ASSESSING THE DRIVERS OF ADAPTIVE RADIATION IN A COMPLEX OF GALL MIDGES: A MULTITROPHIC PERSPECTIVE ON ECOLOGICAL SPECIATION

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ABSTRACT


Natural enemies as selective forces maintaining and shaping morphological, physiological, and behavioral divergence in adaptive radiations have received very little attention. Until recently, the focus has been on primary resource competition as a major driver of trait divergence in adaptive radiations. Here I consider the role of natural enemies specifically in driving trait divergence in a complex of gall midges in the nominal species Asteromyia carbonifera (Diptera: Cecidomyiidae), which appears to be in the throes of an incipient adaptive radiation on its host plant, goldenrod (Solidago). This galler uses a symbiotic fungus (Botryosphaeria dothidae) as a food source and as the major structural component of its gall. Use of this symbiont may be the key innovation that allowed colonization of its host plant and began the process of adaptive radiation in this system. Overall, I find strong evidence that the extended phenotype of these gall midges (i.e., the gall) is experiencing stabilizing, directional, and diversifying selection imposed by natural enemies. I also find that natural enemies appear to be driving divergence in an ovipositional phenotype that may represent a major split in the evolution of these lineages. Furthermore, sequestration or de novo biosynthesis of carotenoids by these midges may provide the precursors that allow gall proliferation, but may also be costly because of the potential attraction of natural enemies. Finally, I find that the host plant has little effect on gall traits, but host plant defenses (e.g., sesquiterpenoids) may limit the general density of galling insects. I conclude that divergence in gall
morphology and ovipositional behaviors are, at least in part, determined by natural
selection imposed by natural enemies and that these forces may help explain the diversity
of gall morphologies and habits found in nature.
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1 INTRODUCTION

1.1 ADAPTIVE RADIATION AND ECOLOGICAL SPECIATION

Adaptive radiation is “…the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage” (Schluter 2000 p. 10). To date, most of the adaptive radiations that have been identified have strong ecological components. In Darwin’s Galapagos finches, character displacement centered on beak morphology has allowed the finches to exploit various niches associated with seed size and hardness (Grant and Grant 2002). In the African cichlid fishes, niche differentiation and subsequent speciation has been facilitated by flexibility in behavior and the pharyngeal jaw apparatus (Hulsey et al. 2006). This adaptive radiation has been promoted by sexual selection on different color morphotypes (Seehausen et al. 1997). The Hawaiian silverswords have colonized nearly every conceivable plant niche in the Hawaiian Islands (Carr 1985). This radiation has been promoted by key innovations in plant structure, morphology, and physiology coupled with a lack of competition.

Ecological opportunity is a recurring theme in adaptive radiations. Ecological opportunity is generally provided by a sudden decrease in competition or by the appearance of a key innovation that opens up otherwise unavailable niches. The rapid diversification of the mammals at the end of the Cretaceous period was probably accompanied by the availability of open niches left by the extinction of the dinosaurs. Long-distance dispersing organisms may frequently find their new habitat devoid of
predators and ripe with exploitable resources. It is likely that a combination of key innovation and release from direct and indirect competition have both played a significant role in driving adaptive radiations (Figure 1.1).

The known and potential driving forces of adaptive radiation and ecological speciation are illustrated in Figure 1.1, which are not mutually exclusive processes and may only be divided semantically by the number of new lineages produced. Adaptive radiations generally involve a great number of new species, while ecological speciation may only involve the splitting of a single species (e.g., *R. pomonella* on apples and hawthorns, see below). Generally, adaptive radiation is thought to begin with colonization of an untapped resource (i.e., ecological opportunity) which may be facilitated by a key innovation or may drive the evolution of such an innovation (Figure 1.1). This puts the evolving lineage in a new environment, which further drives a wedge between the progenitor and sibling lineages. Primary resource competition begins to maintain this genetic divergence and additional selective forces further facilitate the evolution of distinct lineages through pleiotropy or genetic linkage. An example of such a force would be sexual selection on a “magic trait” (MacColl 2009, Servedio et al. 2011). Magic traits provide a selective advantage with respect to natural selection, while simultaneously signaling to mates the presence of this trait allowing sexual selection to act on it as well. However, the role of natural enemies in this whole process has been given very little attention (orange ellipse in Figure 1.1). I have called this potential process secondary resource competition, which refers to the acquisition of enemy free space and the process of apparent competition. If organisms share enemies there exists
the potential for indirect competition because population increases of one prey item will ultimately produce more enemies available to attack the other prey item. In traditional exclusion experiments this process would mirror the results expected from primary competition and therefore it is called apparent competition (Holt 1977, Abrams and Chen 2002).

The criteria for classifying a radiation as adaptive are (1) common ancestry of the component lineages, (2) phenotype-environment correlation, (3) a fitness advantage associated with the phenotypes (also known as trait utility), and (4) rapid speciation (Schluter 2000). These criteria set adaptive radiations apart from other non-adaptive evolutionary processes, such as speciation associated solely with geographical vicariant events, polyploidy, and genetic drift (Schluter 2009). However, these processes undoubtedly also contribute to adaptive radiation and ecological speciation. When environmental differentiation is absent, but the radiation is rapid, it is considered to be a non-adaptive radiation (e.g., Albinaria snails, Gittenberger 1991).

