: Can pressure from natural enemies drive speciation in sympatric populations?.................................................................80

Introduction.............................................................................................................80
Study System and Background Information............................................................87
Biology of Asteromyia carbonifera...........................................................................87
The Host Plant..........................................................................................................90
Pressure from Natural Enemies..............................................................................91

VI. Methods.................................................................................................................93
Study Sites................................................................................................................93
Plots.........................................................................................................................94
Rearing.......................................................................................................................95
Statistical Analyses..................................................................................................96

VII. Results.................................................................................................................98
Parasitism by Gall Morph and Differences in Parasitoid Attack in Time and Space..................98
Parasitism per Gall Morph and Possible Factors Associated with Their Attacks...................102
Density-dependence of Parasitoids.........................................................................106
Density-dependence of Parasitoids at the Plot Level................................................109
Gall Morph Preferences by Particular Parasitoids.......................................................111
Factors Influencing the Rates of Attack by parasitoids............................................122
VIII. Discussion

Parasitism Differences across Gall Morphs and Sites .......................... 125
Parasitism Variance and Density-dependence .................................. 127
Parasitoids and Their Preferences .................................................. 128
Literature Cited ............................................................................. 135

IX. Chapter 4 .................................................................................. 138
Summary ....................................................................................... 138
Literature Cited ............................................................................. 142

X. Appendix A ............................................................................... 143

XI. Appendix B ............................................................................... 148
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 2:</td>
<td></td>
</tr>
<tr>
<td>1. Relative frequencies of gall morphs at each site over the three sampling rounds.</td>
<td>34</td>
</tr>
<tr>
<td>2. Eclosion Patterns of Gall Morphs at each Site over a Twelve week Interval</td>
<td>36</td>
</tr>
<tr>
<td>3. Initiation patterns of Gall Morphs at each Site over a Twelve week Interval</td>
<td>42</td>
</tr>
<tr>
<td>4. Mean Development Times of <em>Asteromyia</em> Gall Morphs</td>
<td>44</td>
</tr>
<tr>
<td>5. Gall Morph Distribution versus Poisson Distribution at GMP</td>
<td>45</td>
</tr>
<tr>
<td>6. Mosaic of Gall Morph Interactions at GMP</td>
<td>49</td>
</tr>
<tr>
<td>7. Mosaic of Gall Morph Interactions at BCW</td>
<td>50</td>
</tr>
<tr>
<td>8. Mosaic of Gall Morph Interactions at SSP</td>
<td>51</td>
</tr>
<tr>
<td>9. GMP Frequency Correlations in Combined Rounds</td>
<td>53</td>
</tr>
<tr>
<td>10. BCW Frequency Correlations in Combined Rounds</td>
<td>54</td>
</tr>
<tr>
<td>11. SSP Frequency Correlations in Combined Rounds</td>
<td>55</td>
</tr>
<tr>
<td>12. Frequency Distribution with Pearson’s Goodness of Fit Values</td>
<td>56</td>
</tr>
<tr>
<td>13. GMP Principal Components</td>
<td>59</td>
</tr>
<tr>
<td>14. BCW Principal Components</td>
<td>61</td>
</tr>
<tr>
<td>15. SSP Principal Components</td>
<td>62</td>
</tr>
</tbody>
</table>
16. Gall Morph Distribution versus Poisson Distribution for GMP (Appendix A)..143
17. Gall Morph Distribution versus Poisson Distribution for BCW (Appendix A)..144
18. Gall Morph Distribution versus Poisson Distribution for SSP (Appendix A)...146
19. BCW Gall Frequency Distribution with Pearson’s Goodness of Fit Values…..148
20. SSP Gall Frequency Distribution with Pearson’s Goodness of Fit Values……150

Chapter 3:

1. Frequency of Parasitism per Gall over Time for each Gall Morph………………100
2. Overall Proportions of Parasitism of Gall Morphs across Sites………………..104
3. Parasitism per Gall Morph for Density-dependence at the Site Level…………..107
4. Gall Morph Preference by the Seven Discovered Parasitoids for GMP………..115
5. Gall Morph Preference by the Seven Discovered Parasitoids for BCW………..118
6. Gall Morph Preference by the Seven Discovered Parasitoids for SSP………..120
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 2</strong></td>
<td></td>
</tr>
<tr>
<td>1. Overall Gall Morph Frequencies Compared to Total Gall Numbers per Site</td>
<td>33</td>
</tr>
<tr>
<td>2. Time of Gall Eclosion Correlations with Pairwise Gall Combinations</td>
<td>40</td>
</tr>
<tr>
<td>3. Number of Generations Estimated by Eclosion Peaks</td>
<td>40</td>
</tr>
<tr>
<td>4. Gall Initiation with Pairwise Gall Combinations at the Three Sampling Sites</td>
<td>41</td>
</tr>
<tr>
<td>5. Results of Two-Way ANOVA of the Effects of Morph, Site, and their interactions with development time</td>
<td>45</td>
</tr>
<tr>
<td>6. All Pairwise Combinations of Gall Morph Interactions per Site</td>
<td>47</td>
</tr>
<tr>
<td>7. Significant Interactions in Final AIC Selected General Linear Models</td>
<td>48</td>
</tr>
<tr>
<td>8. Correlation between Gall Morphs at all Three Testing Sites</td>
<td>58</td>
</tr>
<tr>
<td><strong>Chapter 3</strong></td>
<td></td>
</tr>
<tr>
<td>1. Correlation of Parasitism by Gall Morph per Site</td>
<td>102</td>
</tr>
<tr>
<td>2. ANOVA Table of Gall Morphology, Round, Site, and their Significant Interactions</td>
<td>105</td>
</tr>
</tbody>
</table>
3. Linear Regression Summary for Density-dependence Analyses of Gall
   Parasitism at the Plot Level for GMP.................................110

4. Linear Regression Summary for Density-dependence Analyses of Gall
   Parasitism at the Plot Level for BCW.................................111

5. Linear Regression Summary for Density-dependence Analyses of Gall
   Parasitism at the Plot Level for SSP.................................111

6. Pearson’s Chi-squared Results for Parasitoid Frequencies among Gall
   Morphs.................................................................113

7. Table of Proportions of Parasitism per Gall Morph per Site..........114

8. Effect of Morph*Round*Site on Parasitoid Attacks....................124
Ecological Speciation in a Multi-trophic Context: Goldenrods, Gall Midges, and Parasitoids

Speciation is central to evolutionary biology yet it is one of the least understood processes of evolution (Schluter 2001). Despite many years of study, the causes of speciation and the genetic basis of reproductive isolation remain unclear in most cases (Coyne & Orr 1999). One way researchers have made headway in understanding the long-term process of speciation is by examining instances of rapid ecological and evolutionary diversification of particular clades, or “adaptive radiation” (Schluter 2000). Adaptive radiation incorporates the origin of new species and the evolution of ecological differences between them, and may possibly be the most common pattern in the origin and proliferation of taxa (Schluter 2000).

Adaptive Radiation

Adaptive radiation is a “selection-driven evolution of ecological and phenotypic diversity within a rapidly multiplying lineage” (Schluter 2000). It occurs as a single common ancestor differentiates into an array of species that use a variety of environments and that differ in the traits used to exploit these environments (Schluter 2000). Four key requirements that describe an adaptive radiation include common ancestry, a correlation
between phenotype and environment, trait utility, and rapid speciation. These four key features, as outlined by Schluter (2000), are explained in more detail below:

1. Common Ancestry: A monophyletic clade consisting of a single common ancestor and all of its descendents in contrast to lineages that have evolved several times.

2. Correlation between Phenotype and Environment: Phenotypic suitability of the species in relation to their divergent environments suggests an adaptive shift or radiation.

3. Trait Utility: Morphological and physiological traits of species are advantageous and/or suited to divergent environments. Acquired traits should enhance the species’ ability to adapt to novel environments.

4. Rapid Speciation: This feature of adaptive radiation involves two requirements. First, speciation refers to the evolution of reproductive isolation. This isolation, consequently, allows greater phenotypic divergence among species. Second, the diversification of species is considered “rapid” relative to other lineages or other time periods.

These criteria distinguish an adaptive radiation from non-adaptive radiations. Non-adaptive radiations are also rapid bursts of species; however, the process of differentiation is unrelated to resource use and environmental variation. Genetic drift, bottlenecks, founder effects, and sexual selection are all potential mechanisms of species proliferation through non-adaptive radiation.

A Classic Example: Adaptive Radiation in Cichlid Fishes
Many cases of adaptive radiation have been proposed, most of which focus on either vertebrates or plants. Cichlids fishes, in the East African Lake Malawi, offer one of the best examples of an adaptive radiation in vertebrates. The cichlids of Lake Malawi have demonstrated a large-scale rapid radiation generating 700-1000 species within a limited geographical area (Lake Malawi) and within a fairly short amount of time (0.7-2 mya) (Turner 1999, Salzburger et. al 2004). Although some evolutionary biologists question whether this radiation occurred in response to sexual selection or other non-environmental processes (Salzburger et. al 2004), this radiation of cichlid fishes nicely illustrates the key features of an adaptive radiation. With respect to the common ancestry prerequisite (i.e. Key Feature 1: Common Ancestry), most of the cichlid species form a monophyletic group and are descendants of one riverine species (Turner 1999). In their recent radiation, these cichlids have undergone diverse trophic level transitions within Lake Malawi. These cichlids possess a breadth of feeding habits including detritivores/microbivores, phytoplankton and macrophyte feeders, insectivores, and piscivores illustrating Key Feature 2: Correlation between Phenotype and Environment (Schluter 2000). In addition, some cichlids have evolved more precise dietary specializations such as scale-eaters and molluscivores (Genner et. al 1999, Schluter 2000, Smits 1996). Niche division necessitates utilizable traits for resource acquisition. Several studies (Liem 1986, Nee et. al 1994) have suggested that diversification of the pharyngeal apparatus has allowed divergence into different niches, or trophic levels, providing each species with utilizable feeding apparatus within specific environments (i.e. Key Feature 3: Trait Utility). In association with Key Feature 4: Rapid Speciation, the cichlid radiation has also been relatively ‘rapid’ in Lake Malawi ranging from 0.7-2
mya (Meyer 1983). Unoccupied adaptive zones (open trophic levels) provided a means of niche differentiation in the cichlid fishes of Lake Malawi. Stemming from a single common ancestor, these cichlids have diversified into a highly diverse community.

Although there is considerable evidence for speciation through adaptive radiation in nature, we know little about its causes. Much of the focus on understanding the underlying causes has been on divergent selection-associated with resource competition (Schluter 2001). Little attention has been paid to other ecological factors, such as predators, host or prey defenses, and phenological variation among species interactions, which may influence the probability and extent of adaptive radiations. Because organisms exist in a web of complex species interactions, we need to study other ecological processes aside from merely resource competition that may promote or hinder adaptive radiation.

Ecological Speciation

A key component in adaptive radiation is ecological speciation. In ecological speciation, reproductive isolation evolves as a result of ecologically-based divergent selection between environments in contrast to neutral modes of speciation in which species may arise from genetic drift or founder effects (Schluter 2001). Reproductive isolation may arise as a by-product of adaptation to alternative selection regimes via low hybrid fitness and reinforcement (Schluter 2001). Ecological selection arises through interactions of individuals with their environment leading to a divergence between two populations pulling them in opposite directions and favoring opposite phenotypes within
a single population (Rundle et al 2005). Consequently, speciation can be the end result of divergent natural selection (Schluter 2000). Environmental pressures pushing adaptive divergence may include climate, resources, competition, and predation and the speed of adaptive divergence may depend on ecological differences between populations such as dispersal rate, habitat preference, and selection against migrants or hybrids (Schluter 2001, Hendry et al 2007).

Cases of Ecological Speciation and Phytophagy

Phytophagous, or plant-feeding, insects have several features that make them useful models for ecological speciation (Joy et al 2007). To begin with, phytophagous insects are highly diverse and virtually ubiquitous in terrestrial ecosystems. Next, most phytophagous insects are ecologically specialized on particular host plants, and this specialization may facilitate the evolution of reproductive isolation (Joy et al 2007). Lastly, the developmental timing (phenology) of phytophagous insects may be dependent upon host-plant resources with different phenologies. For instance, adults from populations specialized on distinctive host-plant resources may mature and mate at different times leading to temporal isolation among populations (Joy et al 2007). The apple maggot, Rhagoletis pomonella, complex illustrates how phenological differences have led to divergence. Studies involving sympatric Rhagoletis populations on apple and hawthorn trees have shown that differences in eclosion times may be responsible for reduced gene flow (Filchak et al 2000).
Species interactions such as predation and host-plant preference are ecological factors that can influence speciation. For instance, a study investigating the genus *Timema*, or walking-stick insects provides evidence of how both pre-zygotic and post-zygotic barriers have influenced their divergence. *Timema* are phytophagous, wingless insects that occupy two common host-plants in western North America (Nosil 2004). Host-plant related crypsis has fueled divergence of *Timema* morphs (pre-zygotic barrier) and, as a result of this host-plant related crypsis, *Timema* suffer lower rates of predation (Nosil 2004). Although low levels of gene flow continue between the parental groups of walking sticks, increased hybrid mortality due to predation (post-zygotic barrier) is also causing reproductive isolation between populations using different host plants (Nosil 2004). Intermediate morphs that are not camouflaged by parental host-plant types are likely to exhibit increased predation rates by birds and/or lizards (Nosil 2004). Therefore, predation is likely to reduce interbreeding between the two parental populations leading to an extrinsic cause of reproductive isolation (Nosil 2004). The *Timema* study shows how host-plant preference and predation can encourage reproductive isolation and eventually speciation.

Gall-inducing midges (Diptera: Cecidomyiidae) can be instructive for understanding speciation in phytophagous insects. Galls are abnormal growths formed from plant tissues as a result of parasitic activity by the gall producer, typically an arthropod (Price 2005). Galled structures provide nutrition for the maturing insect, which distinguishes them from other insect-generated shelters such as leaf-rolls which are used for protection (Stone & Schönrogge 2003). Gall-forming midges are highly diverse and
abundant in terrestrial environments. They utilize all plant parts such as the leaves, stems, twigs, buds, flowers, and roots for gall development (Price 2005). Cecidomyiids are also widely distributed across plant taxa such as gymnosperms and angiosperms (Gagne 1968). Although gall midges are widely distributed, nearly all species are highly host-specific and most often feed only on one part of a single host-plant species (Gagne 1968).

Research Focus

In my thesis research, I studied a gall midge-host plant system in Ohio involving the gall midge, Asteromyia carbonifera (Diptera: Cecidomyiidae), and its goldenrod (Solidago) host-plants. The early stages of divergence in this gall midge appear to meet the criteria of Schuler’s definition of adaptive radiation: Common Ancestry, Correlation between Phenotype and Environment, Trait Utility, and Rapid Speciation (Schluter 2000).

Asteromyia carbonifera is a specialist on the genus Solidago, but it is widespread on many different species in this genus (Gagne 1968). Until recently, A. carbonifera was considered one species; however, A. carbonifera gall morphologies vary across the Solidago genus and there is evidence of cryptic host-associated genetic differentiation of populations on different Solidago species (Stireman et al. 2010). In addition, differing gall morphologies have also been found to coexist on a single host plant, Solidago altissima (Late Goldenrod) (Crego et al. 1999, Stireman et al. 2008). These A. carbonifera gall “morphs” are largely sympatric and sometimes coexist on the same stem
(ramet) or even leaf. *A. carbonifera* gall morphs associated with *S. altissima* differ in their number of generations, gall morphologies, larval placement, and number of larvae per gall.

Geographically, *A. carbonifera* is widespread in the United States and southern Canada; however, it is most commonly found in the northeastern United States (Gagne 1968). *A. carbonifera* produce blister-like galls on their *Solidago* hosts formed in part by an apparently mutualistic fungus, a symbiont of the midge. Their widespread distribution and abundance provides an opportunity to investigate how speciation can ensue through adaptive radiation initiated by environmental and ecological pressures other than resource exploitation.

Studying the biology and ecology of *A. carbonifera* and its current radiation across and within host plants may allow us to satisfy gaps in our understanding of adaptive radiation. Most studies of speciation via adaptive radiation present evidence of speciation in allopatry, or geographical division of populations leading to reproductive isolation (Turner 1999, Salzburger et. al 2004). Conversely, studies providing evidence of speciation in sympatric, or geographically overlapping, populations are infrequent. Since *A. carbonifera* are currently undergoing radiation, this system may provide insight into the ecological forces that drive adaptive radiations. While speciation is probably not complete within this *Asteromyia* group, the incipient nature of diversification in thus system enables the study of factors such as predation and mutualism that may promote and/or hinder divergence when they are most detectable and recognizable.
In my research, I considered ecological factors other than resource competition that may be influencing the radiation of four lineages of *A. carbonifera* on a single host plant. The ecological processes that I examined include phenology, or the timing of adult emergence and egg-laying, specialization of *A. carbonifera* established on different host-plant genotypes, and varying sources and intensities of parasitism affecting these gall morphs. I found evidence that there is some difference in eclosion phenology among gall morphs; however, presently, there is still considerable overlap suggesting there is ample ability to mate. Additionally, I found that there are significant effects of the gall morph, the site, and the interaction between the gall morph and site on development time and that development time varies among sites and morphs. Regarding host-plant preference, *A. carbonifera* are not specializing on particular host-plant genotypes, but data suggests that there are some *S. altissima* plants that are optimal hosts for all gall morphs and some that are poor hosts. Parasitism pressure did vary significantly among site, gall morph, and the interaction with the morphs and site. Furthermore, seven different hymenopteran parasitoid species were found to attack these gall midges. This evidence firmly suggests that parasitism is likely a strong selective pressure driving the divergence and possible coexistence of the *Asteromyia carbonifera* gall midge.
Literature Cited


Chapter 2

Phenology and Distribution of a gall-making midge, *Asteromyia carbonifera*

Introduction

*Adaptive Radiation*

When two or more closely related species coexist sympatrically, the geographic mode by which they diverged is not always clear. One means of species divergence is adaptive radiation, or an increase in the number and diversity of species within a single lineage (Schluter 2000). Beginning with a single common ancestor, an adaptive radiation entails the ecological and phenotypic diversification within a rapidly multiplying lineage (Schluter 2000). The traditional idea of adaptive radiations is that an ancestral species colonizes a new area where there are many vacant and resource-rich niches (Gavrilets and Losos 2009). Probably the most commonly cited example of an adaptive radiation is that of Darwin’s finches on the Galapagos Islands (Schluter 2000, Gavrilets and Losos 2009). Since the formation of the Galapagos Islands, the finches diverged rapidly from a common ancestor and diversified into fourteen species with different bill morphologies adapted for different ecological feeding niches (Burns et al. 2002). The divergence of
Darwin’s finches is thought to have been driven by resource utilization and competition (Schluter 2000, Burns 2002). This focus on resource utilization and/or competition as the factors used to shape species divergence is characteristic of most studies investigating adaptive radiations (Schluter 2000, 2001). Only recently have researchers began to consider ecological factors other than resource utilization and/or competition that could possibly promote or hinder species divergence in adaptive radiations. Ecological forces such as predation, disease, and mutualisms may drive adaptive divergence; hence, driving reproductive isolation between contributing species (Hendry et al. 2007, Schluter 2001 & 2005). It is crucial to study the ecology of certain species that may be undergoing adaptive radiation because this may allow us to see which, if any, ecological factors that contribute to species divergence. Ecological speciation, or the evolution of reproductive isolation between populations that differ in environments or ecological niches, is central to adaptive radiation (Schluter 2009). Aside from resource utilization and competition, several studies have shown that ecologically selective forces such as mutualistic relationships and phenological differences based on life history traits and adult longevity have influenced several naturally occurring systems (Langerhans 2007, Stone and Schonrogge 2003, Nosil 2004, Sachet et al. 2009).

Ecological speciation merges the ideas of speciation with divergent selection on traits due to environmental differences (Schluter 2001). This marriage of ideas consequently posits a mechanism for reproductive isolation and for the adaptive divergence of species (Schluter 2001). Insects and their interactions with plants together represent two dominant taxa in terrestrial ecosystems in terms of diversity that can be
studied to understand ecological speciation (Cook & Segar 2010, Labandeira 2002, Marussich & Machado 2007). Several studies have shown the importance of pollinating fig-wasps with their respective host fig species (Ficus sp.) in the coevolution, speciation, and continuance of both mutualists (Labandeira 2002, Jousselin et al. 2006, Marussich & Machado 2007, Cook & Segar 2010). Mutualisms and phenological shifts have shown to be ecological factors associated with the divergence of figs and fig-wasps (Jousselin et al. 2006, Cook & Segar 2010, Labandeira 2002, Marussich & Machado 2007) as well as coneflies (Sachet et al. 2009) and may very well lay the foundation for many other recently occurring radiations.

*Ecological Speciation and Phytophagy*

Phytophagous insects have several characteristics that make them useful models for understanding the process of ecological speciation (Joy et. al 2007, Funk 2010, Matsubayashi et al. 2010). These insects along with their host-plants and natural enemies comprise nearly 75% of the species on Earth (van Veen et al. 2006, Drés & Mallet 2002). This wealth of species provides a diversity of systems at different stages of diversification that can be studied. In addition, most phytophagous insects are ecologically specialized on particular host plants (Drés & Mallet 2002, Caillaud & Via 2000). This specialization may facilitate the evolution of reproductive isolation because insects experience strong divergent selection on alternative host plants due in part to the secondary defensive chemicals of plants (Drés & Mallet 2002, Caillaud & Via 2000, Strong, Lawton, & Southwood 1984).
Insects and their intimate relationships with their host-plants may also provide a strong enough spatial barrier that leads to reproductive isolation (Strong, Lawton, & Southwood 1984). Mopper et al. (1995) argues that the intimate relationship between most phytophagous insects and their host plants could be a critical factor that favors the formation of isolated populations, or demes, in herbivorous insects. For instance, if particular genotypes of herbivores experience increased fitness on particular host plants, and this fitness advantage is heritable, then, over time, populations on different host plants should diverge, become reproductively isolated, and form host races (Stiling and Rossi 1998). These host races are sympatric populations of phytophagous insects that use different hosts but are genetically differentiated and co-existing in space (Tarayre et al. 2008, Drès & Mallet, 2002).

A prime example of an insect that has diverged based on host plant preference is a tephritid fly Eurosta solidaginis (Craig et al. 2000). This fly has developed into two host races that form ball-like galls on the stems of their host plants Solidago altissima and Solidago gigantea (Craig et al. 2000). Craig et al. (1997, 2000) demonstrated that each host race preferred oviposition on its own host plant rather than the alternative host and this preference was associated with increased offspring survival. The formation of the two host races occurred due to a host shift by the altissima group; thereby creating the gigantea population (Craig et al. 1997, 2000). Craig et al. (1993) found that there is assortive mating based on host-plant preference (Craig et al. 1993, 1997). Due to differing emergence times among the adults coupled with assortive mating, E. solidaginis
experiences enough of a barrier to gene flow to allow divergence (Craig et al. 1993, 1997).

*Phenology*

Phenological differences are yet another ecological factor that may influence adaptive radiation. Temporal, or timing, variation in life histories between certain species may lead to less intense competition and the ability of closely related species to coexist on a single host (Sachet et al. 2009). For instance, Sachet et al. (2009) found that several closely related species of large cone flies (*Strobilomyia* sp.) coexist on their host plant larch, *Larix decidua*. The different cone fly species are strict pests of the larch and utilize the host’s cones for nutrition and protection for their maturing larvae; however, cone fly ovipositions between the species are spaced at two-week intervals (Sachet et al. 2009). Larval maturation within the cone is estimated around six weeks suggesting a spatial overlap among the species; however, larvae residing in these cones with varying maturation levels may allow just enough time for a phenological shift between mating in the adults, leading to reproductive isolation (Sachet et al. 2009). This suggests that differing phenologies have allowed the adaptive radiation of the cone flies (Sachet et al. 2009). The developmental timing of phytophagous insects may be dependent upon host-plant resources with different phenologies (Joy et. al 2007, Sachet et al. 2009). For instance, adults from populations specialized on distinctive host-plant resources may mature and mate at different times leading to temporal isolation among populations (Joy et. al 2007). The apple maggot, *Rhagoletis pomonella*, complex illustrates how phenological differences have led to divergence. Studies involving sympatric *Rhagoletis*
populations on apple and hawthorn trees have shown that differences in eclosion times may be responsible for reduced gene flow (Filchak et al. 2000). Ecological speciation through phenological isolation as well as host-plant distribution in host-dependent insects is apparent (Labandeira 2002, Marussich & Machado 2007, Sachet et al. 2009) but may be responsible for an abundance of insect diversity.

The current view of adaptive radiation highlights the role of ecological factors that may drive species divergence (Sachet et al. 2009). These factors include the colonization of new resources or the division of existing niches, although this perspective does not incorporate closely-related species that coexist on the same host (Sachet et al. 2009). In cases like these, phenological shifts, or timing differences within life history traits, may allow species that are living sympatrically to diverge, by reducing opportunities for gene flow. In phytophagous insects, related species may exploit different organs of the same host plant; however, these closely-related species that use the same resource may do so at different times (Sachet et al. 2009).

A key example of host-specific, phytophagous insects that are prone to host race formation due to phenological differences are the seed weevils (Coleoptera: Curculionoidea: Apionidae, *Exapion* spp.) that feed specifically on three gorse species (Fabaceae, *Ulex* spp.) in western France (Tarayre et al. 2008). Tarayre et al. (2008) found that the two closely related weevil species appear morphologically similar, share like life cycles, live sympatrically on genetically closely related host-plants and seem to be reproductively isolated in time. The female weevil lays her eggs in the gorse pods and the hatched larvae feed on the seeds (Tarayre et al. 2008). Full development of the
seed weevils take place within the gorse pod and the adults wait to emerge until pod
dehiscence where they can then feed on the flowers and vegetative parts (Tarayre et al.
2008). This finding demonstrates that host-specificity as well as the life history of the
weevil (egg-laying, eclosion, etc.) is linked to host-plant phenology (Tarayre et al. 2008).

I studied a gall-making midge, Asteromyia carbonifera, and its association with
surrounding species to investigate ecological interactions that may influence speciation.
Exploration of this system will also allow us to understand how closely related species
are able to coexist in the same areas since they occupy a single host plant. Two
ecological factors that I analyzed include phenology and host-plant specialization based
on host-plant genotype to determine whether these ecological factors drove the
divergence in Asteromyia carbonifera.

Diversification in Asteromyia gall midges

Among phytophagous insects, gall-making midges (Diptera: Cecidomyiidae) are
particularly useful models for studying speciation due to their high host specificity and
the potential for documenting selective pressures associated with ecological interactions
due to their confinement within galls (Weis 1982, Askew 1975). Galls are plant-tissue
abnormalities caused by the gall-maker’s ability to control development of plant cells in
order to promote fungal inoculation which aids in gall initiation and growth (Rohfritsch
2008). Cecidomyiids sometimes share their galls with specific fungi that help form the
structure of the gall as well as provide nutrition to the growing larvae (Bissett and
Borkent 1988; Janson et al. 2009). Even though the key role of the fungus is to provide
nutrition (Janson et al. 2009), the gall itself offers shelter and predator protection for immature stages of the gall-maker (Weis 1982). Cecidomyiids form galls on a wide diversity of plant taxa (Gagne 1968); however, individual species tend to be highly specialized. The gall-making midge, Asteromyia carbonifera (Diptera: Cecidomyiidae) only forms galls on species in the genus Solidago (Family Asteraceae) (goldenrods). Although these midges are considered a single species (Gagne 1968), evidence exists of morphologically cryptic host-associated genetic differentiation of populations among different Solidago species (Stireman et al. 2010). Not only are these midges diversifying across Solidago taxa, but there is also evidence of differing gall morphologies on a single host plant, Solidago altissima (Late Goldenrod) (Crego et al. 1990, Stireman et al. 2008). Crego et al. (1990) initially observed three distinct gall morphologies occurring on the same part of the S. altissima plant and later found a fourth type, all of which were genetically distinct (Crego 1990, Stireman et al. 2008). These distinct gall morphologies were coined “crescent”, “cushion”, “flat” and “irregular” based on their general shape and thickness. A. carbonifera gall morphotypes are sympatrically distributed and can even be found on the same ramet as well as the same ramet organ, the leaf. The gall morphs existing on S. altissima differ noticeably in appearance, placement on the leaf, placement of larvae, and their obligate fungal symbiont.

Given that the morphotypes of Asteromyia carbonifera are sympatric and use the same resources, how can they coexist on a single host-plant, S. altissima? Possible ecological factors that could foster the coexistence of this diverging species could be phenology, host-plant choice, and pressure from natural enemies.
Research Hypotheses:

Phenological isolation of Asteromyia gall morphs has allowed their genetic and morphological divergence

Phenologies of the individual gall morph populations may constitute an ecological factor that could contribute to temporal isolation among the A. carbonifera adults. Differences in eclosion times and short life-spans (Gagne 1968, Weis 1983) may force the adults to mate with adults from the same gall types. A. carbonifera gall morph populations may differ phenologically, or in developmental timing. Asteromyia undergo several generations per season (May through October) (Gagne 1968, Weis 1983). Since the adults are short-lived (Gagne 1968, Weis 1983) there is much opportunity for reproductive isolation, which may have facilitated the divergence between the gall morphs. If phenological isolation has played a role in divergence of Asteromyia morphotypes, we expect to observe gall-morph related variation in the timing of adult emergence and egg-laying in the field.

Genetic divergence among gall morphs has been driven by differences in preference for and performance among host plant genotypes

Herbivorous insects often exhibit preferences for particular plant genotypes (Craig 2007). This may lead to local adaptation of insect lineages, encouraging population divergence (Craig 2007). S. altissima is likely to possess high levels of genetic variation due to its broad geographic range and large population size, and is known to consist of three ploidy races (2N, 4N and 6N; Halverson et. al 2007a). Even
though these midges prefer the same host-plant, *Asteromyia carbonifera* morphs may differ in use of host-plant ploidy races or other host-plant genotypes resulting in a nonrandom distribution of gall morphs. This host-plant choice could limit mating due to spatial barriers. If genetic divergence among gall morphs has been driven by differing host-plant genotypes, we expect to observe a non-random distribution, or clumping, of gall morphs in relation to the distributions of their preferred host-plant types. Temporal (phenology) and spatial (host-plant preference) differences may have created the original divergence of *A. carbonifera*, but are these the same forces enforcing genetic isolation among the four host races? If phenology plays a role in this enforcement, then there should be little or no overlap in the emergence of the adults, thereby, allowing reproduction between the gall morphs. In addition, if host-plant preference is not a factor, then gall morphs should overlap in space countering the hypothesis that they experience spatial reproductive barriers.

**Study System**

In central Illinois, *A. carbonifera* have three to five generations per year with each generation (egg-adult) lasting approximately four weeks (Weis 1983). The late larval stage of the last generation overwinters in the gall and falls to the ground in autumn (Gagne 1968). In the spring, the larvae pupate, and the adults emerge shortly thereafter (Gagne 1968). One to five larvae may inhabit each gall and all larvae within the same gall are the progeny of a single female (Weis 1981, Gagne 1968). Females lay eggs in small clutches on the underside of new leaves on a young host-plant (Gagne 1968, Weis 1983; Heath and Stireman 2010). As the female lays eggs, fungal conidia is deposited
with the eggs, which have been identified as *Botryosphaeria dothidea* (Janson *et al.* 2010). First larval instars burrow into the leaf lamina initiating gall development. Fungal spores introduced during oviposition germinate within a few days following oviposition and penetrate the leaf tissue as well (Heath & Stireman 2010, Weis 1983). Larvae remain stationary while fungal mycelia proliferate around it forming a cast around the *Asteromyia* larvae (Gagne 1968). Maturing galls develop a covering of white fungal hyphae that forms just below the leaf epidermis (Crego *et al.* 1990). After the midge pupates, the fungal mycelium rapidly proliferates and forms a thick, hardened, carbonaceous material known as the stroma which encases the pupae for the remainder of the juvenile stages (Batra 1964). Adults emerge approximately 5-7 days after pupation, usually leaving a pupal exuviae partially inside the gall, and fungal growth associated with the gall subsides (Gagne 1968). *A. carbonifera* adults possibly only mate once per lifetime, are naturally short-lived (2-3 days), and are non-feeding as suggested by their reduced mouthparts (Batra 1964, Weis 1983, Gagne 1968). Emerging adults from a single gall are of the same sex (Gagne 1968).

Morphological and genetic differentiation of the midges may be influenced by three major ecological interactions in this system: interactions with the host plant, the gall midge/fungus association, and pressure from natural enemies. Populations of *A. carbonifera* may prefer specific host plant genotypes that may be spatially clustered due to the rhizomatous growth of *S. altissima* (Gagne 1968, Crego *et al.* 1990). This spatial patterning of these host-plants may provide a spatial boundary between *A. carbonifera* morphs. Gene flow between the gall morphs may decrease because of the female’s
tendency not to travel such distances to mate with other morphs or there may be pre-or post-zygotic setbacks where the offspring do not develop or are not viable. The midge’s association with the cohabitant fungus may also play a role in the divergence of this species (Batra 1964, Crego et al. 1990). According to Batra (1964), the fungus is believed to be responsible for differences in gall morphologies. The relationship between the midge and the fungus appears to be mutualistic in that the midge relies upon the fungal mycelium for food while the fungus depends on the female for the transport and inoculation in the host-plant (Bissett and Borkent 1988; Heath & Stireman in Review, Janson et al. 2009). The fungal tissue alters the thickness and texture of the gall which helps protect A. carbonifera from its natural enemies (Weis 1981, Batra 1964).

Hymenopteran parasitoids are known to inflict high levels of attack on A. carbonifera populations, ranging between 40-60% (Stireman et al. 2008, Weis 1982). Increased parasitoid pressure may select for particular gall traits controlled by the midge/fungal symbionts, such as gall thickness or the number of larvae per gall.

The Host Plant

The host plant, Solidago altissima, may shape genetic differentiation of both A. carbonifera and its fungal symbiont. S. altissima is a rhizomatous perennial with a native distribution over much of temperate North America (Semple and Cook 2006). Three known cytotypic variations (ploidies) exist within S. altissima: diploid (2N), tetraploid (4N), and hexaploid (6N) (Halverson et.al 2007a). Genetic variation in dominant plant species has shown to be a major factor in ecosystem biodiversity and function (Genung et al. 2010). For instance, Genung and colleagues(2010) found that intraspecific genetic
variation and genotypic diversity in *Solidago altissima* affect flower visitor abundance and richness suggesting that floral community phenotypes may vary in response to plant genotypic diversity (Genung *et al*. 2010). Cytotypic variation as well as other genetically based traits within *S. altissima* populations may influence plant susceptibility to different gall morphs (Genung *et al*. 2010, Halverson *et al*. 2007b).

**Fungal Mutualism and Asteromyia**

*Asteromyia carbonifera* larvae are dependent upon a fungal symbiont, *Botryosphaeria dothidea* for gall production (Batra 1964, Janson *et al*. 2009; Heath & Stireman 2010). The relationship between the midge and fungus appears to be highly intimate suggesting a symbiotic relationship. The fungus supplies food for the maturing *Asteromyia* and may be responsible for gall morphology (Weis 1983, Crego *et al*. 1990, Janson *et al*. 2009). The fungus, in the absence of the *Asteromyia*, will not differentiate and form the typical thick, hardened, carboniferous stroma (Heath & Stireman 2010). It has been suggested that larval frass (excrement) may provide a supplemental source of nutrients to the fungus and in turn, the fungus provides shelter and food for the maturing midge (Batra 1964). *A. carbonifera* may select for certain fungal types based on the growth rate and structural and nutritional supports offered by the fungus; and, in return, offer the fungus transportation and inoculation into the host-plant (Bissett and Borkent 1988). This mutualism may promote reciprocal co-evolutionary interactions between the mutualists.
It is unclear if the fungal symbiont promotes or hinders host-plant shifts. If the fungal strains differ genetically, they may hinder the midges from mating outside their natal gall types. For instance, females emerging from a cushion gall morph may prefer to mate with a male that has emerged from a cushion gall morph facilitating maintenance of gall characteristics such as nutrition or defense benefits associated with cushion gall morphs. Alternatively, differing fungal strains may allow the midges to rapidly exploit new host plants. The fungus may act as a buffer of host plant defenses that would otherwise deter shifts onto novel hosts; thus, the fungus may represent a key innovation for *A. carbonifera* (Berlocher *et al.* 2002). Further research is needed to determine whether the *Asteromyia-Botryosphaeria* relationship is involved in the division of species within the *Asteromyia carbonifera* group.

**Methods**

In this study, I first investigated phenological differences among the differing gall morphs of *A. carbonifera*. Field transects through fields of *S. altissima* were monitored weekly from June through September to determine the initiation and eclosion of all gall morphs. This allowed us to test if phenological differences exist between gall morphs; thus, leading to partial or total reproductive isolation. Second, to test for non-random distribution of gall morphs among host-plants, we set up ten plots (five along two transects) in each of three field sites and surveyed the number of specific gall morphs within each plot. This allowed us to determine whether gall morphs are distributed randomly among host plant ramets and presumed genets, or whether they appear to exhibit spatial clumping.
Study Sites

All sampling took place at three sites: Germantown Metropark (39°39’51.43”N, 84°24’58.17”W), Sycamore State Park (39°49’00.54”N, 84°21’56.41”W), and Beaver Creek Wetlands (39°45’57.69”N, 84°00’17.26”W) located in Montgomery and Greene counties in Southwest Ohio. Distances between the parks are as follows: Sycamore State Park to Beaver Creek Wetlands = 42.33km, Beaver Creek Wetlands to Germantown Metropark = 54.56km, and Sycamore State Park to Germantown Metropark = 24.94km.

The Germantown Metropark field site consisted of approximately 0.81 hectares of old prairie fields divided by a gravel roadway. The site at Beaver Creek Wetlands was approximately 2.02 hectares of restored prairie surrounded by restored wet prairies, fens, and marshes. Restoration of this prairie began in the early 1990’s. The site at Sycamore State Park was approximately 1.21 hectares of restored prairie bordered by second growth forest stands. Restoration of the Sycamore State Park prairie began in the early 1980’s. Areas were chosen where continuous goldenrod patches could support two 50m transects with surrounding areas of approximately 20m on either side of the transects.

Abbreviations for these sites were used in numbering of galls throughout the surveys and are designated as GMP for Germantown Metropark, BCW for Beaver Creek Wetlands, and SSP for Sycamore State Park.

Transects

GMP: Transect 1 (39°38’45.74”N, 84°24’41.39”W, elevation 273.4m) was oriented diagonally off the shoulder of Conservancy Road near the maintenance building. The second transect would not adequately fit near the first; therefore, Transect 2
(39°38′41.17″N, 84°24′33.10″W, elevation 273.4m) was placed about 112m from the gate at 6791 Conservancy Road in the patch east of the gravel roadway. The distance between the two transects was 0.24 kilometers.