Schluter (2000) argues that even cases with apparent environmental differentiation may not be considered adaptive radiations if there is a lack of morphological or physiological characters associated with an increase in fitness in those environments. Clearly, this narrow view of adaptive radiation eliminates the role of behavioral modifications in spurring this process. I see no reason why the behavior of a particular lineage should be considered non-adaptive. In the early stages of an adaptive radiation, behavioral modifications may provide enough reproductive isolation for subsequent morphological and physiological adaptations to arise and spread, especially in
sympatry (see Chapter 3). It could be argued that the proximate reason for the evolution of the two host races of the apple maggot fly, *R. pomonella*, was host preference via associative learning (Prokopy et al. 1982). This behavioral modification may have led to the subsequent differentiation in diapause phenology that ultimately reduced gene flow between the sympatric host races (Dambroski and Feder 2007). A similar argument can be made for the host races of *Eurosta solidaginis* on *Solidago altissima* and *S. gigantea* (Waring et al. 1990, Craig et al. 1993, Brown et al. 1996).

Figure 1.1  A schematic diagram of adaptive radiation incorporating the known and potential selective forces directly or indirectly responsible for the process. The orange ellipse represents the potential effects of apparent competition and the focus of the current work.
The current work focuses on evaluating an apparent incipient adaptive radiation of gall midges in the *Asteromyia carbonifera* (Osten Sacken) complex. It evaluates the importance of mutualisms and behavioral, physiological, and morphological modifications in this process. The diversification of several groups of gall forming midges may represent adaptive radiations (Joy and Crespi 2007) including the genus *Asteromyia*. Crego et al. (1990) and Stireman et al. (Stireman et al. 2008, Stireman et al. 2010, Stireman et al. 2012) have provided strong genetic evidence of cryptic species hidden within *A. carbonifera*. Stireman et al. (2008) identified four relatively distinct clades associated with different gall morphologies that appear to represent four cryptic species (Figure 1.2). These all occur on *S. altissima* and often on the same individual plant or even leaf (pers. observ.). Stireman et al. (2010) have suggested that this genus as a whole may be quite young and that cryptic species exist on different host plant species. Weis (1983) suggested that fitness varied with clutch size on *S. altissima*. However at the time, he was not aware of the various morphotypes of *A. carbonifera* or the level of genetic structure (Figure 1.2).

Several years of data on parasitism rates (Wells et al., unpublished) have suggested that multiple peaks may exist in the adaptive landscape of *A. carbonifera* on *S. altissima*. The tops of these “peaks” may differ little in overall fitness, but nevertheless minimize indirect competition. That is, parasitism rates among the four morphotypes differ.
depending on which of the parasitoids is considered, but overall parasitism rates vary much less.

The primary goal of this project is to understand the pattern and process of diversification in this system within the framework of the modern concept of adaptive radiation. Specifically, I will examine phenotype-environment correlations and trait utility of the four morphotypes. A. carbonifera is one of many groups within Cecidomyiidae that may help shed light on the mechanisms that drive adaptive radiation, an evolutionary phenomenon that appears to be as dependent on biological diversity as it is responsible for creating it.

1.2 THE STUDY SYSTEM

Cecidomyiids are a diverse family of some 6000 species belonging to the primitive fly infraorder Bibionomorpha (sister group to the “higher” flies) and the superorder Sciaroidea (fungus gnats). The adults are small and delicate and some such as the Hessian fly (Mayetola destructor) cause large losses of crop yield and economic productivity worldwide (Harris et al. 2003). Gall-forming has evolved independently several times, and like most galling insects, cecidomyiids tend to be host plant specialists (Gagné 1989, Raman et al. 2005, Joy and Crespi 2007, Dorchin et al. 2009), but they display an unusually broad-range of feeding-modes. In addition to herbivory, some are predators, and many species have specialized interactions with fungi, as do most of their close relatives in their superfamily Sciaroidea, which includes the fungus gnats. Stem groups of Sciaroidea have been found preserved in amber as far back as the Jurassic but probably diversified during the Cretaceous about 125 to 80 Ma (Blagoderov and Grimaldi
“Ambrosia” gallers are species that derive nutrition or shelter from host plants with the aid of a mutualistic fungus, not unlike some bark beetles or even attine ants. All midges are liquid feeders, lack chewing mouthparts, and digest their food extra-orally by secretion of digestive enzymes and other products (Mamaev 1975, Gagné 1989). Evolutionary change in the digestive system of midges has been reductive, while salivary glands are correspondingly more complex, enlarged and transcriptionally-active (Stuart and Hatchett 1987). In some species, these glands produce the silk used to form cocoons, or for predatory species, the venoms used to paralyze prey.