BCW: Transect 1 (39°45′54.72″N, 84°00′15.86″W, elevation 256m) was laid about 190m from the parking area on New Germany-Trebein Road while transect 2 (39°45′54.92″N, 84°00′15.80″W, elevation 256m) was about 185m away from the parking area. After the original transects were set up (week 1), park officials accidentally mowed portions of both transects. Meters 0-9 and 44-50 were missing from transect 1. Meters 0-9 were missing from transect 2. To compensate, I set up a third transect (39°45′55.11″N, 84°00′15.84″W, elevation 256m) with 25 meters on the north side of transect 2. Monitoring of the third transect began at the beginning of week 2.

SSP: Transects 1 and 2 ran parallel to one another and were placed south of the main pathway approximately 165 meters off of Diamond Mill Road. Coordinates for these transects were as follows: Transect 1, 39°48′35.83″N, 84°22′08.22″W, elevation 294.7m and Transect 2, 39°48′35.63″N, 84°22′08.24″W, elevation 294.7m.

*Phenological isolation of Gall Morphs*

In order to test whether gall morphs differ significantly in their timing of adult eclosion and egg-laying, I monitored gall development on S. altissima in two 50-meter transects at each of the three site for a total of 6 transects or 300 meters. These transects were set up on June 10, 2007 and monitored through the end of August 2007. Four S. *altissima* ramets (stems) were marked at each meter of each transect and monitored
weekly for gall phenology and density for twelve weeks. These four ramets were selected as the four closest to the transect line and were marked with fluorescent-colored flagging tape indicating the plant number, permit number, and the initials BW. As galls appeared on leaves, they were given a number indicating site, plant, and gall. These galls were followed weekly and observed for eclosion holes, herbivory from other insects such as caterpillars, or leaf damage due to environmental stress. This allowed me to follow gall development from initiation through adult emergence to determine whether temporal isolation exists among gall morphotypes. Also, these data were used to determine if the gall-morphs differ in developmental timing.

Host-plant Distribution

To test for differences in host-plant distribution among gall morphs, I surveyed 10 1m$^2$ plots monthly from June-September at each of the three sites for a total of 30 plots per month. In these plots, all stems of *S. altissima* were counted and recorded. Afterwards, all leaves with galls were collected, dissected, and recorded. Gall dissection includes detachment of the fungal hyphae and stroma from the gall exposing the larvae or pupae which can then be removed from the cell. *A. carbonifera* larvae and pupae are found in discrete cells within the fungal matrix. Prior to dissection, each gall was given a unique number representing the site, plot, plant, leaf, and gall. Subsequent data for each gall was also recorded, including the type of insect (parasitoid or midge), life stage (larva, pupa, or adult), number of inhabitants, number of cells (larval chambers) within each gall, and distinguishing remarks about the gall or inhabitant. All undamaged parasitoids were saved in 95% ethanol for later species identification. Collection of these data
permits us to examine gall morph distributions and test for associations between gall morphs at the levels of host-plant ramets (S. altissima), genets (plots), and A. carbonifera populations inside Beaver Creek Wetlands, Sycamore State Park, and Germantown Metropark. Due to the rhizomatous growth of S. altissima, it is difficult to determine the extent of a single genetic individual (genet), thus I examined gall distribution at the ramet level and plot level which were assumed to represent different genets. Of course, multiple genets could exist within these plots and it is possible that samples from different plots could represent the same genet.

**Statistical Analyses**

Frequencies of gall morph occurrence per site and change in these frequencies over time were calculated. First, overall gall morph frequency was calculated from the plot data at all three sites to determine if the relative frequency of gall morphs varied in the study areas. Also, the means and variances of abundance of each gall morph among ramets were calculated within the plots. Second, individual gall frequency over June, July, and August sampling rounds was assessed to see if relative frequencies changed over the season.

**Phenological Isolation**

To establish overall gall phenology among the four gall morphs, gall initiation, gall eclosion and development time of galls were summarized for each site over the two transects. Using R statistical software (version 2.7.1), correlations in gall initiation and eclosion among gall morphs for each site were calculated. Mean development time of
gall morphs was estimated for each site along with standard errors. In order to determine if and how development times differ between the four gall morphs, I used a two-way ANOVA including the explanatory variables of site, morph, and interaction between site and morph.

**Distribution of gall morphs among plants**

To determine if gall morphs vary in preference/performance for host-plant genotypes, I assessed whether gall morphs were randomly distributed among stems. As an additional measure, I also assessed whether gall morphs were randomly distributed among plots. Because *S. altissima* are rhizomatous plants, plots were assumed to represent a genetic individual, or genet. When looking at neighboring ramets of *S. altissima*, plant genotypes may be genetically identical and my goals were to establish if host-plant genotype plays a role in the divergence and ecological maintenance of gall morphs; therefore, analyzing plots may provide a better estimate of gall morph distribution based on host-plant use. Finally, I determined whether the gall morphs co-varied in frequency within these distributions. In order to test whether gall morphs were randomly distributed among stems (ramets), I combined plot collections from June through August (total of 30 per month per site) and compared the distribution of galls to a Poisson distribution with R statistical software (version 2.7.1). These distributions were compared using the Pearson’s Goodness of Fit Test. Also, log-linear models of presence/absence data were used to determine how gall morphs were associated during the months of June through August. Each site was analyzed separately to ascertain if differences existed among the sites. I selected models in two different ways to determine
whether gall morphs were distributed randomly with respect to one another among individual ramets. First, step-wise general linear models were constructed in R to determine the association between the variables, i.e. presence/absence of each gall morph. I began with the most complex model which included all four gall morphs plus all two-way and three-way interactions between them. The goal was to look for interactions between gall morphs which would indicate that they were positively or negatively associated. Negative interactions suggest differential specialization, whereas positive interactions suggest that gall morphs are responding similarly to host-plant variation. From there, I ran ANOVAs on ramet data and compared the deviance between each of the models. I eliminated terms based on p-values lower than 0.05 to see if the strongest relationships between gall morphs still showed significance after being removed from the model. Second, I used the Akaike Information Criterion (AIC) to select the model which best explained the relationships between the gall morphs among ramets.

In order to examine the distribution of galls among plots, I again compared the plot data to a Poisson distribution with the Pearson Goodness of Fit measure in R. To obtain sufficient sample size for estimating the distribution of gall morphs among plots, I combined all of the plots from June through August for a total of 86 plots. Originally, a total of 90 plots were measured and collected; however, plots six through ten of SSP during July were unavailable. Again, I combined all rounds and examined each morph separately per site. To determine if the gall morphs co-vary in frequency, I estimated correlations among gall morphs and made scatter-plot matrices of the correlations between gall morphs for individual rounds (June, July, and August) and combined rounds.
for each site. I used Principal Components Analysis (PCA) in PAST (version 1.90, Hammer et al. 2001) to characterize each plot by the frequency of each of the gall morphs, and to examine if they were grouped by site or were spatially clustered within sites. This was also used to assess the relationships among plots (genets) in terms of the frequency of gall morphs.

Results

I. Gall morph frequencies over space and time

Overall gall morph frequencies varied greatly among sites GMP (908 galls), BCW (719 galls), and SSP (688 galls), but maintained similar relative abundances across the three sites (Table 1). At all three sites, crescent and irregular gall morphs appeared to be the most frequent while flats were consistently the least frequent (Table 1). Gall morph frequencies also fluctuated over time at each site (Fig. 1a-c). Again, at all sites, crescents and irregulars tended to be the most frequent, but their frequencies did not remain the highest throughout the season (Figs. 1a-c). For example, the frequency of cushions tended to be a little higher than those of crescents, but not irregulars, in Round 2 (July) of GMP (Fig. 1a). Irregular galls were the only morph that tended to increase in frequency over the season at all sites (Fig. 1a-c). Crescents showed a decrease over time, but began to increase after Round 2 at SSP (Fig. 1c). The decrease in crescent frequencies was substantial: at GMP from 0.39 in July to 0.32 in August, at BCW crescents decreased from 0.6 to 0.2, and at SSP, they decreased from 0.4 to 0.33. Cushions were generally lower in frequency at the beginning of the season, but normally showed an increase in the middle of the season (July) (Figs. 1a-c). This pattern was the same at all three sites.
After July (Round 2), however, cushions seemed to react differently at the three sites.

For example, at GMP, cushions began to decrease dramatically after July; however, at BCW, cushions increased (Figs. 1a and 1b). At SSP cushions had the same initial pattern, but began to level off after July (Fig. 1c). Flats were always the least frequent morph and generally decreased throughout the season (Fig. 1a-c).

Table 1: Relative gall morph frequencies per site. Shown below are the most frequent as well as the least frequent gall morphs among the sites Germantown Metropark (GMP), Beaver Creek Wetlands (BCW), and Sycamore State Park (SSP). The (N) represents the total of all gall morphs found per site. The numbers in bold are the most frequent gall morphs found per site.

<table>
<thead>
<tr>
<th>Site</th>
<th>Gall Morph</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP</td>
<td>Crescent</td>
<td>0.323</td>
</tr>
<tr>
<td></td>
<td>Cushion</td>
<td>0.239</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.074</td>
</tr>
<tr>
<td></td>
<td>Irregular</td>
<td>0.364</td>
</tr>
<tr>
<td>N = 908</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCW</td>
<td>Crescent</td>
<td>0.426</td>
</tr>
<tr>
<td></td>
<td>Cushion</td>
<td>0.192</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>Irregular</td>
<td>0.335</td>
</tr>
<tr>
<td>N = 719</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP</td>
<td>Crescent</td>
<td>0.342</td>
</tr>
<tr>
<td></td>
<td>Cushion</td>
<td>0.183</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.078</td>
</tr>
<tr>
<td></td>
<td>Irregular</td>
<td>0.397</td>
</tr>
<tr>
<td>N = 688</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: Relative frequencies of gall morphs at each site over the three sampling rounds (Round 1 = mid June, Round 2 = mid July, Round 3 = mid August). The (N) for the GMP collection is 2498 galls (a), BCW is 1107 (b), and SSP is 974 (c).
c) Phenological isolation of Asteromyia gall morphs

Correlations of gall eclosion among all gall morphs are shown in Table 2. At BCW, no gall morph eclosions were significantly correlated (Table 2); however, the other two sites showed some significant correlations in eclosion times (Table 2). Eclosions of flat and irregular galls were significantly positively correlated at both GMP and SSP (Table 2), and although it is not statistically significant, flat and irregular eclosions at BCW exhibited one of the highest correlation coefficients (Table 2). The other combinations of gall morph eclosions were not nearly as consistent. For example, at GMP crescent and cushion gall eclosions were correlated while at SSP cushions and
irregulars as well as flat and irregular gall combinations were significantly positively correlated (Table 2). Most of these pairwise gall combinations exhibited positive correlation coefficients in their eclosion timing; however, most were not significant, and a few were slightly negative.

**Figure 2:** Eclosion patterns of gall morphs at each site over a twelve week interval (June-August). The lines for each gall morph indicate when the galls eclosed over the season. The figures are as follows: GMP gall eclosions (a), BCW gall eclosions (b), and SSP gall eclosions (c).

a)
None of the gall morph eclosion patterns appeared to be predictable within sites or across sites. From graphs of eclosion over time, we estimated generations by counting

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b) 

[Graph showing number of galls over weeks for different gall morphs]

c) 

[Graph showing number of galls over weeks for different gall morphs]
the peaks (Fig. 2a-c). For instance, at GMP, irregulars possibly peaked 3-4 times during the twelve week period, once near week six, once possibly at week eight, and once around week 10 (Figure 2a). However, at BCW, irregulars appeared to peak 3-4 times, near weeks 3, 5, 8, and 11 (Figure 2b) and SSP showed the potential for four generations over the summer season for irregulars (Figure 2c). Potential generation times of morphs at each site based on the number of peaks from the eclosion graphs (Figure 2c) are given in Table 3. All generations may not have been assessed in the sampling period due to the start date being in early June. By this time, it is possible that the first generation of gall midges had already mated, laid eggs, and died.

Cushion and irregular gall initiations were significantly positively correlated at all three sites (Table 4). In addition, cushions and flat and irregular and flat gall combinations were positively correlated at both GMP and BCW (Table 4). At both GMP and BCW sites, crescents were not correlated with any other gall morph (Tables 4); however, at SSP, the correlation of crescent and cushion gall initiation was highly significant (Table 4). Little predictability was seen in gall initiation patterns within sites and across sites (Fig. 5). At GMP, crescents were more prevalent in the beginning of the season and do not seem to correlate with other gall morphs; whereas, the other gall morphs appeared to be initiated near week four (Figure 5a). At BCW, cushions, irregulars, and flats all tended to have similar initiation patterns throughout the season (Figure 5b). Crescents at BCW also were active towards the beginning of the season and declined gradually throughout the season until a brief initiation near week eight (Figure 5b). At SSP, all four of the gall morphs shared similar initiation times during the season
(Figure 5c). All morphs seemed to stop or slow gall initiation near week 5 as well (Figure 5c). Across all sites, crescents seemed to have 4 generations per season, cushions had about 3 generations, flats had approximately 3-4, and irregulars had 3-4 generations. For both the initiation and eclosion data, there were very few, if any, periods that had no activity for a particular gall morph indicating that the generations were not entirely discrete (Figs. 4 and 5, Table 3).

Development time varied among the gall morphs. Overall, crescents had the longest development time with a mean of 4.3 weeks followed by irregulars with a mean development time of 3.9 weeks (Figure 6). Cushions and flats had shorter mean development times (Figure 6). At BCW, cushions had the longest development time compared to other gall morphs with crescents being second (mean dev. time = 4.703 weeks, s.d. = 2.25). These conflicting results indicated variability in development time of the gall morphs across sites (see discussion). A two-way ANOVA indicated that gall morph, site, and the interaction between the site and gall morph all significantly affected the development times of gall morphs (Table 5).
Table 2: Time of gall eclosion correlations with pairwise gall combinations. Gall eclosions were measured in weeks. The table indicates the correlation coefficients for each site below the diagonal while the p-values are shown above the diagonal.

<table>
<thead>
<tr>
<th>Site</th>
<th>Morph</th>
<th>cres</th>
<th>cush</th>
<th>flat</th>
<th>Irr</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP</td>
<td>Cres</td>
<td>1</td>
<td><strong>0.004</strong></td>
<td>0.509</td>
<td>0.641</td>
</tr>
<tr>
<td></td>
<td>Cush</td>
<td>0.791</td>
<td>1</td>
<td>0.2973</td>
<td>0.697</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.223</td>
<td>0.346</td>
<td>1</td>
<td><strong>0.015</strong></td>
</tr>
<tr>
<td></td>
<td>Irr</td>
<td>0.159</td>
<td>0.133</td>
<td>0.707</td>
<td>1</td>
</tr>
<tr>
<td>BCW</td>
<td>Cres</td>
<td>1</td>
<td>0.235</td>
<td>0.327</td>
<td>0.878</td>
</tr>
<tr>
<td></td>
<td>Cush</td>
<td>0.371</td>
<td>1</td>
<td>0.989</td>
<td>0.259</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.310</td>
<td>0.004</td>
<td>1</td>
<td>0.275</td>
</tr>
<tr>
<td></td>
<td>Irr</td>
<td>-0.050</td>
<td>0.354</td>
<td>0.343</td>
<td>1</td>
</tr>
<tr>
<td>SSP</td>
<td>Cres</td>
<td>1</td>
<td>0.393</td>
<td>0.212</td>
<td>0.679</td>
</tr>
<tr>
<td></td>
<td>Cush</td>
<td>0.287</td>
<td>1</td>
<td>0.686</td>
<td><strong>0.036</strong></td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>-0.408</td>
<td>0.138</td>
<td>1</td>
<td><strong>0.015</strong></td>
</tr>
<tr>
<td></td>
<td>Irr</td>
<td>-0.141</td>
<td>0.634</td>
<td>0.709</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3: Number of generations estimated by eclosion peaks. Table 3 shows the approximate number of generation times for each gall morph based on the counted number of eclosion peaks in Figure 2.

<table>
<thead>
<tr>
<th>Site</th>
<th>Morph</th>
<th>Possible Generations</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP</td>
<td>Irregular</td>
<td>3-4</td>
</tr>
<tr>
<td></td>
<td>Crescent</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Cushion</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>3-4</td>
</tr>
<tr>
<td>BCW</td>
<td>Irregular</td>
<td>3-4</td>
</tr>
<tr>
<td></td>
<td>Crescent</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Cushion</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>3-4</td>
</tr>
<tr>
<td>SSP</td>
<td>Irregular</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Crescent</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Cushion</td>
<td>3-4</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>3-4</td>
</tr>
</tbody>
</table>
Table 4: Gall initiations with pairwise gall combinations at the three sampling sites.

Correlation coefficients for each site are below the diagonal, p-values are shown above the diagonal.

<table>
<thead>
<tr>
<th>Morph</th>
<th>cres</th>
<th>cush</th>
<th>flat</th>
<th>Irr</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP</td>
<td>Cres</td>
<td>1</td>
<td>0.939</td>
<td>0.768</td>
</tr>
<tr>
<td></td>
<td>Cush</td>
<td>-0.025</td>
<td>1</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.095</td>
<td>0.807</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Irr</td>
<td>0.311</td>
<td>0.821</td>
<td>0.693</td>
</tr>
<tr>
<td>BCW</td>
<td>Cres</td>
<td>1</td>
<td>0.152</td>
<td>0.971</td>
</tr>
<tr>
<td></td>
<td>Cush</td>
<td>0.440</td>
<td>1</td>
<td><strong>0.036</strong></td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>-0.012</td>
<td>0.608</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Irr</td>
<td>0.274</td>
<td>0.885</td>
<td>0.741</td>
</tr>
<tr>
<td>SSP</td>
<td>Cres</td>
<td>1</td>
<td><strong>0.005</strong></td>
<td>0.208</td>
</tr>
<tr>
<td></td>
<td>Cush</td>
<td>0.776</td>
<td>1</td>
<td>0.198</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.412</td>
<td>0.420</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Irr</td>
<td>0.478</td>
<td>0.724</td>
<td>0.281</td>
</tr>
</tbody>
</table>
**Figure 5:** Initiation patterns of gall morphs at each site over a twelve week interval (June-August). Peaks indicate when galls are initiated. Week 1 was ignored because galls were previously initiated and already fully present. The figures are as follows: GMP gall initiations (a), BCW gall initiations (b), and SSP gall initiations (c).

a)
**Figure 6**: Mean development times of *Asteromyia* gall morphs. This figure shows the mean development time of gall morphs across sites (or with all three sites combined).

![Bar chart showing development times of Asteromyia gall morphs](chart.png)

**Table 5**: Results of Two-Way ANOVA of the effects of morph, site, and their interactions with development time of *Asteromyia* gall morphs on *Solidago altissima* across three sites in southwestern Ohio.

<table>
<thead>
<tr>
<th></th>
<th>SumSq</th>
<th>Df</th>
<th>F value</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morph</td>
<td>26.83</td>
<td>3</td>
<td>3.4219</td>
<td>0.01683</td>
</tr>
<tr>
<td>Site</td>
<td>16.21</td>
<td>2</td>
<td>3.1017</td>
<td>0.04544</td>
</tr>
<tr>
<td>Morph:Site</td>
<td>37.67</td>
<td>6</td>
<td>2.4021</td>
<td>0.02613</td>
</tr>
</tbody>
</table>
Distribution of gall morphs among plants

Among ramets:

Gall morphs were distributed non-randomly among stems. In almost all cases, stems having just one or a few galls were found much less than expected (Fig. 7). The probabilities for all morphs at each site were far less than 0.001.