The North American genus, Asteromyia, is a complex of eight mostly North American species on composites. A. carbonifera forms “ambrosia galls” on goldenrod (Solidago spp.) with the aid of Botryosphaeria dothidea (Ascomycota: Dothideomycetes), a phytopathogen whose anamorphs are found worldwide on monocots, dicots and gymnosperms (Gagné 1968, Weis 1982a, Weis 1982b, Figure 1.3. The life cycle of A. carbonifera and its fungal mutualist. 1. B. dothidea is a free-living plant pathogen. 2. Gall midge females presumably acquire fungal conidia and vector them to the plant. However, fungi may be present on the plant prior to the gall midge. 3. Mated females lay eggs, wounding the plant, and fungal hyphae invade the wound, overgrowing the developing larvae in the process. 4. Larva feed within the fungal mat, which assume different morphologies, depending on the genotype of the gall midge within (inset: two representative examples of gall morphs). 5. After eclosion, adults emerge, mate, and seek out new host plants.
Weis et al. 1983, Weis 1983, Borkent and Bissett 1985, Bissett and Borkent 1988, Gagné 1989, Crous et al. 2006, Schoch et al. 2009). Like other gall midges, Asteromyia is attacked by a diverse array of hymenopteran parasitoids, and parasitism rates can be high (30-50% or higher).

Interestingly, interactions with parasitoids appear to be mediated by a polymorphism in gall morphology. Weis (1982a) showed that parasitism by the specialist parasitoid Torymus capite was dramatically reduced by the development of hard, fungal stromata in mature galls. Discrete polymorphisms in gall thickness and other traits (“morphotypes”) are commonly found on a single host plant (Figures 1.2 and 1.3). And different combinations of morphs can be found across many hosts in the goldenrod genus Solidago (Stireman et al. 2012). The purpose of the different fungal morphologies appears to be related to parasitoid attack as parasitoids have different length ovipositors that may vary in their ability to reach and/or ovipuncture sessile larvae (Weis 1982b, Jeffries and Lawton 1984).

One might expect that these different fungal morphs correspond to reproductively-isolated fungi (e.g., Bultman et al. 2011); however, there is no evidence of specialization by the fungus itself: all morphs of A. carbonifera, and probably all species in the genus, utilize the same fungus, indicating that fungal gall morphology is an “extended phenotype” of gall midges (Janson et al. 2010). Furthermore, other genera also appear to use the same fungus (Adair et al. 2009). In essence, A. carbonifera appear to be fungal architects which transform the fungal mycelia into chambers that provide
varying degrees of protection from natural enemies and environmental factors. However, evidence for this conjecture is limited.

1.3 OUTLINE AND OBJECTIVES

The objectives of this work were four-fold. First, was to characterize the nature of the symbiosis Asteromyia has with its obligate fungal partner, Botryosphaeria dothidea. This acquisition may have been the major key innovation that allowed colonization of the host-plants. Second was to begin to explore the functional significance of carotenoid sequestration and biosynthesis in A. carbonifera and insects in general in order to evaluate the potential role carotenoids may have played in this adaptive radiation. Third was to understand the distribution of chemical defenses in S. altissima to begin to explain the patterns of within plant differences in galling insect attack. The final goal was to assess the potential of natural enemies as a major driving force of adaptive radiation. The second chapter addresses the first objective as well as the primary source of the fungal conidia collected by the midges. The third chapter addresses a newly discovered ovipositional phenotype associated with one of the morphotypes and its relationship to carotenoid sequestration. The fourth chapter reviews the known physiological and ecological roles that carotenoids have in insects with the goal of finding potential benefits that must be present to offset their apparent costs found in chapter three. The fifth chapter addresses plant defense strategies with the goal of understanding additional costs associated with the behavioral phenotype found in chapter three. Finally, selection gradients are generated to evaluate the role that natural enemies
may play in shaping and maintaining gall morphology, which itself is hypothesized to be under the proximate control of carotenoids and (or) their derivatives.
1.4 REFERENCES


Borkent A, Bissett J (1985) Gall midges (Diptera: Cecidomyiidae) are vectors of their fungal symbionts. Symbiosis 1:185-194


Weis AE (1982a) Use of a symbiotic fungus by the gall maker *Asteromyia carbonifera* to inhibit attack by the parasitoid *Torymus capite*. Ecology 63:1602-1605

DISSECTING THE ASSOCIATION BETWEEN A GALL MIDGE, *ASTEROMYIA CARBONIFERA*, AND ITS SYMBIOTIC FUNGUS, *BOTRYOSPHAERIA DOTHIDEA*

2.1 ABSTRACT

The Ambrosia gall midge [*Asteromyia carbonifera* (Osten Sacken) (Diptera: Cecidomyiidae: Alycaulini)] consists, in part, of a complex of genetically differentiated populations that have diverged in gall morphology on the host plant *Solidago altissima* L. (Asteraceae). This divergence appears to be an incipient adaptive radiation that may be driven by parasitoid pressure. Understanding the mechanisms driving this genetic and phenotypic diversification requires a close examination of the relationship between the midge and its fungal associate *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (Ascomycota: Dothideomycetes), whose mycelia actually form the protective gall structure. I used manipulative experiments to test the degree of interdependency of the fungus and the midge, and I employed field and laboratory studies to gain insight into the source of fungal conidia, which my data and observations indicate are collected by females and stored in specialized pockets (mycangia) on the ovipositor. Manipulative experiments demonstrate that fungal proliferation on the host plant is dependent on the midge larvae and larvae exhibit significant growth on the fungus alone. Field observations and experiments were unable to identify the source of mycangial conidia; however, analyses of conidia shape suggest a biotrophic source. I conclude that this association is an obligatory mutualism with respect to successful gall formation. These
findings corroborate recent findings that the primary food source of the midge is the gall fungus.

2.2 INTRODUCTION

Mutualistic associations with microbes have likely played an important role in the phenomenal evolutionary and ecological success of the Insecta (Moran, 2002; Janson et al., 2008). Virtually every insect species that has been examined closely has been found to be engaged in some form of microbial mutualism, most frequently in the form of gut-associated bacteria (e.g., Buchner, 1965; Douglas, 1998). However, insect-fungal symbioses are also widespread across many insect groups including beetles (Scolytinae: Bentz & Six 2006), ants (Formicidae; Mikheyev et al., 2006), moths (Tortricidae: Fermaud & Le Menn, 1989), and flies (Cecidomyiidae: Borkent & Bisset, 1985; Gagné, 1989; Anthomyiidae: Schiestl et al., 2006). In these associations, the insect typically benefits from using the fungus as a food source (leafcutter ants: Cherrett et al., 1989; gall midges: Bisset & Borkent, 1988; Ambrosia beetles: Farrell & Sequeira 2001) in exchange for dispersing the fungus or promoting fungal outcrossing (Schiestl et al., 2006).

One of the most diverse and widespread groups of insects known to engage in symbiotic associations with fungi are the ‘Ambrosia’ gall midges, which represent a significant portion of the family Cecidomyiidae. The galls that these midges induce on their host plants are typically lined internally with fungal hyphae, which the developing larva(e) may feed upon (Haridass, 1987; Bisset & Borkent, 1988). Borkent & Bissett (1985) have provided tantalizing morphological evidence that at least some of these species actively transport fungi in specialized pockets, or mycangia, associated with the
terminal abdominal segments. However, the nature of the Ambrosia midge-fungus interaction is largely speculative and there has been little experimental analysis of the association and the degree to which it represents an obligate mutualism. In fact, it has been argued that at least in some cases the fungi may represent opportunistic colonization rather than a strict mutualism. This controversy has been thoroughly reviewed (Haridass, 1987; Rohfritsch, 2008; Adair et al., 2009). Here, I employ observational and manipulative experiments to dissect the association between the gall midge Asteromyia carbonifera (Osten Sacken) (Diptera: Cecidomyiidae: Alycaulini) and its associated fungus Botryosphaeria dothidea (Moug.) Ces. & De Not. (Ascomycota: Dothideomycetes) in order to classify this symbiosis and understand its consequences for the ecology and evolution of the gall midge.

The galls induced by A. carbonifera and its associated fungus on host plants in the genus Solidago (goldenrod; Asteraceae) were observed over a century ago (Trelease, 1884; Batra, 1964). However, despite the efforts of several researchers (e.g., Batra, 1964; Gagné, 1968; Weis, 1982a,b; Bisset & Borkent, 1988), the biology of A. carbonifera is still poorly understood. Populations of A. carbonifera use a wide range of goldenrod species as hosts (Gagné, 1968), and preliminary analysis indicates that many of these populations exhibit host associated genetic differentiation (Stireman et al., 2010). Furthermore, within the single host plant Solidago altissima L., at least four genetically distinct gall morphotypes coexist, suggesting that A. carbonifera is adaptively radiating on this host (Crego et al., 1990; Stireman et al., 2008).

Understanding the details of the interaction between A. carbonifera and its fungal associate is likely key to understanding the adaptive diversification in this system.
Borkent & Bissett (1985) mentioned that *A. carbonifera* transport fungal conidia, but only supplied photographs of the mycangia of *A. tumifica* and related species. Gagné (1968) attempted to initiate *A. carbonifera* galls in screen cages. Galls developed in his experiment, but I suspect that there were eggs already on the plants before they were caged. In fact, Gagné (1968, p. 13) does not reject this possibility when he states, ‘It is possible also that…some newly laid eggs were overlooked.’ Weis (1982a,b) clearly illustrated that larvae are variously protected from parasitoid attack by the fungal stroma and *A. carbonifera* has never been found in plant tissue not associated with the fungus (Trelease, 1884; Batra, 1964); nevertheless, some still question whether this association represents a mutualism. Therefore, the main goal of this study was to investigate the nature of this intimate association between the midge and the fungus. That is, is it truly a mutualism? If so, is it an obligatory relationship?

In particular, I was interested in (1) verifying earlier claims that adult females harbor fungal conidia in specialized structures on their ovipositor called mycangia and whether these conidia are present on eggs, (2) determining whether the midge is necessary for fungal growth and vice versa, (3) initiating galls under controlled conditions, (4) understanding where midges mate and obtain fungal conidia, and (5) determining the time required for fungus to appear on the top of the leaf after oviposition has occurred.