Figure 7: Gall morph distributions of each gall morph among stems at each site (red bars) versus expected Poisson distributions (blue line) at GMP. Only the distributions of the two most common gall morphs, crescents and irregulars, are shown here. Appendix A includes the figures for cushions and flats from GMP as well as all of the other gall morphs from BCW and SSP.
Pair-wise General Linear Models and AIC at the Ramet Level

Pair-wise general linear models of the presence/absence of gall morphs among ramets showed that all two-way interactions between all four gall morphs were highly significant in almost all cases. This suggests there are associations between the gall morphs at the ramet level (Tables 6 and 7, Figs. 8-10). In general, the general linear models and the models chosen by the AIC were similar (Table 7). None of the three-way interactions turned out highly significant; however, the final AIC models for SSP and GMP suggest the existence of positive three-way interactions between cushions, crescents, and irregulars (1:2:3) and between cushions, irregulars, and flats (1:3:4; Table 7, Figs. 8 and 10). A three-way interaction, for example, could be where cushions and flats are present, irregulars tend to grow as well; or where irregulars and cushions are present, flats are likely to also be present and so on. Mosaic plots provide a visual interpretation of the two- and three-way interactions between the four gall morphs (Figs. 8-10). The zeros in the perimeter of the matrices indicate gall absence while the ones signify gall presence (Figs. 8-10). The perimeter also contains the names of each of the gall morphs. In the colored bar next to the mosaic plot, gray symbolizes no association, blue indicates that the combination of gall morphs occurs more than expected, and red signifies that the combination of gall morphs occurs less than expected. For example at GMP stems with no galls, stems with irregular and crescent morphs, stems with irregulars, crescents, and cushions and stems with all four gall morphs occur more frequently than expected, however, stems with only a single gall morph of any type are less frequent than expected (Figure 8). At all three sites, the occurrence of all four gall
morphs happened more than expected as did the absence of all four gall morphs (Figs. 8-10). The mosaics also provide likely three-way interactions of flats, irregulars, and crescents, and irregulars, crescents, and cushions across the three sites (Figs. 8-10). The positive interactions suggested by these models indicate *Asteromyia carbonifera* are responding similarly to plant ramets (and possibly genotype) or some associated underlying environmental variable.

**Table 6:** All pairwise combinations of gall morph interactions per site. The table shows p-values from general linear models of all pairwise combinations at each of the three sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>Interaction</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP</td>
<td>Irregular, flat</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Crescent, flat</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Crescent, irregular</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cushion, flat</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>Cushion, irregular</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cushion, crescent</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BCW</td>
<td>Irregular, flat</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Crescent, flat</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Crescent, irregular</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cushion, flat</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td>Cushion, irregular</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cushion, crescent</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SSP</td>
<td>Irregular, flat</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Crescent, flat</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>Crescent, irregular</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cushion, flat</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cushion, irregular</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cushion, crescent</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 7: Significant interactions in final AIC selected general linear models. Key: 1= cushion, 2= crescent, 3= irregular, 4= flat. The paired numbers in the table represent the two-way combination of gall morphs that were the most significant at each site.

<table>
<thead>
<tr>
<th>Site</th>
<th>Significant Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP</td>
<td>1:2+1:3+1:4+2:4+3:4+1:2:3+1:3:4</td>
</tr>
<tr>
<td>BCW</td>
<td>1:2+1:3+2:4+3:4</td>
</tr>
<tr>
<td>SSP</td>
<td>1:2+1:3+1:4+2:3+3:4+1:2:3+1:3:4</td>
</tr>
</tbody>
</table>
**Figure 8:** Mosaic of gall morph interactions at GMP. The large blue box in the top left hand corner represented stems with no gall morphs (symbolized by the zero for each type of gall morph on every axis). The smaller blue box in the lower right hand corner symbolized plants where all gall morphs are present. Associations: Gray=no association, blue=association occurs more than expected, pink=association occurs less than expected.
Figure 9: Mosaic of gall morph interactions at BCW. The same patterns as seen at GMP were also found at BCW.
Figure 10: Mosaic of gall morph interactions at SSP. Again, the pattern of a small portion of goldenrods is chosen by *A. carbonifera* to place their galls.
Distribution of gall morphs among plots (genets)

Scatter plots of morph frequency for the combined data set (all sampling dates) indicate that at least some of the gall morphs positively co-vary in frequency at the plot level (Figs. 11-13). At all three sites, numbers of cushions and irregulars consistently and positively co-vary in number (Figs. 11-13). Numbers of crescents and irregulars are also strongly correlated in frequency at the plot level at the sites GMP and SSP (Figs. 11 and 13), while flats and irregulars co-varied in frequency at BCW and SSP (Figs. 12 and 13). Gall morph frequency distributions among plots vary depending on the morph. Gall morphs were non-randomly distributed among plots (Figure 14, Appendix A) and differed significantly from random distributions (Poisson). At each site, crescents and irregulars were the most abundant gall morphs while flats were the least (Figure 14 and Appendix A). Figure 14 shows only gall morph abundances from GMP; however the gall morphs were distributed similarly at BCW and SSP (see Appendix A).
**Figure 11**: GMP frequency correlations in combined rounds. Both axes for the scatter plot matrix indicate gall morph frequency per plot (plots were assumed to be genetically individual *S. altissima* plants).
Figure 12: Plots of gall morph abundances among sampled plots at BCW for all sampling rounds.
Figure 13: Plots of gall morph abundances among sampled plots at SSP for all sampling rounds.
**Figure 14:** Frequency Distributions with Pearson’s Goodness of Fit Values. The figures show the numbers of each gall morph contained in certain plots. The figures below are for the Germantown Metropark site only and show the distributions of all four gall morphs. The figures are as follows: crescents (a), cushions (b), flats (c), and irregulars (d). Distributions of each gall morph for BCW and SSP are shown in Appendix B.

a) 

![Graph of crescents distribution](image1)

Crescents: $P(>X^2) = 0$

b) 

![Graph of cushions distribution](image2)

Cushions: $P(>X^2) = 0$
Correlations of the number of galls of each morph within plots (Table 8) show that associations between gall morphs are again positive. Those stronger correlations include crescents and cushions as well as crescent and irregulars at all three sites. However, at GMP, the relationship between cushions and irregulars is stronger than at BCW and SSP (Table 8).
Table 8: Correlations between the Gall Morphs at all three testing sites. Correlation coefficients are below the diagonal while p-values for the correlation coefficients are above the diagonal. Bolded values have the highest correlation coefficients and p-values.

<table>
<thead>
<tr>
<th>Site</th>
<th>Morph</th>
<th>cres</th>
<th>Cush</th>
<th>flat</th>
<th>Irr</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP</td>
<td>Cres</td>
<td>1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cush</td>
<td>0.268</td>
<td>1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.092</td>
<td>0.153</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Irr</td>
<td>0.273</td>
<td>0.443</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td>BCW</td>
<td>Cres</td>
<td>1</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cush</td>
<td>0.123</td>
<td>1</td>
<td>0.006</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.071</td>
<td>0.076</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Irr</td>
<td>0.171</td>
<td>&lt;0.001</td>
<td>0.325</td>
<td>1</td>
</tr>
<tr>
<td>SSP</td>
<td>Cres</td>
<td>1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Cush</td>
<td>0.226</td>
<td>1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.116</td>
<td>0.121</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Irr</td>
<td>0.434</td>
<td>0.003</td>
<td>0.157</td>
<td>1</td>
</tr>
</tbody>
</table>

Principal Components Analyses

The main point of the PCA analyses was to examine how plots are distributed with respect to gall morph frequencies. Because of their high variance in abundance, crescent and irregulars contribute most to principal components while flats contribute the least. The first principal component of gall morph abundance for GMP explains approximately 75% of the variance in gall morph composition among plots, and is largely a function of the abundance of irregulars and cushions (Fig. 15). The second component (15% of the variance), has high loadings for cushion and crescent abundance (Fig. 15). At this site, cushion morphs seem to be responding differently to the environment than the other gall morphs (Fig. 15). Crescents and flats are distributed similarly among plots, as are irregular morphs to a lesser extent (Fig. 15). In Figure 15b, in component 1,
irregulars are responding differently than all other morphs. However, cushions are responding differently to the environment than the other morphs in component 2 (Figure 15). In the PCA for BCW, approximately 90% of the variation can be explained by components one and two. For component 1 (55%), crescent abundance contributes strongly and uniquely; while for component 2 (35%), gall morphs exhibit eigenvectors of similar direction but varying magnitude (Fig. 16). At SSP, 85% of the variance of the PCA is explained by components 1 and 2 (Figs. 17 and 29). Sixty-one percent of the variance is explained by component 1, which is strongly influenced by abundance of irregulars and crescents (Figs. 28 and 29). For component 2, approximately 25% of the variance is explained, a strong influence of crescent abundance (Figs. 28 and 29). Irregulars, again, play a part due to their negative loading on component 2.

**Figure 15: GMP Principal Components**

a) GMP distribution of plots in gall morph space using principle components analyses

<table>
<thead>
<tr>
<th>Component</th>
<th>Eigenvalue</th>
<th>Percent Variance Explained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1322.68</td>
<td>74.718</td>
</tr>
<tr>
<td>2</td>
<td>276.949</td>
<td>15.645</td>
</tr>
<tr>
<td>3</td>
<td>101.505</td>
<td>5.734</td>
</tr>
<tr>
<td>4</td>
<td>69.0987</td>
<td>3.9034</td>
</tr>
</tbody>
</table>
b) Biplot of the PCA of gall morph frequencies at the GMP site.
Figure 16: BCW Principal Components

a) Distribution of Galls in Plots using Principle Components Analysis of BCW

<table>
<thead>
<tr>
<th>Component</th>
<th>Eigenvalue</th>
<th>Percent Variance Explained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>420.702</td>
<td>55.037</td>
</tr>
<tr>
<td>2</td>
<td>268.493</td>
<td>35.125</td>
</tr>
<tr>
<td>3</td>
<td>62.0828</td>
<td>8.1217</td>
</tr>
<tr>
<td>4</td>
<td>13.124</td>
<td>1.7169</td>
</tr>
</tbody>
</table>

Component 1

Component 2
b) Biplot of the BCW PCA

**Figure 17**: SSP Principal Components

a) Spatial Distribution of Galls in Plots using Principle Components Analysis of SSP

<table>
<thead>
<tr>
<th>Component</th>
<th>Eigenvalue</th>
<th>Percent Variance Explained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>387.091</td>
<td>61.632</td>
</tr>
<tr>
<td>2</td>
<td>155.604</td>
<td>24.775</td>
</tr>
<tr>
<td>3</td>
<td>77.2827</td>
<td>12.305</td>
</tr>
<tr>
<td>4</td>
<td>8.09207</td>
<td>1.2884</td>
</tr>
</tbody>
</table>
b) Biplot of the SSP PCA
Discussion

Several ecological factors or interactions could be facilitating the radiation of *Asteromyia carbonifera* into genetically distinct morphotypes. Two of these factors were explored in this study, phenology and host-plant choice. In relative numbers alone, *A. carbonifera* gall morphs differ greatly in abundance at all sites visited: GMP, BCW, and SSP. Crescents and irregulars, undoubtedly, were the most frequent gall morphs at all sites while flats were the rarest. Although this outcome was fairly consistent across space and time, frequencies varied substantially over time among gall morphs and sites, indicating that these gall morph populations exhibit distinct population dynamics. This further supports the genetic and morphological data that indicates that these gall morphs represent discrete populations if not incipient species (Crego et al. 1990, Stireman et al. 2008).

Phenology

Because the morphotypes of *A. carbonifera* considered here are living on the same host-plant species, on the same part of the host-plant, and frequently on the same host individuals, species divergence in these sympatric conditions would be impossible without some sort of isolating mechanism. One mode of sympatric speciation is allochronic speciation where differentiation of populations is due to a phenological shift without habitat or host change (Santos et al. 2007). Phenology has been implicated as an important mechanism of genetic isolation in some insect species and may be an important factor in insect diversification. For example, in *Apocryptophagus* species,
hymenopteran parasites of *Ficus* sp., phenological differences allow many species to utilize the same host plant (Weiblen and Bush 2002). Several other studies have focused on this type of speciation as well; however, the literature remains scarce (Santos *et al.* 2007, Weiblen & Bush 2002, Abbot & Withgott 2004). Because *A. carbonifera* adults are so short-lived (2-3 days), phenological isolation may be one possible mechanism that may reduce gene flow and allow genetic differentiation on phenotypic traits in sympatry (Santos *et al.* 2007, Weiblen & Bush 2002).

In this study, I analyzed phenology of the four *A. carbonifera* gall morphs to assess the likelihood of phenological isolation. None of the gall morphs was so strictly isolated as to indicate that phenology is a likely isolating mechanism. The low frequencies of strong positive eclosion correlation coefficients confirm genetic data indicating that these populations are independent (Crego *et al.* 1990, Stireman *et al.* 2008). At GMP, crescents and cushions were highly correlated in eclosion time as well as flats and irregulars. At SSP, cushions and irregulars and flats and irregulars were correlated in eclosion time; however, no pairs of gall morphs were correlated at BCW. Flats and irregulars were the only combination of gall morphs that was correlated at two sites, GMP and SSP. According to genetic marker (AFLP) data, flat and cushion morphs are closely related while irregulars and crescents are closer to one another (Stireman *et al.* 2008). One reason that cushions and flats differ in phenology even though they are closely related could be reinforcement, selection for pre-zygotic isolation due to low hybrid viability (Funk 2010, Santos *et al.* 2007). There may be gene flow between the two gall types, but their offspring may not be able to produce optimal gall morphologies.
for defense. These results suggest that though phenological isolation could have played a role in isolating in the past, it does not represent a strong barrier to gene flow currently and was probably not the primary mode of isolation within this species complex. It may be that during the initial divergence of gall morphs, phenological isolation was greater, perhaps due to selection for pre-zygotic isolation, but after other isolating mechanisms evolved, A. carbonifera converged again. Although phenology has been known to create divergence in other species (Sachet et al. 2009), it does not appear to be a highly influential force in A. carbonifera divergence or coexistence.

There were marked differences in the estimated number of generations due to variance in development times between the four gall morphs. The generations of each of the gall morphs did not appear to be discrete. For the most abundant morphs, new galls were initiated and older ones eclosed during every sampling period examined. This overlapping of populations of A. carbonifera in time and space in the sampling periods makes it difficult to accurately assess the number of generations occurring over the season. The lack of discrete generations is surprising. Because adults are so short-lived (<1 week), I would expect eclosion to be tightly synchronized (Yukawa & Akimoto 2006), but it does not appear to be. Generation number and time estimates could also be affected by parasitoid eclosion. Parasitoids of A. carbonifera often have a more extended period of emergence than their midge hosts (B. Wells, pers. obs.) and differ in development time from A. carbonifera, and this could result in error in documenting midge eclosions. I made an effort to differentiate between eclosion of midges and parasitoids by examining the eclosion holes. Eclosion holes of A. carbonifera are often
jagged, not perfectly circular, and usually contain the pupal exuviae, while those of the parasitoids are nearly circular and rarely include the pupal exuviae.

Gall initiation was more correlated among morphs than adult eclosion. Cushions, flats, and irregulars were highly correlated with one another at two of the sites. Perhaps the reason that they are more correlated is that gall initiation is a better indicator of phenology than eclosion because it would give more accurate estimates of generation and development time. The morphs may be more correlated in phenology than it appears which makes phenological isolation even less likely. The final aspect of phenology analyzed was duration of development. Development times varied significantly among morphs, but differences were relatively small. Overall, crescents exhibited the longest development times compared to the other morphs; however, cushions, flats, and irregulars were very similar in development time: The two-way ANOVA indicated that gall morph, the site, and the interaction between the gall morph and site all influence gall development times. Site may have an effect on development time through interactions with the gall morph. For example, if one site produces healthier host-plants or differs in plant genotypes, midge larvae may develop more rapidly.

Placement of the galls on the host plant may also affect development. For example, crescents tend to form galls on older leaves instead of the newer growth preferred by the other gall morphs (B. Wells, pers. obs., Heath unpub. data). Crescents also take longer to develop than the other gall morphs perhaps due to this association with older leaves (see below). This longer development time may result in a temporal barrier in relation to the other gall morphs. Furthermore, crescent placement on older
leaves may lead to spatial isolation in these tiny insects. These differences may explain why crescents were rarely correlated with other gall morphs in gall initiation. However, the eclosion data suggests ample opportunity for crescents to mate with the other gall morphs. In terms of an isolating mechanism, phenological differences between the four gall morphs do not appear to present a strong barrier to gene flow or a major cause of reproductive isolation in this system. Although timing may play a role, it does not appear to be sufficient to explain the divergence of gall morph races in Asteromyia carbonifera.

Gall morph distribution

Results of the gall morph analyses are consistent with the hypothesis that some plant ramets and/or genets are more suitable for gall induction and individuals of a given morph are generally clumped together in a non-random fashion while other plant genotypes are less suitable for any gall induction. However, the clumping pattern could also reflect short-distance movements of females depositing eggs. This result was consistent among all gall morphs at all three visited sites (Figs. 11-13).

Because phytophagous insects are generally host-plant specific (Drés & Mallet 2002, Caillaud & Via 2000), it seems reasonable that preference for a particular host plant could cause reproductive isolation of populations. In the case of A. carbonifera, however, all four gall morph races coexist on a single host-plant, Solidago altissima which is remarkable since they live in such a close proximity on the host plant. Because of this nearness, some gene flow may still exist, but they are still diverging suggesting another ecological factor could be responsible. In Southwestern Ohio, S. altissima consists of three ploidy races (2N, 4N, and 6N) that may differ in their suitability for A.
carbonifera. Although, I was not able to assess ploidy or genotype of S. altissima plants in my research, I studied the distributions of gall morphs among plant ramets and plots (~genets) to indirectly assess whether there was evidence of differential host-plant use by the four gall morphs.

First, I analyzed gall morph distributions among ramets (stems) to determine if each of the morphs exhibited a non-random distribution. Among ramets, none of the gall morphs showed a random distribution. Galls were generally found in a ‘clumped’ pattern. This result is consistent with the hypothesis that ramets vary in their suitability for gall development. Although females have been known to travel many meters away from their natal site (Heath, J. pers. obs.), females may have a tendency to limit their dispersal and frequently lay eggs in a clustered arrangement even if all ramets are equally suitable. Limited movement between oviposition events may allow the female the extra energy to produce more eggs and reduce the risk of adult mortality. This result was consistent among all gall morphs at all three visited sites (Figs. 15-17).

Next, I analyzed gall morphs distributions among plots to as an indirect means to determine if the morphs prefer certain plant genotypes over others, resulting in a non-random distribution. Because S. altissima are rhizomatous, they are probably in clumped, clonal distributions themselves. This may influence gall morph distributions if there is a preference for a particular host-plant; in this case, a particular host-plant genotype. Again, the results revealed that the gall morph distributions among plots differed significantly from random. The gall morphs exhibited a clumped distribution, but, because I did not determine plant genotypes, I cannot determine what the preferred
genotypes were. Again, these results could also be explained by limited dispersal of females. However, analyses of the co-distribution of gall morphs in terms of presence/absence (General linear model interactions and mosaic plots) provide evidence that some plant genotypes are better for multiple gall types while other plant genotypes are not suitable for any of the galls. At GMP and BCW, crescents and cushions appear to be most concordantly distributed on the plants while flats and irregulars were regularly found together at GMP and SSP. Crescents and irregulars at all three sites were least likely to be found near one another. Three tentative conclusions can be made from these results: First, gall morphs are not exclusively specialized on different host plant genets. Second, there appears to be variation among host-plants in their suitability for *A. carbonifera*. This variation may mediate interactions between these midges and their chosen host plants (Härri *et al.* 2009). For example, plant quality and plant chemistry may also be altered by the presence of microorganisms such as fungal endosymbionts that play a major role in gall formation (Härri *et al.* 2009). Third, the gall morphs tend to respond similarly to this variation in host plant suitability.