### 2.2.1 Study system

*Asteromyia carbonifera* is involved in complex interactions involving many interacting
species. The four most prominent players are goldenrod (*Solidago* spp.), the gall midge *A. carbonifera*, the fungus *B. dothidea* (Bisset & Borkent, 1988; Janson et al., in press), and at least nine associated parasitoids, predators, and inquilines. Together, the fungus and midge larva produce at least four morphologically distinct galls on the leaves of *S. altissima*. Crego et al. (1990) described these four gall morphotypes on *S. altissima*. These were named crescents, flats, irregulars, and cushions in accordance with their overall morphology. Stireman et al. (2008) provided photographs and descriptions of these morphotypes. These galls are not deformations of plant tissue, but rather a gall formed mainly of fungus. Aside from chlorosis, the gall causes very little physical change at the cellular level of the plant (Camp, 1981). Other *Asteromyia* spp. also form similar Ambrosia galls on related host plants (Gagné, 1968, 1989; Stireman et al., 2010) and many *Asphondylia* spp. (Diptera: Cecidomyiidae) harbor the same fungal species (Bisset & Borkent, 1988; Adair et al., 2009); therefore, understanding this symbiosis in *A. carbonifera* may provide insight concerning other potential adaptive radiations.

### 2.3 MATERIALS AND METHODS

#### 2.3.1 Biology and life history traits

Unless otherwise specified, the biology and life history traits were generally inferred from field observations at all the field sites (Table 2.1) over the summers of 2007-2009. To investigate the time for fungus to appear on the top of the leaves, *A. carbonifera* egg clutches were located on the most susceptible *S. altissima* clone in a common garden and marked (see subsequent section for details on the common garden). These clutches were
checked daily until at least one position within the clutch began to show fungal growth on the top of the leaf (i.e., when the growth was about 0.3 mm in diameter). The leaf the eggs were found on was given an estimate of age in days between 0 and 3. The plants grow about 1 cm day\(^{-1}\) and the distance between pairs of leaves is roughly 1 cm at the top of the plant. Age 0 leaves were on the outside of the whorl and had not dropped to a horizontal position. Age 1 leaves were still connected to the whorl, but were horizontal. Age 2 and 3 leaves were one and two nodes lower than the base of the whorl, respectively. Eggs were only found on age 0-3 leaves, but only the new growth was searched. Females were observed laying eggs in three instances in the field and these eggs were tracked as above. These clutches were followed until they formed mature, identifiable morphotypes (clutch sample sizes per morph: cushion: 22, flat: 4, irregular: 13, crescents: and 0, unknown: 1). A linear model with leaf age as a covariate was used to test for differences between the morphotypes in the time for fungus to appear on the top of the leaf.

2.3.2 Nature of the midge-fungus interaction

Two types of experiments were conducted to test the interdependency of the midge larva and fungus. The first experiment tested whether the growth of the gall fungus depends on the presence of the midge larva by removing larvae from very young galls and assessing fungal growth. The second experiment tested whether the midge larva feeds on the fungus by isolating the larva and fungus on growth media and tracking larval growth.

To test whether gall fungal growth is dependent on the presence of the midge larva, the larvae from very young galls were removed and fungal growth measured. *Solidago*
altissima stems with very young blister galls (<2 mm in diameter) were collected from the Beavercreek Wildlife Management Area (BCWMA) site (Table 2.1). The stems were re-cut under water in the field and immediately placed in a container of cut-plant solution (Aquaplus; Syndicate Sales, Kokomo, IN, USA). On sunny days stems were placed in the shade during collection. The galls on the stems were randomly processed in the laboratory as negative controls, mocks, or removals. Controls were left untouched and intact. Mock removals (‘mocks’) controlled for possible confounding effects of the removal process; galls were dissected from the bottom of the leaf to the removal point and the larva was either touched with the forceps as if it was going to be removed or not touched. The removal treatment was conducted in the same manner as the mock treatment, but the larva was permanently removed from the gall. In both mocks and removals the disturbed fungal layer and leaf tissue was carefully placed back in its original position. These treatments were repeated in five independent experiments with a balanced sample size within each experiment (n = 6-9 per treatment). All galls were photographed with identical camera and magnification settings at the time of processing and after 10 days of incubation at room temperature. Fungal growth before and after incubation was measured in pixels in Photoshop (version 9.0), converted to net fungal growth per day, and standardized to the number of larvae present in the final gall. Standardization was necessary because during the initial stages of gall development each larva initiates a separate tiny gall. If the initial galls are close enough they will eventually merge leading to the appearance of a single large gall. In some cases immature galls processed as negative controls had neighboring galls close enough that they eventually merged leading to the need to standardize net fungal growth to the number of larvae
To test whether the midge larva feeds on the fungus, immature galls were collected from the BCWMA site, dissected, and a small portion (ca. 10 mm³) of black fungal stroma was transferred to malt-extract agar plates along with a young larva (n = 18), which was placed atop the portion of stroma. Negative controls without fungal transfer were also included (n = 17). The length of larvae was measured with an ocular micrometer mounted on a Nikon SMZ1000 stereomicroscope (Nikon Instruments, Melville, NY, USA) on the day of transfer and after 2 weeks of incubation at room temperature.