This system is ecologically complex. Differing dynamics of gall morphs are consistent with genetic evidence that they represent distinct populations or races, but why are they diverging? Two possible isolating mechanisms, phenology and host-plant preference, were assessed in natural populations to determine if they may play a role in the recent divergence of *A. carbonifera* gall morph populations. Both phenological differences and host-plant genotype preference among the gall morphs are evident in this system; however, neither factor appears to be responsible for the separation of this species. This study was conducted in the natural arena of *Asteromyia carbonifera*;
nevertheless, controlled laboratory experiments may be needed to assess whether
differences in phenology and host-plant choice are significant. For phenology,
collections of each gall morph in late May could be set up in a closed setting such as a
greenhouse filled with approximately 100 ramets of *Solidago altissima*. Weekly
monitoring (for 12-14 weeks) of all galls may help establish more accurate estimates of
gall initiation, eclosion, and development times. Collecting samples of the 2N, 4N, and
6N populations of *S. altissima* and also arranging them in a closed environment may
facilitate in assessing the importance of host-plant ploidy in determining their suitability
for gall development. All gall types could be placed in this closed setting and
observations of gall placement on the different ploidy races of *S. altissima* may provide
more direct evidence of host plant preference and suitability. Regardless, results from this
study show that further research is needed and other possible separating mechanisms
should be explored.

My observations suggest that each of the gall morph populations has the
opportunity to mate with the other morphs, and it is quite possible that some gene flow is
still happening. Hybrid galls may be forming but may have reduced viability. All two-
way interactions between gall morph presence/absence were significant and most
interactions are positive. These positive interactions provide no indication of competition
among the different gall morphs; however there may be indirect competition. This
indirect competition may be due to parasitoids. Natural enemies represent a substantial
source of herbivore mortality and can sometimes lead to local population extinction
Gall morph variation could influence the mortality imposed by hymenopteran parasitoids (Bailey et al. 2009).

Ecological theory would suggest that two species sharing the same niche cannot coexist (Salomon et al. 2010); yet, these midges appear to be doing exactly that. Perhaps, enemy pressure keeps the populations from building up to the point where competition becomes important. This could work if the “enemy dimension” of their niches has diverged. Resource utilization and competition, the forces which most studied adaptive radiations concentrate on (Schluter 2000, Gavrilets and Losos 2009), are not evident in this system. *A. carbonifera* exploit the same resource, *Solidago altissima*. More specifically, they use the same part of the resource, the leaves. They do not necessarily compete for this resource because goldenrods are plentiful, most ramets are ungalled, and galls do not appear to significantly harm the host-plant. Therefore, other ecological forces may have influenced the divergence and sympatric coexistence of these midges.

Although there was some variation in the phenology of the gall morphs, it did not appear to be a compelling isolating factor because simultaneous emergence of all morphs was evident. Based on the non-random distributions of *A. carbonifera* gall morphs, host-plant genotype preference seems to play a small role in this divergence. Limited movement of female between oviposition sites may create spatial structures that isolate populations. However, anecdotal observations of the rapid colonization of isolated goldenrod plants suggest that females are capable of reasonably long distance dispersal, at least on the scale of hundreds of meters (Heath J. pers. obs).
Further studies of phenology and host-plant preference as well as the pressure from parasitoids are needed to assess the importance of ecological interactions in adaptive radiations. Deeper observations into radiation across flora and fauna may indicate that species interactions and life histories may be largely responsible for speciation and coexistence of closely related populations.
Literature Cited


Chapter 3

Divergence and Coexistence of a gall-making midge, *Asteromyia carbonifera*: Can pressure from natural enemies promote speciation in sympatric populations?

Introduction

Inter-specific interactions provide strong selective pressures that can play an important role in population divergence and speciation (Stireman *et al.* 2008, Bailey *et al.* 2009). Often, interactions such as parasitism and symbiosis can be difficult to observe among populations of organisms (van Veen *et al.* 2006, Sadedin *et al.* 2009). Determining which interactions contribute to population divergence and speciation can aid in understanding the genetic origins of community composition and contribute novel insight into the ecological basis of speciation. Competition for resources has long been the focus of studies seeking to understand both the adaptive divergence of populations and their subsequent coexistence (Schluter 2000, Joy & Crespi 2007); however, other ecological interactions can create divergence besides the traditional competition and resource use theories (Schluter 2000, Orr & Smith 1998, Hendry *et al.* 2007). Interest in alternative types of interactions and their selective impact is growing. For instance, interactions such as the influence of enemies on population dynamics have recently garnered considerable attention (Bailey *et al.* 2009, Schluter 2000, 2001Rundle and Nosil 2005).

An emerging theme in ecology is that adaptive trait variation at one trophic level may force change in other trophic levels (Bailey *et al.* 2009, Forbes *et al.* 2009, Futuyma & Agrawal 2009, Janson *et al.* 2008). For instance, in order to escape herbivory, plants
may evolve a highly potent secondary chemical defense that may evade most, if not all, herbivores allowing the plants to radiate into several species that share the same defense (Ehrlich & Raven 1984, Futuyma & Agrawal 2009). Over time, herbivores may begin to expand older niches to colonize the ‘empty niches’ of the chemically-transformed plants. Similarly, beneficial variants in the insect herbivores are selected for creating a divergence in their adaptations to these newer chemical defenses (Ehrlich & Raven 1984, Futuyma & Agrawal 2009). This chain of speciation events across trophic levels can create a great deal of biodiversity (Stireman et al. 2005).

In the present study, I will focus on ecological selective forces that may have promoted the divergence of Asteromyia carbonifera (Cecidomyiidae), a gall-making midge. These midges form convex and ellipsoid galls on the leaves of various Solidago species (Asteraceae) (Weis 1982, Crego et al. 1990). This species consists distinct lineages that differ in host plant use and gall morphology. Currently, there are four gall morphs coexisting on the goldenrod species, Solidago altissima, that are morphologically discernible by their gall structures (Weis 1982, Crego et al. 1990, Stireman et al. 2008). Several cecidomyiid taxa, including A. carbonifera, have an obligate fungal symbiont that is possibly responsible for differences in gall morphology (Gagne 1968, Bissett & Borkent 1988. Rohfritsch 2008). In initial studies, Crego et al. (1990) demonstrated that three of these gall types were morphologically and genetically distinct based on gall measurements and allozyme frequencies. These gall types (morphs) were assigned the names ‘cushion’, ‘flat’, and ‘irregular’ based on their morphology (Crego et al. 1990). A noted existence of a fourth type, ‘crescent’, was also observed in the same study by
Crego et al. (1990) but was unable to test if it was truly different from the other morphs due to low abundances of these crescents in this study. More recent studies (Stireman et al. 2008) confirmed the previous work of Crego et al. (1990) through mtDNA and AFLP data by showing that A. carbonifera consists of four genetically differentiated populations on the host plant S. altissima. Given their ecological similarity, how are these four populations able to coexist? How did they initially diverge? Why do they differ in morphology? The first phase of speciation, the evolution of genetic differences, has taken place in A. carbonifera, but what ecological factor fostered this radiation? More importantly the second phase, barriers to gene flow amongst the four gall morphs has also occurred on some level, but what created these barriers? Could reproductive isolation among gall morphs evolve in sympathy? This paper will focus on whether natural enemies of A. carbonifera (Hymenopteran parasitoids) have played a role in gall morph divergence and coexistence.

The immense radiation of phytophagous insects provides excellent systems for understanding how ecological selective pressures due to host-plants, mutualists, and enemies may facilitate population divergence. Plant-feeding insects, their host-plants, and insect parasitoids form some of the most complex and species-rich food webs and amount to nearly two-thirds of terrestrial species (Nyman et al. 2007, van Veen et al. 2006, Joy & Crespi 2006, Strong et al. 1984, Bailey et al. 2009, May 1990). Most phytophagous insects are highly specialized on particular host plants (Strong et al. 1984) while some insect parasitoids are highly specialized on their insect hosts (Stilmant et al. 2008). Singer & Stireman (2005) argue that research involving these three trophic levels
collectively rather than the traditional bi-trophic studies will better help in understanding herbivore community structure, population divergence, and evolutionary diversification. More tri-trophic type studies will also further establish the association between ecological and evolutionary processes that drive biodiversity in many natural systems (Singer & Stireman 2005).

The concept of enemy-free space driving host shifts is also possible whereby interactions between enemies and host plants could result in ecological adaptive changes in herbivores and potentially speciation (Jeffries & Lawton 1984). Shifts in host plant use by herbivores as a means to possibly evade enemy pressure is described under the concept of enemy-free space (Jeffries & Lawton 1984). Does transferring to novel hosts relieve the herbivore from extreme enemy pressure, at least temporarily? Gratton & Welter (1999) experimentally created host shifts in the Dipteran leaf mining species *Liriomyza helianthi* and found that this shift decreased larval mortality from parasitoids; yet, these researchers agree that there are only narrow windows of opportunity for host shifting and predation relief and it is only likely in some circumstances (Gratton & Welter 1999).

Galling insect herbivores offer a prime opportunity to test whether intense enemy pressure can cause divergence, via the search for enemy-free space, among these ‘closed’ communities of herbivores (Weis 1982). There are over 5000 gall-inducing species of Cecidomyiidae, and each species produces a structurally distinct gall (Rohfritsch 2008). Galls are considered extended phenotypes (Bailey *et al.* 2009) of the galling midges and have evolved complex morphologies that serve a number of functions. Galls provide
rearing chambers, nutrition, and protection from elemental stresses as well as enemies (Rohfritsch 1997, 2008, Bailey *et al.* 2009). Cecidomyiids construct their galls by controlling the development of plant cells. Gall morphology differs in a variety of ways; some are spiny, rough, thick, and some galls are made partially of fungi (Batra 1964, Rohfritsch 2008). Some Cecidomyiids, the ambrosia gall midges, craft galls where the exterior of the gall chamber is coated with a layer of fungal hyphae in which maturing larvae feed (Rohfritsch 1997, Janson *et al.* 2009). Although the main function of gall may be to provide nutrition, some level of protection is offered by structural traits of the gall (i.e. thickness, hardness) (Bailey *et al.* 2009). Despite any degree of protection that galls may provide, Cecidomyiids often fall victim to intense parasitoid attack sometimes ranging from 50-90% mortality (Weis 1982, Stireman *et al.* 2008, Bailey *et al.* 2009). If parasitism runs high among certain gallers, selection should favor variable host traits that may lead to a reduction in parasitism (Stireman *et al.* 2008, Bailey *et al.* 2009). For instance, often oviposition by the female parasitoid occurs through the gall tissues and into or onto the host (Bailey *et al.* 2009); therefore, galls that better resist pressures from parasitoid attack due to the exterior structures should be favored through natural selection, an idea consistent with the Enemy Hypothesis (Stone & Schönrogge 2003, Bailey *et al.* 2009). Bailey *et al.* (2009) studied parasitoid wasp communities linked with a large radiation of cynipid wasps (Hymenoptera, Cynipidae) on oak trees. They found that gall defensive properties as well as gall location on the host plant influenced parasitoid community structure, thereby supporting the Enemy Hypothesis which, in this case, suggests that the extended phenotypes of the galler’s genes have evolved to better defend against their parasitoids (Bailey *et al.* 2009).
Aside from initial divergence of insect herbivores, the ability of closely-related species to coexist in sympatry is another interesting phenomenon in natural ecosystems. Parasitoid pressure may offer a means of coexistence in their insect herbivores. For example, density-dependent parasitism may produce a stable coexistence amongst their preferred insect hosts. As gall morph populations fluctuate, parasitoids may focus on the most abundant gall morph which, over time, may stabilize the gall morph populations. Conversely, density-dependent parasitoids that focus on the most abundant gall morph could cause higher parasitism of other nearby morphs, or apparent competition (van Nouhuys & Hanski 2000), which should inhibit coexistence among the gall morphs. Apparent competition refers to a mutually harmful relationship between species at the same trophic level that share the same enemies rather than resources which could eventually lead to the elimination of one of the species involved (van Nouhuys & Hanski 2000).

In this study, my focus is to understand the potential role of parasitoids in driving the within-host adaptive radiation of *A. carbonifera* on *Solidago altissima* and to understand if and how parasitoids play a role in the coexistence of the four gall morphs on a single host plant species. Furthermore, I tested if *A. carbonifera* parasitoids exhibit density-dependence in order to see if this contributes to the coexistence of the *A. carbonifera* gall morphs. Together, these studies will provide insight into whether *A. carbonifera* enemies may have selected for differences in gall morphs and whether they play a role in maintaining the coexistence of these morphs that apparently possess very similar ecological niches. Due to the similarity and close relationship of the gall midges
in their sympatrically-distributed habitat, studying *A. carbonifera*, their host-plants, and associated parasitoids presents an excellent opportunity to observe the effects of parasitism in the early stages of ecological speciation.

Differences in gall morphotypes may have evolved in response to selective pressure by parasitoids. I hypothesize that selective pressure by parasitoids is causing differences in gall traits such as larval placement, clutch size, and gall thickness. In addition, I predict that parasitoid community composition and frequency varies with host-plants and sites. My research is aimed to answer the following specific questions:

1. Have parasitoids contributed to divergence of gall morphs?

Parasitism rates are high and could present a strong selective force for *A. carbonifera*. To establish parasitism patterns, parasitoids were examined across the season and across sites to get an overall estimate of parasitoid levels rather than just a point estimate. *Asteromyia carbonifera* densities generally start at a low level in June and continue to rise until the populations reach diapause in late August-mid September. If parasitoids are density-dependent, *A. carbonifera* densities may force low parasitism early on and increased parasitism towards the end of the season. Given that these are very closely related populations and are likely to share parasitoids, we might be able to see a correlation between gall defensive traits and parasitism. Furthermore, has “apparent” competition through shared parasitoids selected for different gall morphologies? Apparent competition (Holt 1977) might occur where the combined density of two species increasingly attracts the attention of certain parasitoids. Thus, both species would suffer from the other’s presence and abundance. I expect that
parasitism among patches and/or sites may be correlated due to shared parasitoid preferences among the gall morphs. I hypothesize that not all gall morphs are equally armored to avoid pressure from enemies and that gall morphs differ in susceptibility to different parasitoids.

2. Do parasitoids contribute to the coexistence of gall morphs?

The presence of generalist and specialist parasitoids and high parasitism rates could contribute to the coexistence of *Asteromyia* gall morphs. For example, Teder & Tammaru (2003) studied the coexistence between two moth species mediated by the presence of shared parasitoids. Their results indicated that the dominant moth species actually benefitted from reduced parasitoid levels by the presence of the lower density moth (Teder & Tammaru 2003); and the host choice of parasitoids has possibly promoted numerical stability and coexistence of the two moth species (Teder & Tammaru 2003). With the *Asteromyia* gall morphs, I hypothesize that parasitism within the morphs is density dependent and the most common morphs are attacked more so than the lower density morphs.

Study System and background Information

*Biology of Asteromyia carbonifera*

The gall midge species *Asteromyia carbonifera* forms blister galls on leaves of *Solidago altissima* that provide nutrition for the maturing larvae and offer shelter and protection from enemies while preventing desiccation (Batra 1964). *A. carbonifera* undergo three to five generations per year with each generation (egg-adult) lasting
approximately four weeks (Weis 1982, 1983). The late larval stage of the last generation overwinters in the gall (Gagne 1968). In the spring, the larvae pupate, and the adults emerge shortly thereafter; however, the adults survive only approximately 2-3 days in nature (Weis, Price, & Lynch 1983, Gagne 1968). Depending on the gall morph, one to five larvae may inhabit each gall (Gagne 1968, Weis 1981). Females lay eggs in small clutches on the underside of new leaves on a young host-plant; however, crescent females tend to lay eggs on older plants. However, all morphs are ultimately associated with *Botryosphaeria dothidea*, an endophytic fungus which female midges carries in mycangia, or hair-like pockets used for transporting fungal spores, and deposits during oviposition (Gagne 1968, Weis 1983; Heath and Stireman in Review, Janson et al. in Review). Newly hatched larvae burrow into the leaf lamina initiating gall development. The fungal spores germinate within a few days following oviposition, enter through a larval opening created by the female, and then enter into the leaf tissue as well (Heath & Stireman in Review, Weis 1983). Larvae navigate through the gall until they reach their desired location while fungal mycelia proliferates surrounding and forming a cast around the *Asteromyia* larvae (Gagne 1968). After the midge pupates, the fungal mycelium rapidly proliferates and forms a thick, hardened, carbonaceous material known as the stroma that encases the pupae for the remainder of development (Batra 1964). All larvae within the gall are the offspring of a single female and emerge approximately 5-7 days after pupation, usually leaving a pupal exuviae partially inside the gall and fungal growth associated with the gall ceases (Gagne 1968, Weis 1982). *A. carbonifera* adults are very short-lived (2-3 days) and do not feed as suggested by their reduced mouthparts (Batra 1964, Weis 1983, Gagne 1968).
Previous studies have suggested that *A. carbonifera* is undergoing a rapid, within-host radiation on the host-plant species *Solidago altissima* (Late goldenrod) (Crego *et al.* 1990, Stireman *et al.* 2008). Through analysis of gall morphology and allozymes, Crego *et al.* (1990) discovered four gall types, crescent, cushion, flat, and irregular coexisting on the host plant *Solidago altissima*. These galls differed not only in shape, but also in the number of larvae, placement of larvae, location on the plant, and gall thickness (Crego *et al.* 1990, Stireman *et al.* 2008). Stireman *et al.* (2005), using mtDNA and AFLP markers, provided additional evidence that genetically distinct, morphologically cryptic lineages of *A. carbonifera* are, in fact, coexisting on their host plant *S. altissima*. Morphological and genetic differentiation of the midges may be occurring because of pressure from natural enemies.

The midge’s association with the mutualistic fungus (*B. dothidea*) may play a role in the morphological transformations (Batra 1964, Crego *et al.* 1990). The relationship between the midge and the fungus appears obligate in that the midge relies upon the fungal mycelium for food while the fungus depends on the female for the transport and implantation into the host-plant (Bissett and Borkent 1988; Heath & Stireman, in press, Janson *et al.* 2009). Aside from the nutritional value, the fungal symbiont supplies shelter and protection (Weis 1982). The fungal tissue alters the thickness and texture of the gall, which helps protect *A. carbonifera* from its natural enemies (Weis 1981, Weis 1983). *Asteromyia carbonifera* larvae are dependent upon a fungal symbiont, *Botryosphaeria dothidea* for gall production (Janson *et al.* 2009). The relationship between the midge and fungus appears to be obligate (Heath & Stireman 2010). In the
absence of the *Asteromyia*, the fungus will not differentiate and form the typical thick, hardened, carboniferous stroma (Heath & Stireman 2010). *A. carbonifera* may depend on the structural and nutritional supports offered by the fungus *Botryosphaeria dothidea*; and, in return, offer the fungus transportation and inoculation into the host-plant (Heath & Stireman 2010, Weis 1983, Crego *et al*. 1990, Batra 1964, Bissett and Borkent 1988).

Hymenopteran parasitoids inflict high levels of attack on *A. carbonifera* populations, ranging between 40-60% (Stireman *et al*. 2008, Weis 1982). Intense parasitoid pressure may select for gall traits controlled by the midge/fungus symbionts such as gall thickness or the number of larvae per gall.