Preliminary experiments had shown that larvae grew significantly larger when transferred with the fungus as above, but the growth was marginal (i.e., a mean increase over the control of only 51 µm). Furthermore, in preliminary experiments most of the control plates were also contaminated with gall fungus. Therefore, about 5 mg of medical grade Nystatin ointment (a fungicide, 100 000 USP g⁻¹; E. Fougera & Co, Melville, NY, USA) was applied to a 1-cm² area of both the treatment and control plates. The larva and fungus or larva alone (in the case of controls) was transferred atop this paste. The paste completely prevented fungal contamination in the controls and substantially reduced fungal growth in the treatments. The paste did not appear to affect the behavior or survival of the transferred larvae.

A linear model in R (version 2.8.1; R Development Core Team, 2007) with ‘larval end length’ as the response and ‘start length’ as a covariate was used to determine fungal treatment effects. The full model included the following explanatory variables: larval start length, fungal treatment (gall fungus added or not), and their interaction.
2.3.3 Asteromyia eclosion behavior

To understand the reproductive behavior and to gain insight into where and how the adults obtain fungal conidia, mature irregular galls on S. altissima (SC-2 site; Table 2.1) and rugosa galls on Solidago rugosa P. Mill. (SC-1 site; Table 2.1) were marked and monitored daily in the field from 10 July to 7 August 2007 (see Gagné, 1968, for a description of S. rugosa-type galls). As adults emerged from the galls, their behavior was recorded until they either flew off or otherwise became unobservable. On average, they were observed for 2.5 h. Ethograms were generated from these data and the relative frequency of the transition between behaviors calculated by dividing the number of times a transition from behavior x to y occurred by the total number of behavioral transitions.

2.3.4 Conidia morphology

To gain insight into where fungal conidia are obtained, I compared the shape of egg-associated conidia to conidia obtained from other sources. Eggs of presumably different morphotypes were collected from the BCWMA and Koogler Wetland Preserve (KWP) sites (Table 2.1), mounted on microscope slides in EMD™ lactophenol cotton blue (Fisher Scientific, Pittsburgh, PA, USA), and photographed at 400× with a Nikon Coolpix 8800 VR camera mounted on a Nikon Optiphot compound microscope. The width and length of the conidia were measured from photographs in ImageJ (version 1.39u; National Institute of Health) after calibration with a photograph of a stage micrometer (2 mm, ruled to 0.01 mm; Micromaster, Fisher Scientific, Hampton, NH, USA). The length and width of these egg-associated conidia were compared to conidia from six other sources: (1) gall-isolated fungus grown at room temperature on oatmeal
agar under cool white fluorescent lights (six each, Philips, F40T12/CW Plus, 40-W bulbs, suspended 60 cm above the plates), (2) gall-isolated fungus grown on fresh-cut autoclaved goldenrod stems placed on the surface of water agar plates, (3) egg-conidia isolates grown on oatmeal agar, (4) egg-conidia isolates grown on fresh-cut autoclaved goldenrod stems on water agar, (5) conidia collected from pycnidia found on field-collected *S. altissima* stems, or (6) conidia found in the mycangia of malaise-trapped adult *A. carbonifera* females from the HS site (Table 2.1). The gall and egg-conidia isolates (1-4 above) were all grown at the same time under the same conditions in a randomized complete block design. Two linear ANCOVA (analysis of covariance) models with ‘width’ as a covariate were used to test for significant differences in conidia length (R, version 2.8.1; R Development Core Team, 2007). The standardized residuals from these models were roughly normal, distributed mostly between the mean ± 2 standard deviations, and appeared randomly associated with the fitted values. The first model included three explanatory variables: (a) fungal isolate source (i.e., egg-conidia isolate or gall-isolate), (b) growth media (i.e., fresh-cut *S. altissima* stems on water agar or oatmeal agar only), and (c) width as a covariate; plus (d) all the two- and three-way interactions. This model included only the first four treatments (1-4 above). The second model included all seven conidia sources with only two explanatory variables (i.e., width as a covariate, conidia source, and their interaction).

### 2.3.5 Gall initiation in field plots

Two experiments were conducted in an attempt to initiate galls on *S. altissima* accessions grown up in the greenhouse and transplanted to a common garden (WSU-1; Table 2.1).
Solidago altissima rhizomes were collected from three sites [BCWMA, KWP, and Varner Road (VS) sites; Table 2.1] in early April and 10 clones of 10 source plants were started in a greenhouse and later transplanted to the common garden. Large conical tomato cages were placed over half of the plants in the field plot (n = 50, five replications of each accession). The cages were covered with fine-mesh sleeves (194 holes cm\(^2\)) and buried about 10 cm deep. Mature galls were collected from S. altissima from various field sites on 9 July 2008, separated by morphotype, and placed on the ground inside each of 40 cages (10 plants were controls) in the same morphotype proportion as the total collected. Each cage received 44 irregular, seven crescent, five cushion, and four flat galls. Recently cut dried goldenrod stems, old goldenrod stems (previous year’s growth), and extraneous ground litter was cut into 10-cm sections and placed in the bottom of each of the cages. It was thought that this debris might provide a source of fungal conidia for emerging adult females to collect. The caged plants were checked periodically for the formation of new galls and the presence of emerging adults. After 24 days all plants were cut to 30 cm and checked thoroughly for the presence of galls.