*The Host Plant*

The host plant, *Solidago altissima*, may shape genetic differentiation of both *A. carbonifera* and its fungal symbiont. More specifically, parasitoids could interact with certain plant genotypes to favor gall morph specialization and differentiation. *Solidago altissima* is a rhizomatous perennial with a native distribution over much of temperate North America (Semple and Cook 2006). Three known cytotypic variations (ploidies) exist within *S. altissima*: diploid (2N), tetraploid (4N), and hexaploid (6N) (Halverson *et al*. 2007a). Genetic variation in dominant plant species has been shown to be a major factor in ecosystem biodiversity and function (Genung *et al*. 2010). Perhaps, *A. carbonifera* search for certain plant genotypes for manipulation of plant resources to their advantage (Stone & Schönrogge 2003). For example, gall midges can attract plant nutrients and metabolites in desired gall tissues by elevating photosynthetic rates in the
affected areas of the plants as well as gather nutrients from neighboring plant tissues (Stone & Schönrogge 2003). This allocation of plant nutrients towards gall formation may allow the midges to create stronger, more parasitoid-resistant galls. On the other hand, parasitoids could interact with plant genotype to favor morph specialization and differentiation. Because the A. carbonifera populations are so closely related and are likely to share parasitoids, we may see a correlation between gall defensive traits and parasitism.

Pressure from Natural Enemies

A diverse array of natural enemies is known to attack gall-inducing midges (Bailey et al. 2009). A. carbonifera construct ‘closed-community’ galls, thus attack by predators such as birds or larger insects occur less often than by parasitoids (Bailey et al. 2009). High rates of parasitoid attack are the primary causes of mortality on A. carbonifera populations, with hymenopteran parasitoids being the principal natural enemies of these gall makers (Weis 1982a). Parasitoid population densities may increase as those of A. carbonifera increase temporally creating stronger pressures on A. carbonifera populations; hence, creating a density-dependent relationship. Seven parasitoid species have been found to attack A. carbonifera. Taxonomically, these species include Torymus capite (Hymenoptera: Torymidae), Baryscapus fumipennis, Aprostocetus tesserus, and Aprostocetus homeri (originally classified as Tetra 2s in the realm of this study), Aprostocetus sp. (originally classified as Tetra 1s in this study), Neocrysocharis sp. (originally classified as Green Tetra 2s), and a Platygaster sp. (Hymenoptera: Platygasteridae). Weis showed morphological and behavioral limitations
often restrict attack by parasitoid species to certain stages of gall development (Weis 1982b). The rate and timing of parasitism rates on *A. carbonifera* is often constrained by the development of the fungal stroma suggesting that the fungus provides defensive benefits (Weis 1982a). Given that stromal development occurs later in the midge’s larval stages, early developmental periods of galls are more susceptible to parasitoid attack (Weis 1982a,b). For example, *P. solidiginis* has a relatively short ovipositor and is probably an egg-larval parasitoid. In other words, the female oviposits into the host’s egg and parasitoid development is completed after the host has reached the third larval stage (Weis 1982b). Due to the short ovipositor, the *P. solidiginis* female is unable to penetrate both mature gall tissues and the host (Weis 1982b). *T. tesserus* has a long ovipositor and is able to attack galls later in development (Weis 1982b). Parasitoid oviposition behavior may select for the host’s clutch size or possibly the length of diapause leading to differences in development time of *A. carbonifera* (Weis 1982, Härry et al. 2009).

Some gall morphs may be more susceptible to parasitoid attack than others. Alternatively, recent analyses indicate that the flat gall morph exhibits the highest rates of attack per gall (Stireman et al. 2009). In this case, parasitoids may concentrate on areas with high host densities (i.e., galls with large clutches) instead of increased time spent moving between patches with lower *A. carbonifera* densities.

Previous studies have shown that increased gall thickness and hardness significantly reduce the vulnerability of galling midges to enemy attack (Stone & Schönrogge 2003). For example, in *A. carbonifera* galls, cushions are the thickest of the gall morphs. Recent analyses show that cushions exhibit the second lowest parasitism
rates behind irregular morphs (Wells & Stireman, unpub. data). Although irregulars are
not thick, larval location may aid in reducing parasitoid attack. Larval placement in
irregulars is usually near the perimeter of the gall, whereas the other gall morphs have
larvae near the center of the gall (Crego et. al 1990).

**Methods**

**Study Sites**

All sampling of galls took place at three sites: Germantown Metropark, Sycamore
State Park, and Beaver Creek Wetlands located in Southwest Ohio. Distances between
the parks are as follows: Sycamore State Park to Beaver Creek Wetlands = 42.3km,
Beaver Creek Wetlands to Germantown Metropark = 54.6km, and Sycamore State Park
to Germantown Metropark = 24.5km. The Germantown Metropark field site consisted of
approximately 0.81 hectares of old prairie fields divided by a gravel roadway. The site at
Beaver Creek Wetlands was approximately two hectares of restored prairie surrounded
by restored wet prairies, fens, and marshes. Restoration of this prairie began in the early
1990’s. The field site at Sycamore State Park was approximately 1.2 hectares of restored
prairie bordered by second growth forest stands. Restoration of the Sycamore State Park
prairie began in the early 1980’s. Areas were chosen where continuous goldenrod
patches could support two 50m transects with surrounding areas of approximately 20m
on either side of the transects. Abbreviations for these sites were used in numbering of
galls throughout the surveys and are designated as GMP for Germantown Metropark,
BCW for Beaver Creek Wetlands, and SSP for Sycamore State Park.
To determine whether parasitism rates vary temporally and per gall morph, I surveyed 10 1m$^2$ plots monthly from June-September at each of the three sites for 30 plots per month. In these plots, all stems of *S. altissima* were counted and recorded. Afterwards, all leaves with galls were collected, dissected, and recorded. Prior to dissection, each gall was given a unique number representing the site, plot, plant, leaf, and gall. Gall dissection included detachment of the fungal hyphae and stroma from the gall exposing either the *A. carbonifera* or parasitoid larvae or pupae which can then be removed from the cell. Larvae and pupae were found in discrete cells within the fungal matrix of the gall. For each gall, I recorded the type of insect (parasitoid or midge), life stage (larva, pupa, or adult), number of inhabitants, number of cells (larval chambers) within each gall, and distinguishing remarks about the gall or inhabitant. All undamaged parasitoids were identified to genus and saved in 100% ethanol for further identification. For preservation, the parasitoids were placed in small vials with 100% ethanol, labeled by noting the site and morph from which they came, and originally identified by Weis’ description of previously found parasitoids (Weis 1982). It was not possible to identify all the larval parasitoids and many endoparasitoids were likely missed due to their tiny size within the *Asteromyia* larvae; however, the focus is on the relative rates of parasitism among gall morphs. Collection of these data permitted me to examine parasitoid frequency relative to gall morph distribution at a large spatial scale (sites), a small spatial scale (plots within a site), and a temporal scale (June-September).
Rearing

One hundred individuals of each gall morph were collected haphazardly at each site each month from June to September for a total of four collections (1200 galls). *S. altissima* leaves bearing mature galls were placed in vials and capped with a cotton swab to prevent leaf desiccation. These vials were then placed on ventilated platforms within closed plastic containers with approximately an inch of saturated solution (water and NaCl) covering the bottom of the containers. Saturated solutions maintain constant relative humidities in small closed spaces and prevent desiccation and inundation of the organism (Winston 1960). Each container was marked with the site location and the appropriate gall morph; crescent, cushion, flat, and irregular. All containers were kept at room temperature, approximately 24°C, and placed under fluorescent lights which were programmed relative to day length for Southwest Ohio. For instance, in June, relative day length was 14-15 hours day length, while July and August had approximately 12-13 hours of day length. From galls where eclosion occurred, identification of either midges or parasitoids was noted. As adults of either parasitoid or midge emerged, the galls were given a number including site, morphotype, leaf, and gall. Galls capable of housing more than one inhabitant such as cushions, flats, and irregulars were held in these growth chambers for exactly one week after the emergence of the first adult (either midge or parasitoid) to observe all eclosions from that particular gall. After one week, the vial was removed from the growth chamber and placed in the freezer. All uneclosed galls were dissected to discover if any occupants that may have failed to eclose were present. Again, all intact parasitoids were saved in 100% ethanol for later species identification.
These data allowed me to determine parasitism rates per gall morph, per plant, per site, and per midge generation as well as permitting me to see if gall morphs differed in their parasitism by particular parasitoid species. If the galls do not seem to differ in resource use in any major way, then maybe these gall-morph populations are diversifying in their ‘enemy niches’.

**Statistical Analyses**

I used the parasitism data collected from the 30 1m² plots per morph, per season (June-August), and per site to assess overall levels of parasitism over space and time and to determine whether gall morphs were attacked in similar frequencies. Using R statistical software (version 2.7.1, (C) R Foundation, from http://www.r-project.org), correlations of parasitoid attacks were assessed to determine whether any gall morphs may share potential enemies. I used generalized linear models plus ANOVAs to establish if gall morph, round (time during season), site, and interactions between all of these factors may play a role in the rates of parasitism. In this study, both gall data and cell data (individuals within a gall) were used for analyses of the effects of parasitism on *A. carbonifera*. I examined the effects of gall morph on parasitism frequency. I analyzed both gall and cell data where gall data are the actual galls themselves (i.e. crescent, cushion, flat, and irregular) and the cell data involved looking at the individual inside each cell of each of the gall morphs. Analyzing cell data is a better estimate of how parasitism affects individuals within a population of *A. carbonifera*; yet, in this study, I am trying to identify the effects of the gall morphs. Therefore, I focus on the gall–level data.
Again, using the plot data, linear regressions were conducted in R for each gall morph to assess whether rates of parasitism vary with gall densities. In these regressions, each site*sampling date was included as a data point for both density and parasitism frequency. Because each site is represented three times (sampling dates), the points are not statistically independent and are pseudoreplicated. However, because variation in density among sampling dates was similar in magnitude to variation among sites, and each sampling date represents a different generation of gall midges and parasitoids, this analysis still provides a rough assessment of the effect of density per se on parasitism at the site level. Regressions were also carried out to determine if density dependence existed at the plot level as well as the site level. Scatterplots were constructed in R (Rcmdr: R commander package) for only the site level density-dependence. Again, cell data was also analyzed to gain a better understanding of how parasitism affects individual Asteromyia within populations. However, cell data again will not be shown.

To determine gall preference for each parasitoid taxon, I used the rearing transect data that was collected over the 3 month span (1200 total galls). I first categorized the dissected parasitoids into species or morphospecies. These were originally categorized by dividing wasps into 5 groups: Tetra 2, Tetra 1, Platygaster, Green Tetra 2, and Torymus sp. (these categories were maintained throughout this study), but were later found to be seven different species: Torymus capite (Hymenoptera: Torymidae), Baryscapus Fumipennis, Aprostocetus tesserus, and Aprostocetus Homeri (grouped under Tetra 2s category), Aprostocetus sp. (Tetra 1s), Neocrysocharis sp. (Green Tetra 2s, Hymenoptera: Eulophidae), and a Platygaster sp. (Hymenoptera: Platygasteridae).
Afterwards, I organized the number of galls collected by site into parasitized versus non-parasitized for each parasitoid taxon and tested if this deviated from random expectation using Pearson’s chi-squared tests. This was completed for all three sites, all four gall morphs and all parasitoid types. Generalized linear models (GLM) were also conducted to assess the relationships between the frequency of each parasitoid and gall morph and round (time). For each site, I ran GLMs on all parasitoids investigating whether gall morphology, time of season (June-August), and the interaction between morph and round had an effect on the rates of their attacks.

Results

*Parasitism by gall morph and differences in parasitoid attack in time and space*

Parasitism rates among the *Asteromyia* gall morphs showed considerable variation in time, space, and across morphs. Overall parasitism rates for all gall morphs varied over the season from June through August. For instance, in June, thirty-six percent of all galls were parasitized; in July, thirty-five percent were parasitized; and in August forty-six percent were parasitized. Parasitism also varied per site and across morphs. SSP had the highest total parasitism at 46% (N=974), GMP had 37% total parasitism (N=2498), and BCW had the lowest total parasitism at 34% (N=1107). Across morphs, cushions experienced the highest parasitism at 45%, crescents and flats suffered similar attack rates at 40% parasitism and 39%, respectively, and irregulars had the least parasitism at 33%. Crescents were generally one of the most attacked morphs by parasitoids in June as well as being the most abundant gall morph in June (Figures 1a-c, see Chapter 2: Phenology and Distribution, Table 1) while flats suffered their highest parasitism towards...
the middle and end of the season (July & August) (Figures 1a-c). At BCW and SSP, some patterns of parasitism attack rates were fairly similar. For example, cushions showed a relatively steady rate of parasitism throughout the season at both sites while flats peaked in parasitism in July (Figures 1b-c). At GMP, however, cushions nearly doubled their rates of attack from June through August and flats had their lowest parasitism in June (Figure 1a). Although some similarities among sites are noted, parasitism seemed to be somewhat inconsistent and unpredictable as far as which gall morphs are favored and when they are favored. Overall parasitism rates did not seem to be consistently lower in June when fewer Asteromyia galls were present. At some sites on particular morphs, parasitism may be lower in June, but this may have been due to the densities of particular morphs. For example, flats at all three sites, tended to have the lowest density of all morphs in June (Chapter 2: Phenology and Distribution). In conjunction with these lower densities, parasitism was also lower (Figures 1a-c). As expected, the other gall morphs were more abundant than flats in June and seemed to endure more parasitism. Gall densities and parasitism was variable over the season. For example, irregulars began with fairly high densities at GMP and steadily rose throughout the season (Chapter 2, Figure 1: red line). Conversely, irregulars suffered the least amount of parasitism overall. Crescents began with the highest density of gall morphs at GMP, then dropped somewhat in July, and slowly began to rise again in August (Chapter 2, Figure 1); yet, the pattern of parasitism among crescents at GMP nearly paralleled the frequency of crescents over the season (Figure 1a, Chapter 2: Phenology and Distribution, Figure 1).
Some of the gall morphs were correlated in parasitism suggesting they may share enemies and exhibit “apparent” competition through these shared enemies. For example, at GMP, three gall morph combinations were correlated (Table 1): crescents and irregulars (r=0.387, p=0.035), cushions and irregulars (r=0.410, p=0.025), and flats and irregulars (r=0.486, p=0.006). At SSP, flats and irregulars were also correlated (Table 1, r=0.553, p=0.003). BCW had no strong correlations between parasitism among gall morphs (Table 1).

**Figure 1**: Frequency of parasitism per gall over time for each gall morph at: a. Germantown Metropark (GMP) b. Beaver Creek Wetlands (BCW) and c. Sycamore State Park (SSP), respectively.
b)

![Graph showing the proportion of parasitism for different months and egg shapes.](image)

- Crescent
- Cushion
- Flat
- Irregular

Proportion of Parasitism

Month

June, July, August

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

June, July, August

Month

c)
Table 1: Correlations of parasitism by gall morph per site. The correlation coefficients are shown below the diagonal (empty boxes) while p-values are noted above the diagonal. These numbers show the correlations of parasitoid attacks among all four gall types.

<table>
<thead>
<tr>
<th>Site</th>
<th>Crescent</th>
<th>Cushion</th>
<th>Flat</th>
<th>Irregular</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP</td>
<td>Crescent</td>
<td>0.189</td>
<td>0.628</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>Cushion</td>
<td>0.247</td>
<td>0.158</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.092</td>
<td>0.264</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Irregular</td>
<td>0.387</td>
<td>0.410</td>
<td>0.486</td>
</tr>
<tr>
<td>BCW</td>
<td>Crescent</td>
<td>0.598</td>
<td>0.410</td>
<td>0.571</td>
</tr>
<tr>
<td></td>
<td>Cushion</td>
<td>0.100</td>
<td>0.551</td>
<td>0.562</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>0.156</td>
<td>-0.113</td>
<td>0.874</td>
</tr>
<tr>
<td></td>
<td>Irregular</td>
<td>0.108</td>
<td>0.110</td>
<td>-0.030</td>
</tr>
<tr>
<td>SSP</td>
<td>Crescent</td>
<td>0.062</td>
<td>0.918</td>
<td>0.891</td>
</tr>
<tr>
<td></td>
<td>Cushion</td>
<td>-0.371</td>
<td>0.127</td>
<td>0.074</td>
</tr>
<tr>
<td></td>
<td>Flat</td>
<td>-0.021</td>
<td>0.307</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Irregular</td>
<td>-0.028</td>
<td>0.357</td>
<td>0.553</td>
</tr>
</tbody>
</table>

Parasitism varied strongly among gall morphs. Cushions had the highest overall parasitism among the gall morphs except for the site SSP where crescents had the highest rate of parasitoid attack (Figures 2a-c). Crescents and flats experienced similar
parasitism rates at GMP and BCW but there are some slight differences such as flats experiencing more attacks at BCW (Figures 2a-c). Irregulars were the least attacked gall morph overall at all three sites (Figures 2a-c). The cell parasitism data paralleled these results. Not only is gall morphology a factor in parasitism rates of galls, but sampling date (temporal variation) and site (spatial variation) also played significant roles in parasitism frequency across Asteromyia populations (Table 2). Interactions between morph, round, and site also had significant effects on parasitism among the gall morphs suggesting that all three factors and their interactions with one another influenced survival of A. carbonifera populations (Table 2).

Each gall morph, for the most part, seemed to have its own rate of parasitoid attack that is not necessarily consistent throughout all of the morphs (Table 2). However, irregulars across the sites, consistently had the lowest parasitoid attacks compared to the other three gall morphs, while cushions were usually the most attacked with a slight exception at Sycamore State Park (Figures 2a-c, Table 2).
Figure 2: Overall proportion of parasitism of gall morphs across sites: a. Germantown Metropark (GMP) b. Beaver Creek Wetlands (BCW) and c. Sycamore State Park (SSP), respectively.

a)

![Graph a](image1)

b)

![Graph b](image2)
Table 2: ANOVA table of gall morphology, round, and site, and their significant interactions as factors influencing parasitism from the generalized linear model

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>Deviance</th>
<th>Resid. Df</th>
<th>Resid. Dev</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td>5462</td>
<td>7275.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morph</td>
<td>3</td>
<td>44.3</td>
<td>5459</td>
<td>7231.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Round</td>
<td>2</td>
<td>82.9</td>
<td>5457</td>
<td>7148.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Site</td>
<td>2</td>
<td>29.4</td>
<td>5455</td>
<td>7119.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Morph:Round</td>
<td>6</td>
<td>55.3</td>
<td>5449</td>
<td>7063.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Morph:Site</td>
<td>6</td>
<td>37.3</td>
<td>5443</td>
<td>7026.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Round:Site</td>
<td>4</td>
<td>59.9</td>
<td>5439</td>
<td>6999.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Morph:Round:Site</td>
<td>12</td>
<td>33.2</td>
<td>5427</td>
<td>6933.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Density-dependence of parasitoids

At the site level, the linear regression results confirmed that parasitoid attacks on all gall morphs were not necessarily predictable and consistent (Figures 3a-d). For example, crescents at GMP and BCW (Fig. 3a) generally had higher densities in June and July but endured lower parasitism. However, as crescent densities decreased over the season, the level of parasitism intensified suggesting strong density-dependence of parasitoids at the site by sampling date level (Figures 3a-d). This pattern was not consistent with all morphs at all sites, however. Cushions at SSP showed very low densities in June and suffered low parasitism; yet, in August, cushion densities still remained fairly low but the level of parasitism escalated (Fig. 3b).
Figure 3: Parasitism per gall morph for density dependence at site level. These figures contain marginal box-plot along both (x) and (y) axes and are fitted with a least-squares line. These data included all 3 rounds (June, July, and August) across all 3 sites (BCW, GMP, and SSP). Hence, the 9 points on the graph. The points go as follows on the figures 3a-d: BCW: (1,2,3) 1=June, 2=July, 3=August, GMP: (4,5,6) 4=June, 5=July, 6=August, SSP: (7,8,9) 7=June, 8=July, 9=August.

a) Crescents
b) Cushions

![Graph showing the relationship between Gall Density and Proportion of Parasitism for Cushions.](image)

Data points are scattered along a linear trend, indicating a positive correlation between Gall Density and Proportion of Parasitism.

c) Flats

![Graph showing the relationship between Gall Density and Proportion of Parasitism for Flats.](image)

Data points are scattered along a linear trend, indicating an inverse correlation between Gall Density and Proportion of Parasitism.
d) Irregulars

Density-dependence of parasitoids at the plot level

At the plot level, the overall relationship at all three sites between parasitoids and all four gall morphs demonstrated negative density dependence with a slight exception of irregulars at BCW (Tables 3-5). At GMP, crescents showed significant negative density dependence (p = 0.0129) during Round 3 (August) when crescents tended to have some of the lowest densities at GMP (Table 4, Chapter 2: Phenology and Distribution). This pattern was also seen when density dependence was measured at the site level (Figure 3a). Irregulars also exhibited a significant negative density dependence value in Round 2 (July) when irregulars displayed fairly moderate densities (Table 3, Chapter 2: Phenology and Distribution). At SSP, the linear regression results showed a negative density dependence with all four gall morphs; however, with crescents, the
overall results were significant (Table 6, p = 0.01). Crescents also show significant negative density dependence (p= 0.0352) during Round 1 (June), again when crescent density was at its highest (Table 6, Chapter 2: Phenology and Distribution). Flats, at SSP, revealed negative density dependence during Round 3 (August) when flat densities were at their lowest (Table 6, Chapter 2: Phenology and Distribution).

**Table 3:** Linear regression summary for density dependence analyses of gall parasitism versus gall morph density at the plot level for GMP. Linear regressions were run for overall density-dependence at the plot level as well as for individual rounds. In the table below, overall p-value and Adj. R² shows results for all months combined. For the individual rounds, the abbreviations go as follows: R1 = June, R2 = July, R3 = August.

<table>
<thead>
<tr>
<th>Gall</th>
<th>t-value</th>
<th>Overall p-value</th>
<th>Adj.R²</th>
<th>R1 p-value</th>
<th>R1 Adj.R²</th>
<th>R2 p-value</th>
<th>R2 Adj.R²</th>
<th>R3 p-value</th>
<th>R3 Adj.R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cres</td>
<td>-0.545</td>
<td>0.590</td>
<td>-0.025</td>
<td>0.164</td>
<td>0.131</td>
<td>0.216</td>
<td>0.082</td>
<td>0.013</td>
<td>0.513</td>
</tr>
<tr>
<td>Cush</td>
<td>-0.640</td>
<td>0.528</td>
<td>-0.021</td>
<td>0.567</td>
<td>-0.077</td>
<td>0.157</td>
<td>0.138</td>
<td>0.184</td>
<td>0.110</td>
</tr>
<tr>
<td>Flat</td>
<td>-1.158</td>
<td>0.258</td>
<td>0.013</td>
<td>0.828</td>
<td>-0.135</td>
<td>0.966</td>
<td>-0.166</td>
<td>0.426</td>
<td>-0.034</td>
</tr>
<tr>
<td>Irr</td>
<td>-1.739</td>
<td>0.094</td>
<td>0.067</td>
<td>0.960</td>
<td>-0.125</td>
<td>0.018</td>
<td>0.515</td>
<td>0.912</td>
<td>-0.123</td>
</tr>
</tbody>
</table>
**Table 4: Linear Regression Summary of Gall Parasitism v. Density at the Plot Level at BCW.** See description for this table above table 4.

<table>
<thead>
<tr>
<th>Gall</th>
<th>t-value</th>
<th>Overall p-value</th>
<th>Adj.R²</th>
<th>R1 p-value</th>
<th>Adj.R²</th>
<th>R2 p-value</th>
<th>R3 p-value</th>
<th>Adj.R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cres</td>
<td>-0.27</td>
<td>0.789</td>
<td>-0.033</td>
<td>0.515</td>
<td>-0.063</td>
<td>0.109</td>
<td>0.201</td>
<td>0.877</td>
</tr>
<tr>
<td>Cush</td>
<td>-0.587</td>
<td>0.562</td>
<td>-0.025</td>
<td>0.981</td>
<td>-0.125</td>
<td>0.226</td>
<td>0.087</td>
<td>0.942</td>
</tr>
<tr>
<td>Flat</td>
<td>-0.133</td>
<td>0.896</td>
<td>-0.058</td>
<td>0.596</td>
<td>-0.084</td>
<td>N/A</td>
<td>N/A</td>
<td>0.219</td>
</tr>
<tr>
<td>Irr</td>
<td>0.060</td>
<td>0.952</td>
<td>-0.038</td>
<td>0.541</td>
<td>-0.090</td>
<td>0.078</td>
<td>0.255</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Table 5: Linear Regression Summary of Gall Parasitism v. Density at the Plot Level at SSP.** See description for this table above table 4.

<table>
<thead>
<tr>
<th>Gall</th>
<th>t-value</th>
<th>Overall p-value</th>
<th>Adj.R²</th>
<th>R1 p-value</th>
<th>Adj.R²</th>
<th>R2 p-value</th>
<th>R3 p-value</th>
<th>Adj.R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cres</td>
<td>-2.658</td>
<td>0.014</td>
<td>0.209</td>
<td>0.035</td>
<td>0.419</td>
<td>0.721</td>
<td>-0.268</td>
<td>0.110</td>
</tr>
<tr>
<td>Cush</td>
<td>-1.098</td>
<td>0.284</td>
<td>0.0085</td>
<td>0.0375</td>
<td>-0.013</td>
<td>0.099</td>
<td>0.418</td>
<td>0.360</td>
</tr>
<tr>
<td>Flat</td>
<td>-0.536</td>
<td>0.597</td>
<td>-0.032</td>
<td>0.390</td>
<td>-0.020</td>
<td>0.321</td>
<td>0.053</td>
<td>0.024</td>
</tr>
<tr>
<td>Irr</td>
<td>-0.590</td>
<td>0.561</td>
<td>-0.027</td>
<td>0.996</td>
<td>-0.125</td>
<td>0.436</td>
<td>-0.053</td>
<td>0.530</td>
</tr>
</tbody>
</table>

*Gall morph preferences by particular parasitoids*

The Pearson’s chi-squared test results showed that parasitoid frequencies among the four gall morphs significantly deviated from expected frequencies (Table 6).

Crescents experienced relatively high parasitism by the collective group *Tetra* 2 (which actually contained the species *Baryscapus fumipennis*, *Aprostocetus tesserus*, and *Aprostocetus homeri*) at all 3 sites (Table 7, Figures 4a, 5a, 6a). According to the
proportions listed in Table 8, *Tetra* 2 were fairly widespread among gall morphs and moderately attacked most of the gall morphs. *Tetra* 2s avoided irregulars at GMP and SSP and flats at BCW (Table 8, Figures 4a, 5a, 6a) and were the most common *A. carbonifera* parasitoid at the 3 sampled sites (Table 7).

*Aprostocetus sp.* (Tetra 1s) preferred flats at GMP and BCW; however, they attacked crescents more at SSP (Table 7, Figures 4b, 5b, 6b). This was possibly due to the very low abundance of flats found at SSP (Chapter 2: Phenology and Distribution). Flat densities were consistently the lowest of all morphs at all three sites. At the sites where flats are preferred, crescents incurred the lowest parasitism even though they were the most abundant gall morph early on (Table 7, Figures 4b, 5b). At SSP where crescents are favored, cushions experienced relatively low parasitism by *Tetra* 1s (Table 7, Graph 6b). *Platygaster* mainly attacked crescents and avoided irregulars completely at all 3 sites (Table 7, Figures 4c, 5c, and 6c). They had the most distinct pattern of gall morph invasion compared to other parasitoids attacking *A. carbonifera*.

*Neochrysocharis* (Green *Tetra* 2) frequencies tended to be highly biased towards crescents, but this varied among sites, and in some cases, flats or cushions had relatively high frequencies of parasitism (Table 7, Figures 4d, 5d, 6d). For example, flats were the preferred morph at GMP, but crescents were a very close second (Figure 4d). *Torymus* mostly attacked cushions overall (Table 7, Figures 4e, 5e, 6e). At BCW though, their frequency on cushions was equivalent to flats (Table 7, Figure 6e). *Torymus* avoided crescents and irregulars at all 3 sites (Table 7, Figures 4e, 5e, 6e).
Table 6: Pearson’s chi-squared results for parasitoid frequency among gall morphs showing that parasitoid frequencies among the four gall morphs differed significantly from expected frequencies. The names of the parasitoids are still kept in their original and collective (Tetra 2) categories.

<table>
<thead>
<tr>
<th>Parasitoid</th>
<th>$X^2$</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetra 2</td>
<td>42.925</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tetra 1</td>
<td>42.7118</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platygaster</td>
<td>123.3752</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Green Tetra 2</td>
<td>40.9515</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Torymus</td>
<td>80.7414</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 7: Table of Proportions of Parasitism per Gall Morph per Site. The numbers in the table below indicate the proportions of parasitism (number of galls parasitized/total number of galls (N)) per gall morph at each of the three studied sites. Red indicates the parasitoid’s preference towards gall morphs based on the higher proportions. Based on lower proportion numbers, Green indicates the parasitoid’s indifference of certain gall morphs. The **** denotes the highest total parasitism over all morphs while the ^^^^ signifies the lowest total parasitism between the gall morphs.

<table>
<thead>
<tr>
<th>Site</th>
<th>GMP</th>
<th>BCW</th>
<th>SSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetra 2</td>
<td>0.34</td>
<td>0.22</td>
<td>0.27</td>
</tr>
<tr>
<td>Tetra 1</td>
<td>0.00</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Platy</td>
<td>0.08</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Grn. T2</td>
<td>0.05</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Tory</td>
<td>0.01</td>
<td>0.13</td>
<td>0.03</td>
</tr>
<tr>
<td>Tot. Para.</td>
<td>0.48***</td>
<td>0.41</td>
<td>0.42</td>
</tr>
</tbody>
</table>

* Indicates the parasitoid’s preference towards gall morphs based on the higher proportions. Based on lower proportion numbers, Green indicates the parasitoid’s indifference of certain gall morphs. The **** denotes the highest total parasitism over all morphs while the ^^^^ signifies the lowest total parasitism between the gall morphs.
**Figure 4**: Gall morph preference by the seven (categorically five for this study) discovered parasitoids for GMP. The Pearson Residual (y-axis) is proportional to the difference between the observed and expected values in the chi-squared test. Any values above the x-axis (zero) indicate the parasitoid’s preference towards that particular gall morph whereas any value below the axis suggests the parasitoid’s avoidance of that morph.

a)
b) 

**Aprostocetus sp. Parasitism per Morph**

<table>
<thead>
<tr>
<th>Gall Morph</th>
<th>Pearson Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cres</td>
<td>-1.8326</td>
</tr>
<tr>
<td>Cush</td>
<td>-1.4252</td>
</tr>
<tr>
<td>Flat</td>
<td>3.6125</td>
</tr>
<tr>
<td>Irr</td>
<td>-0.1671</td>
</tr>
</tbody>
</table>


c) 

**Neochrysocharis sp.**

<table>
<thead>
<tr>
<th>Gall Morph</th>
<th>Pearson Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cres</td>
<td>2.0555</td>
</tr>
<tr>
<td>Cush</td>
<td>0.2482</td>
</tr>
<tr>
<td>Flat</td>
<td>2.3885</td>
</tr>
<tr>
<td></td>
<td>-3.1042</td>
</tr>
</tbody>
</table>
d) **Platygaster sp.**

![Bar chart for Platygaster sp. showingPearson Residuals.]

- Cres: 5.9031
- Cush: 0.0937
- Flat: -1.0921
- Irr: -3.0488

e) **Torymus capite**

![Bar chart for Torymus capite showing Pearson Residuals.]

- Cres: -2.0606
- Cush: 5.8797
- Flat: -1.1367
- Irr: -2.5694
**Figure 5**: Gall morph preference by the seven (categorically five for this study) discovered parasitoids for BCW. See figure 4 for full description.

a)

![Graph for Tetra 2 spp.]

b)

![Graph for Aprostocetus sp.]
c) Neochrysocharis sp.

![Chart for Neochrysocharis sp.]

- Pearson Residuals:
  - Cres: 2.4410757
  - Cush: -0.034189283
  - Flat: -1.039849867
  - Irr: -0.788956812

Gall Morph

---

d) Platygaster sp.

![Chart for Platygaster sp.]

- Pearson Residuals:
  - Cres: 5.34599278
  - Cush: 0.47606571
  - Flat: 0.14680742
  - Irr: -3.8255778

Gall Morph
Figure 6: Gall morph preference by the seven (categorically five for this study) discovered parasitoids for SSP. See figure 4 for a full description.
b) 

![Aprostocetus sp.](image)

**Aprostocetus sp.**

- Pearson Residual: 3.0637684
- Pearson Residual: 1.1973604
- Pearson Residual: -1.9515178
- Pearson Residual: -1.0664873

**Gall Morph:** Cres, Cush, Flat, Irr

---

c) 

![Neochrysocharis sp.](image)

**Neochrysocharis sp.**

- Pearson Residual: 1.3676131
- Pearson Residual: 2.9185354
- Pearson Residual: 0.9920828
- Pearson Residual: -3.7647165

**Gall Morph:** Cres, Cush, Flat, Irr
Factors influencing the rates of attack by parasitoids

The GLM results for each parasitoid. Only significant results or nearly significant results for the GLM results for each parasitoid are shown in this table (Table 8). For each parasitoid, ‘round’ appeared to be the only consistent factor that influenced the rates of
parasitism (Table 8). When the GLM was conducted, the test used particular factors as intercepts for comparison. For example, for Tetra 2s, crescents were used as the intercept, or a means to compare all the other morphs. Tetra 2s preferred crescents at all three sites (Table 7, Figures 4a, 5a, 6a); however, cushions, flats, and irregulars had highly significant numbers considered in the factors that may influence parasitoid attack. However, the estimates in Table 8 were all negative numbers when compared to the intercept or the crescent gall morph (Table 8), indicated relatively lower parasitism for these morphs. *Platygaster* sp. also favored crescents and almost totally avoided cushions and flats (Table 8); yet, cushions and flats showed up in the summary table as the significant factors influencing how *Platygaster* sp. attacked *A. carbonifera*. Other than round, all parasitoids seemed to differ in what affects their rates of attack (Table 8). Crescents seemed to be the preferred gall morph of Tetra 2s, *Platygaster*, and Green Tetra 2s (Figures 5-7). Tetra 2s and Green Tetra 2s appeared to be a more generalist parasitoids compared to *Platygaster* that specialize on crescents (Tables 7, 8, Figures 4-6). *Torymus* sp. preferred cushions and will attack flats but avoided crescents and irregulars (Tables 7, 8, Figures 4-6). Tetra 1s selected flats at two of the sites, GMP and BCW, but attacked crescents at SSP (Tables 7, 8, Figures 4-6).
**Table 8:** Effects of morph, sampling round, and site on parasitoid attack frequency

The table below highlights only the significant results from the GLM tests ran for each parasitoid. These GLMs were used to test which factors between morph, round, and site played a role in the rate of parasitoid attacks against *A. carbonifera*. Those numbers denoted with this symbol (*) shows numbers that are nearly significant.

<table>
<thead>
<tr>
<th>Parasitoid</th>
<th>Factor</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetra 2</td>
<td>Cushion</td>
<td>-1.422</td>
<td>0.291</td>
<td>-4.894</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tetra 2</td>
<td>Flat</td>
<td>-0.942</td>
<td>0.327</td>
<td>-2.882</td>
<td>0.004</td>
</tr>
<tr>
<td>Tetra 2</td>
<td>Irregular</td>
<td>-0.554</td>
<td>0.267</td>
<td>-2.073</td>
<td>0.038</td>
</tr>
<tr>
<td>Tetra 2</td>
<td>Round</td>
<td>-0.521</td>
<td>0.124</td>
<td>-4.215</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tetra 2</td>
<td>SSP</td>
<td>0.554</td>
<td>0.245</td>
<td>2.259</td>
<td>0.0224</td>
</tr>
<tr>
<td>Tetra 2</td>
<td>Cush: Round</td>
<td>0.478</td>
<td>0.133</td>
<td>0.608</td>
<td>0.544</td>
</tr>
<tr>
<td>Tetra 2</td>
<td>Flat: GMP</td>
<td>0.579</td>
<td>0.285</td>
<td>2.030</td>
<td>0.042</td>
</tr>
<tr>
<td>Tetra 2</td>
<td>Cush: SSP</td>
<td>0.684</td>
<td>0.253</td>
<td>2.706</td>
<td>0.007</td>
</tr>
<tr>
<td>Tetra 2</td>
<td>Flat: SSP</td>
<td>0.769</td>
<td>0.285</td>
<td>2.704</td>
<td>0.007</td>
</tr>
<tr>
<td>Tetra 2</td>
<td>Round: SSP</td>
<td>-0.401</td>
<td>0.117</td>
<td>-3.435</td>
<td>0.001</td>
</tr>
<tr>
<td>Tetra 1</td>
<td>Cushion</td>
<td>-2.222</td>
<td>1.098</td>
<td>-2.024</td>
<td>0.043</td>
</tr>
<tr>
<td>Tetra 1</td>
<td>Round</td>
<td>-3.281</td>
<td>1.010</td>
<td>-3.248</td>
<td>0.001</td>
</tr>
<tr>
<td>Tetra 1</td>
<td>Cush: Round</td>
<td>2.474</td>
<td>1.016</td>
<td>2.434</td>
<td>0.015</td>
</tr>
<tr>
<td>Tetra 1</td>
<td>Cush: SSP</td>
<td>-2.276</td>
<td>0.629</td>
<td>-3.616</td>
<td>0.000</td>
</tr>
<tr>
<td>Tetra 1</td>
<td>Flat: SSP</td>
<td>-1.678</td>
<td>0.513</td>
<td>-3.270</td>
<td>0.001</td>
</tr>
<tr>
<td>Tetra 1</td>
<td>Irr: SSP</td>
<td>-1.263</td>
<td>0.478</td>
<td>-2.644</td>
<td>0.008</td>
</tr>
<tr>
<td>Tetra 1</td>
<td>Round: GMP</td>
<td>0.809</td>
<td>0.389</td>
<td>2.080</td>
<td>0.038</td>
</tr>
<tr>
<td>Platygaster</td>
<td>Cushion</td>
<td>-3.528</td>
<td>0.853</td>
<td>-4.136</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platygaster</td>
<td>Flat</td>
<td>-5.072</td>
<td>1.081</td>
<td>-4.694</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platygaster</td>
<td>Round</td>
<td>-2.580</td>
<td>0.665</td>
<td>-3.881</td>
<td>0.000</td>
</tr>
<tr>
<td>Platygaster</td>
<td>SSP</td>
<td>-1.155</td>
<td>0.318</td>
<td>-3.635</td>
<td>0.000</td>
</tr>
<tr>
<td>Platygaster</td>
<td>Cush: Round</td>
<td>1.963</td>
<td>0.722</td>
<td>2.719</td>
<td>0.007</td>
</tr>
<tr>
<td>Platygaster</td>
<td>Flat: Round</td>
<td>2.624</td>
<td>0.773</td>
<td>3.393</td>
<td>0.001</td>
</tr>
<tr>
<td>Green T2</td>
<td>Flat</td>
<td>-1.950</td>
<td>1.080</td>
<td>-1.806</td>
<td>0.071*</td>
</tr>
<tr>
<td>Green T2</td>
<td>Irregular</td>
<td>-1.299</td>
<td>0.646</td>
<td>-2.013</td>
<td>0.044</td>
</tr>
<tr>
<td>Green T2</td>
<td>Round</td>
<td>-0.776</td>
<td>0.151</td>
<td>-5.130</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Green T2</td>
<td>Flat: GMP</td>
<td>2.029</td>
<td>1.162</td>
<td>1.746</td>
<td>0.081*</td>
</tr>
<tr>
<td>Torymus</td>
<td>Cushion</td>
<td>1.298</td>
<td>0.311</td>
<td>4.173</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Torymus</td>
<td>Round</td>
<td>0.408</td>
<td>0.107</td>
<td>3.824</td>
<td>0.000</td>
</tr>
<tr>
<td>Torymus</td>
<td>GMP</td>
<td>1.150</td>
<td>0.255</td>
<td>4.513</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Torymus</td>
<td>SSP</td>
<td>0.745</td>
<td>0.263</td>
<td>2.836</td>
<td>0.005</td>
</tr>
</tbody>
</table>
Discussion

A renewed interest in ecological speciation or ecologically-based processes that drive reproductive isolation and eventually speciation has recently sparked new interest among many researchers (Schluter 2005). Until recently, little evidence had been found that supported ecological speciation; however, an explosion of newer studies have begun to investigate abiotic (climate, resources) and biotic (predation, inter-specific interactions) elements that may have contributed to numerous speciation events (Schluter 2001, Stone & Schönrogge 2003, Nosil 2004, Gow et al. 2007). In this study, I investigated whether pressure from natural enemies could be promoting the divergence and coexistence of a gall-making midge, Asteromyia carbonifera, in southwestern Ohio. I examined if parasitism varies by gall morph, by site, and whether any of the A. carbonifera gall morphs were correlated in their parasitoid attacks. I also examined how parasitism varies over time and if these parasitoids were density-dependent upon A. carbonifera populations at the plot and site levels. Furthermore, I assessed whether parasitoid rates by particular parasitoids vary among the four gall morphs. Finally, I discovered how many parasitoids were attacking A. carbonifera populations and if they were exerting equal pressures on the gall morphs.