In a second experiment, a subset (n = 20) of the same caged plants were allowed to re-grow for 17 days and then mature galls were added to the cages as above (23 irregular, 16 cushion, 11 crescent, and six flat galled leaves per cage) on 19 August 2008, but covered with freshly cut hay. To each of these cages was added a single S. altissima stem obviously infected with a pycnidia-forming fungus (presumably Botryosphaeria spec.) collected from the BCWMA site. Each infected stem was placed in a bottle of cut-plant solution, which was not allowed to go dry. The plants were monitored periodically for the formation of galls and after 29 days all the plants were cut to 30 cm and the cut stems
checked thoroughly for galls.

2.3.6 Gall initiation in screen tents

Two experiments were conducted in an attempt to initiate galls on *S. altissima* accessions grown up in the greenhouse and moved to outdoor screen tents (WSU-2 site; Table 2.1). Thirty goldenrod accessions (presumably different genotypes), 14 of which were the same as those in the field plot experiments, were divided equally and placed in each of two 1.8 × 1.8 × 1.8-m screen tents with the floor covered with black plastic (n = 72 potted plants per tent). The tents were set up adjacent to one another and the plants allowed to stand for 8 days to ensure no galls were initiated during transport from the greenhouse to the screen tents. One tent (treated) had the floor covered with old goldenrod stems (previous year’s growth) collected from a field site. The other tent was a negative control. To provide gall-initiating adults, each tent was randomly supplied with 48 *S. altissima* stems infested with a mixture of mature galls in a 20-l bucket filled with cut-plant solution on 2 July 2008. During collection of these stems, any pupal exuvia found lodged in the galls were removed. The plants were checked periodically for galls and finally after 23 days the plants were cut to 30 cm and the stems and leaves checked thoroughly for galls. During these periodic checks many adult midges were seen alighted on the screening in both tents. The total number of exuvia on the initiating cut-stem galls was tallied to reveal that at least 111 irregular, zero cushion, zero crescent, and one flat adult had emerged in the treated tent. In the control tent at least, 107 irregular, four cushion, two crescent, and zero flat adults emerged.

In a second experiment started on 13 August 2008, the same set of plants and tents
were used as above, but different fungal sources were added to each tent. To one tent were added three 40-l plastic tubs (15 cm deep) filled with a plant-fungus-topsoil mixture. Holes were drilled in the plastic covers and they were elevated 15 cm above the tubs to provide shade, but allow rain water and female access to the soil surface. The soil in each of the tubs contained a mixture of 70 fresh *S. altissima* stems cut to about 5 cm long, the agar from 30 oatmeal-agar plates (100 × 15 mm) of gall-isolated cultured fungus, three handfuls of triple-ground mulch, and about 10 l of old dried goldenrod leaves. This mixture was allowed to stand for 2 weeks before the experiment started. The second tent was supplied with 54 cut *S. altissima* stems infested with pycnidia-producing fungus (presumably *Botryosphaeria* spec.) and each tent was also supplied with 100 gall-infested *S. altissima* cut stems. All cut stems were kept in 20-l buckets of cut-plant solution. Periodically and after 48 days the plants were checked thoroughly for galls. The total number of exuvia found on the cut stems was tallied to reveal that at least 29 cushion, four crescent, five flat, and nine irregular adults had emerged in the plastic-tub treated tent, and 13 cushion, four crescent, four flat, and one irregular adult had emerged in the fungus-infested-cut-stem tent.

### 2.4 RESULTS

#### 2.4.1 Biology and life history

The eggs of *A. carbonifera* are laid on the underside of the leaf in the vicinity of the meristem (Figure 2.1B). Females have up to 300 eggs at the time of emergence and fecundity appears to differ by morphotype (JJ Heath, unpubl.). The larvae (Figure 2.1C)
hatch and the fungal spores germinate (Figure 2.3) within a few days of oviposition and begin to burrow/grow into the leaf tissue within a few millimetres of the ovipositional site. Once the larva has penetrated the leaf tissue, fungal growth becomes evident on the bottom and then the top of the leaf. The mean time for fungus to appear on the top of the leaf was 6.7, 8.0, and 8.2 days for cushions, flats, and irregulars, respectively. The linear model with leaf age the eggs were found on as a covariate provided estimates of the time for fungus to appear on the top of the leaf, assuming oviposition occurred on zero-aged leaves. This linear model indicated that there was a significant interaction between the age of the leaf oviposited on and the time for fungus to appear on the top of the leaf as well as significant main effects of leaf age and morphotype. The intercepts from this model were 8.6, 8.0, and 7.6 days for cushions, flats, and irregulars, respectively.