Parasitism differences across gall morphs and sites

Standardized sampling along transects showed that, overall, parasitism varies by gall morph. At GMP and BCW, cushions experience the highest parasitism overall while crescents are most attacked by parasitoids at SSP. At all three sites, irregulars undergo the lowest parasitism among all gall morphs. Frequencies of gall morphs as well as
parasitism frequencies vary greatly among generations of *A. carbonifera* from June through August. Natural enemies comprise a large source of herbivore mortality (Hawkins *et al.* 1997); however, not all of these *A. carbonifera* populations appear to be attacked at the same rate. Concealment of these midges within galls makes them immobile during immature stages and highly vulnerable to parasitoids (Hawkins *et al.* 1997). Differential gall morphologies may allow for variance in attack rates even if the outcome still results in high midge mortality (Hawkins *et al.* 1997). Total parasitism patterns across sites have some similarities such as parasitoids favoring certain gall morphs but parasitism attack rates seem to be somewhat unpredictable among gall morphs.

Some of the gall morphs are correlated in their rates of attack. For example, flats and irregulars are correlated at both GMP and SSP and crescents and irregulars and flats and irregulars are also correlated at GMP. BCW has no significant correlations in gall morph attacks. Correlations such as these may suggest that these particular gall morphs are experiencing apparent competition. This type of competition may have severe consequences for species assemblages and may even eliminate one of the competitors over time (Nouhuys & Hanski 2000). Nouhuys & Hanski (2000) suggests that evidence of apparent competition generally involves herbivores with a shared generalist parasitoid that aggregate within one generation where one of the host densities are high. This would make sense in *A. carbonifera* populations where flats and irregulars are correlated in abundance and parasitism. Generally, irregular gall morph densities are huge in comparison to flat densities (Chapter 2: Phenology and Distribution). Because these two
gall morphs share a parasitoid (Tetra 2s), this may be having an impact on flat frequencies through apparent competition. However, all of these gall morphs are not correlated in their rates of attack suggesting that all are not involved in apparent competition. This implies that there are marked differences between parasitoid preference towards gall morphs and that the parasitoids tend to specialize on certain morphs. Because gall morphs vary in parasitism as well as their own frequencies over time, parasitoid attack rates may vary over time and respond to variation in gall morph density.

The selective impact of parasitoids is apparent. However, the role of parasitoids in A. carbonifera divergence and coexistence needs further investigation. If apparent competition were taking place in this system, this would hasten divergence of these midges; perhaps, though, apparent competition may have already encouraged divergence in these midges and still somehow plays a small role due to shared parasitoids. Perhaps, these midges have diverged along the enemy axis of their niche to allow coexistence and the parasitoids may be holding down the populations of these gall midges so that actual competition is not important.

*Parasitism variance and density-dependence*

Parasitoid attack rates vary over time in conjunction with preferred gall morphs. At the site level, parasitoid attacks increase as the densities of the gall morphs decrease in most cases suggesting negative density dependence. However, the increase in gall morph densities is not uniform such as being low early in the season and higher towards the end. Some gall morphs, such as crescents, begin at high densities and decrease over the season.
whereas irregulars start with lower densities and increase over time (Chapter 2: Phenology and Distribution). Therefore, if the parasitoids select certain gall morphs, then their rates of attack would vary over time; hence, they are density–dependent at least at the site level. At the plot level, parasitoids also exhibit some negative density dependence towards certain gall morphs possibly due to the non-random distribution of gall morphs. Similar patterns were observed in both the site and plot levels with crescents. For instance, early in the season, crescents densities were highest and experienced less parasitism; however, as densities decreased, parasitism of crescents grew. Perhaps, the initially high crescent densities attracted parasitoids to that particular area and parasitoid pressure decreased crescent populations towards the end of the season. Alternatively, parasitoids may be attracted to certain host-plant genotypes to ensure better chances of survival for their offspring and have a tendency to search for these host plants. Regardless, gall morphs are clustered in space (Chapter 2: Phenology and Distribution) and plots may share several types of gall morphs in close range of one another. This suggests that parasitoids are using some cues, associated with either gall morph or host-plant genotype, in order to select an optimal environment for their offspring to develop to maturity.

*Parasitoids and their preferences*

When this study was completed, seven parasitoids identified to morphospecies had been associated with *A. carbonifera*. The last part of this study explored whether these parasitoids apply equal pressures to the gall morphs and if they are overrepresented in certain gall morphs. These parasitoids do not respond equally towards the morphs.
Crescents are the most attacked gall morphs by several parasitoids. For example, at GMP, 48% of crescents were parasitized, 52% of crescents were parasitized at BCW, and 46% of crescents were attacked at SSP. Weis (1982) addresses that since fungal stromal development within the gall occurs later in the midge’s larval stages, early developmental periods of galls are more susceptible to parasitoid attack (Weis 1982a, b). It makes sense that crescents are attacked by almost all of the parasitoids due to their early presence (Chapter 2: Phenology and Distribution). If some these parasitoids overwinter as larvae, crescents are often the first, most plentiful gall morph available and these parasitoids may compete for hosts (Chapter 2: Phenology and Distribution). Also, because the majority of the parasitoids present have short ovipositors, crescents may offer the most penetrable galls (Weis 1983, B. Wells, pers. obs.). Cushions and flats are nearly equal in their rates of attack. For instance, both gall morphs average around 40% parasitoid attack rates across sites (Table 8). Overall, it appears that irregulars are the least favored gall morph among all parasitoids attacking A. carbonifera. Even though Tetra 2s will attack irregulars, they experience the lowest parasitism by this somewhat versatile parasitoid.

Platygaster sp. are egg parasitoids and they complete their life cycle within the Asteromyia hosts (Sandanayaka & Charles 2006). Across the three sites, these parasitoids strictly attack crescents and avoid irregulars. Platygaster sp. are possibly a specialist parasitoid. Crescents may be favored by Platygaster because of their initially high gall abundance, their relatively thin fungal layers, or easy detection of the crescent’s eggs on the periphery of the leaf. According to Castelo & Corley (2010), host specificity
may have a negative impact on parasitoid-host systems because specialist parasitoids may cause an unstable population of hosts due to temporal differences. For instance, initial *Platygaster* sp. populations and their strong inclination towards crescents may drive down crescent populations leaving later generations of *Platygaster* sp. as well as other crescent parasitoids without adequate gall morph resources.

*Torymus* sp. are ectoparasitoids meaning they live and feed externally on their hosts and eventually kill them. These parasitoids also may be a hyper-parasitoid meaning they have the ability to attack galls that have already been parasitized by another parasitoid species or even another *Torymus*. *Torymus* sp. prefer mostly cushions and sometimes flats at all three sites. Both gall types have central larval placement and thick layers of fungal hyphae; yet, *Torymus* have long ovipositors that enable them to penetrate these gall layers and place their eggs atop the *A. carbonifera* larvae. This species has a relatively long ovipositor in which they insert into the periphery of galls and penetrate the central chambers (Weis 1983). Again, these parasitoids may be considered loose specialists since they mainly attack cushions. Cushions having the highest parasitism overall at two of the three sites may be due to high parasitism by *Torymus* sp. which are the most obvious parasitoids (ectoparasitoids) and easiest to identify.

For the most part, *Tetra* 2s attack crescents, cushions, and flats but tend to avoid irregulars. In this study, three morphologically similar species of parasitoids, *Baryscapus fumipennis*, *Aprostocetus tesserus*, and *Aprostocetus homeri*, were originally lumped into the collective group of Tetra 2 and this could affect interpretation of gall usage by these parasitoids. Crescents seem to be the most attacked morph overall by *Tetra* 2s. Crescent
galls are more abundant than cushions and flats; however, when crescents morphs wane over the season, *Tetra* 2s may begin attacking cushions and flats as the densities of these gall morphs increase. *Tetra* 1s strictly attack flats at GMP and BCW; however, crescents are their preferred morph at SSP even though they still attack some flats. Again, these parasitoids seem to be specialists at two of the sites. Green *Tetra* 2s also attack crescents at both GMP and BCW, but prefer cushions at SSP. They avoid irregulars at all sites. This could be because irregulars have peripheral larval placement whereas the other morphs have more of a central larval placement. Green *Tetra* 2 frequencies tend to be highly biased towards crescents, but this varies among sites, and in some cases, flats or cushions have relatively high frequencies of parasitism (Table 8, Figures 4d, 5d, 6d). For example, flats are their preference at GMP, but crescents are a very close second (Figure 4d). They possibly could have been crescent parasitoids; however, because other parasitoids strongly attack crescents, such as *Tetra* 2s and *Platygaster*, they may be exploiting other gall morphs to avoid competition or mortality from facultative hyperparasitoids like *Torymus* sp.

These parasitoids are somewhat specialized on certain gall morphs but some do attack the other gall morphs. Castelo & Corley (2010) mention that, in spatial density-dependence, a system with two or more hosts with overlapping niches, a switching generalist parasitoid could contribute towards a more stable population and allow species coexistence (Castelo & Corley 2010). The parasitoids attacking *A. carbonifera* appear as if they are mostly generalists, meaning they may promote coexistence of gall-morph populations of *A. carbonifera*.
Although these galls incur high mortality due to parasitism, some galls like irregualrs seem to evade parasitoid attack. A possibility here is that irregualrs have peripheral larval placement whereas the other morphs have central larval placement. It is possible that parasitoids have yet to adapt to the change in larval positioning and this is why crescents, cushions, and flats are attacked more frequently. The Enemy Hypothesis states that galls that better resist parasitoid pressures should be favored by selection (Bailey et al. 2009, Stone & Schönrogge 2003). Even with completion of this study, many questions remain like: Are irregualrs the ‘new wave’ of galls to avoid parasitoid attack? Are crescents the original gall morph that housed *A. carbonifera* and overtime became too easily accessible for parasitoids causing extinction of local communities? Are cushions and flats somewhere between the other two gall morphs in previous attempts to avoid parasitism? Irregualrs are one of the most abundant gall morphs, suffer the least parasitism of all gall morphs suggesting that they may be favored and have found some sort of enemy free space (Jeffries & Lawton 1984). Speciation within *A. carbonifera* will eventually promote changes within the parasitoids as well, if it has not already. In accordance with the EAR hypothesis, as herbivores transition to new ‘space’, interaction with higher trophic levels may also change (Nyman et al. 2007, Gratton & Welter 1999). Perhaps, eventually, *A. carbonifera* will develop another morphotype as a means to avoid parasitism which, in turn, will drive more changes in parasitism and these changes will create a wealth of biodiversity (Nyman et al. 2007, Gratton & Welter 1999).

Further research needs to be completed to understand how these parasitoids locate galls. First, definitive identifications of each of these parasitoids need to be done in order
to learn more about their behaviors. Also, behavioral studies of these parasitoids and their oviposition practices may help us better understand how galls are detected and parasitized. For example, collections of leaves bearing galls could be reared to eclosion and all parasitoids collected from these galls would be identified and offered all galls. Each parasitoid would be monitored to ascertain certain behaviors prior to and during oviposition. Also, galls of varying ages could also be offered to each parasitoid to determine whether the parasitoid oviposits in younger versus older galls.

In summary, *A. carbonifera* consists of several distinct lineages that coexist on a single host plant, *S. altissima*. The results here suggest that variation in gall traits influence parasitism, and thus parasitoids may be responsible for the divergent morphologies we observe in this species. It is unclear how genetic barriers arose – perhaps they arose with due to neutral genetic processes in allopatry or shifts to alternate host plants and subsequent recolonization. Regardless, these genetic barriers made it possible for the morphs to evolve along their own morphological trajectories in response to selection pressure by parasitoids. Thus, they may explain the “adaptive” part in adaptive radiation in *A. carbonifera*. Temporal density dependence and gall morph preference of parasitoids do not provide a strong enough pressure to promote changes in *Asteromyia* gall morphology and their coexistence, but there still may be other relationships that need to be studied to fully understand how this system diverged and is successfully coexisting. For example, the relationship between the midge and its associated fungus, *Botryosphaeria* sp., may have triggered the radiation of *A. carbonifera*; however, natural enemies may promote their coexistence. Parasitoid behavioral studies as well as understanding the symbiotic relationships between *A.*
carbonifera, Botryosphaeria sp., and S. altissima would provide further insight into this complex, remarkable system and allow us to grasp how adaptive radiation and species coexistence can occur in natural systems.
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Chapter 4

Summary

Although speciation is crucial to evolutionary biology, it remains one of the least understood processes still today (Schluter 2001). Speciation is an ongoing process that is often noticed after it has transpired; however, in order to better understand speciation, researchers have begun to examine adaptive radiations, or rapid ecological and evolutionary diversification of particular clades (Schluter 2000). Adaptive radiations balance the inclusion of new species and the evolution of evolutionary differences among them (Schluter 2000).

*Asteromyia carbonifera* offer an ecologically complex system that is currently undergoing an adaptive radiation. Resource utilization and competition, the forces which most studied adaptive radiations consider (Schluter 2000, Gavrilets and Losos 2009), are not apparent in this system. For example, all four gall morphologies of *A. carbonifera* exploit the same resource, *Solidago altissima*; more specifically, they reside on the same leaves of their host plant *S. altissima*. They do not necessarily compete for this resource because goldenrods are plentiful and most ramets are left ungalled. Furthermore, ecological theory advocates that two species sharing the same niche cannot coexist (Salomon et al. 2010); yet, these midges appear to be doing exactly that. Therefore, strong ecological forces may have influenced the divergence and sympatric coexistence of these midges. This system provides numerous, intricate relationships that can be used to study the means of which ecological speciation and coexistence could be occurring. Diverging morphologies of *A. carbonifera* galls are consistent with genetic evidence that
these midges represent distinct populations or races, but why are they diverging and, more importantly, how are they coexisting? Three possible isolating mechanisms, phenology, host-plant preference, and pressure from natural enemies were assessed in natural populations to determine if they may play a role in the recent divergence of *A. carbonifera* gall morph populations. Both phenological differences and host-plant genotype preference among the gall morphs are evident in this system; however, neither factor appears to be responsible for the separation of this species. My analyses of eclosion times provided no evidence of phenological differences among the galls in the field. Furthermore, I found that the emergence times of particular gall morphs were not strictly coordinated with one another. In other words, all types of gall morphs were present simultaneously and had sufficient opportunities to mate even though abundances of each of these gall morphs varied throughout the season. My observations as well as mtDNA and AFLP data (Stireman *et al.* 2008) suggest the existence of gene flow between the gall morph populations. Hybrid galls may be forming but may have reduced viability; for example, between the closely-related cushions and flats where selection for pre-zygotic isolation due to low hybrid viability is further reinforcing the divergence (Funk 2010, Santos *et al.* 2007). Many galls collected from the field were either empty or showed deceased early to mid larval stages of the midges without any distress to the gall itself. Perhaps, these were hybrid midges that lacked the capability to construct nutritionally-adequate galls or galls unable to cope with parasitoid pressure. Spatial differences also do not present a reproductive barrier to *A. carbonifera* populations. The results of this study concluded that some plant ramets are better suited for gall induction than others. All four gall morphs tended to exhibit a non-random, clumping, distribution
on certain members of *S. altissima*. In analyses of both plant ramets and genets, all galls were generally found in this clumped state suggesting that no spatial barriers exist for gene flow. Because *S. altissima* are rhizomatous, this non-random pattern of gall distribution suggests that certain plant genotypes are more suitable for gall creation whereas other plant genotypes are less preferred. Due to the host-plant preferences, divergence may have been possible with this avenue; however, all of the gall morphs seem to be reacting to host-plant preferences in the same manner.

I also examined whether pressure from natural enemies could promote divergence and coexistence in *A. carbonifera*. Hymenopteran parasitoids are the primary cause of mortality on these gall-making midges and can sometimes cause local extinctions of the gallers (Weis 1982). Gall morphs do suffer differing amounts of attack by parasitoids where cushions are the most attacked and irregulars are attacked the least. Parasitoids also display density-dependence at the site and plot levels and have preference over certain gall morphs than others. Perhaps, pressures on certain gall morphs are causing genetic differentiation in the gall morphs; or possibly, pressure from parasitoids are merely keeping *A. carbonifera* populations controlled.

Phenology, host-plant choice, and parasitoid pressures have been examined in this study to explore the divergence and coexistence of *A. carbonifera*. Although all of these ecological factors affect these midges in some way, I believe that these factors may have only been a small part of the divergence and coexistence in these midges. Further research into the symbiotic relationship with the fungus *Botryosphaeria dothidea* needs to be completed. A detailed investigation into how these two species rely upon one
another, how they may function devoid of one another, and how they came to coexist may fill in unanswered questions still remaining in the complex system of *Asteromyia carbonifera*. 
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Appendix A

Gall morph distribution versus Poisson distribution for GMP. The figures for crescents and irregulars are found in the text.
Gall morph distribution versus Poisson distribution for BCW.
Gall morph distribution versus Poisson distribution for SSP.
Appendix B

BCW Gall Frequency Distributions with Pearson’s Goodness of Fit Values-Plots. The figures are as follows: Crescents (a), cushions (b), flats (c), and irregulars (d).

a) Crescents: $P(>X^2) < 0.001$

b) Cushions: $P(>X^2) < 0.001$
c) Flats: \( P (X^2) < 0.001 \)

![Frequency of Flats among Plots](chart1.png)

Number of Flats

d) Irregulars: \( P (X^2) < 0.001 \)

![Frequency of Irregulars among Plots](chart2.png)

Number of Crescents
SSP Gall Frequency Distributions with Pearson’s Goodness of Fit Values-Plots. The figures are as follows: Crescents (a), cushions (b), flats (c), and irregulars (d).

a) Crescents: $P(>X^2) < 0.001$

![Crescents plot](image)

b) Cushions: $P(>X^2) < 0.001$

![Cushions plot](image)
c) Flats: $P(>X^2) < 0.001$

d) Irregulars: $P(>X^2) < 0.001$