Adults (Figure 2.1A) emerge from the galls approximately 3 weeks after fungal growth is evident on the top of the leaf. The entire life cycle from egg to adult is about 4-5 weeks. Galls are unisexual with only rare instances of galls with mixed sexes; this is consistent with the findings reported by Weis et al. (1983) for this species and for cecidomyiids in general. In Ohio, larvae may begin to enter diapause as early as the 1st week of September, but galls continue to be initiated as late as the 1st week of October. *Solidago rugosa* galls marked in the field (SC-1 site) in July and collected in December the same year still had late instars within them, indicating a very early initiation of diapause in some morphotypes. However, the physiology of these larvae may have been altered by undetected parasitoids. Larvae pupate in the spring and new galls can be seen forming in late May, but larger populations are not realized until mid to late June in Ohio. The timing and details of certain aspects of their biology may vary with gall morphotype.
For instance, controlled experiments indicate that crescents oviposit on mature tissue (see Chapter 3).

### 2.4.2 Asteromyia-fungus interdependency

In the experimental trials designed to test whether fungal growth and gall development is dependent on the midge larvae, I found that the galls in which the larva was removed ceased to grow (Figure 2.4), whereas the mock removals and controls were unaffected and continued to develop normally (Figure 2.4). However, in those trials where the larvae were touched with forceps during the mock removals the larva often died, causing these galls to cease development (Figure 2.4). Dissection of the galls at the end of the experiment revealed that all the galls that failed to develop contained dead larvae, whereas developing galls had healthy larvae.

*Asteromyia* larvae that were dissected from galls and placed on agar plates with a portion of their gall fungus (with no host-plant material) grew more than controls with no fungal inoculation ($F_{1,20} = 5.80, P = 0.026$; Figure 2.6). In preliminary experiments, some of the larvae appeared to form gall-like structures on the agar plates (Figure 2.5A-C). As expected, the difference between the treatments was smaller when the larvae were larger at the beginning of the experiment (i.e., a significant interaction between start length and treatment: $F_{1,20} = 6.60, P = 0.018$; Figure 2.6). Some larvae died and became completely deteriorated making a final measurement impossible; therefore, the sample sizes decreased (larva and fungus transferred, $n = 9$; controls, $n = 15$).
2.4.3 Adult behavior

Field observations of 15 *S. rugosa* midges, observed for on average 2.7 h (maximum: 4.4 h), and 31 irregular midges, observed for on average 2.3 h (max: 3.8 h), provided no evidence of mating or conidia collection for either population (Figure 2.7). However, it is clear that females collected fungal spores somewhere in their environment, as the mycangia of Malaise-trapped females always contain conidia (Figure 2.2). This suggests that these activities occur sometime after the period of my observations (Figure 2.7). A rare behavior consisting of touching or dragging the ovipositor on the leaf surface was observed in two irregular females, which may be associated with conidia collection (Figure 2.7B). The leaves were inspected where this behavior occurred and no eggs were found. Males and females began eclosing in the early morning as the last of the night’s dew was dried from the leaves (range of eclosion times: irregulars = 06:05-08:25 hours Eastern Daylight Savings Time (EDST), rugosa = 07:39-09:00 hours EDST). Males and females of both morphotypes spent approximately 2.5 h on the bottom of the leaf from which they eclosed before flying off (mean ± SEM, males: 2.31 ± 0.34 h, n = 6; females: 2.47 ± 0.18 h, n = 22). The marked galls of both morphotypes were always checked for missed eclosions before leaving the field site for the day. In only a few cases were new exuvia or emergence holes found the following day, indicating that the majority of adults eclosed during the early morning hours. At most 5% of the marked galls had eclosions on any given day, but this was more generally 0-1%. The series of behaviors I describe (Figure 2.7) are nearly identical to those provided in the photographs of Gagné (1989; Plate 2, C-F) for a different gall midge species.
2.4.4 Fungal acquisition and gall initiation

The examination of conidia morphology from different sources indicated a high degree of phenotypic plasticity in the shape of *B. dothidea* fungal conidia. All sources of conidia (e.g., gall, stem, agar cultures) produced conidia with a range of sizes overlapping with those found on *A. carbonifera* eggs or in their mycangia (Figures 2.2, 2.3 and 2.8), but there were differences in shape between conidia obtained from cultured fungus and those obtained directly from midge eggs or adults. In a full ANCOVA model with all two- and three-way interactions (first model), the only significant term was the growth media (i.e., goldenrod stems or oatmeal agar), with conidia being slightly shorter when grown saprophytically on goldenrod stems (Figure 2.8H, top four lines; $F_{1,403} = 14.0$, $P<0.001$). Tests for positional effects were not significant. With all seven conidia sources included in the analysis (second model), width covaried with length ($F_{1,552} = 155.4$, $P<0.001$) and the effect of conidia source was highly significant ($F_{6,552} = 190.4$, $P<0.001$). There was no significant interaction of conidia width and source, indicating that the slopes were homogeneous (Figure 2.8). A priori orthogonal decomposition comparing wild conidia sources (Figure 2.8H, bottom three lines) to cultured sources (Figure 2.8H, top four lines) revealed that wild conidia were significantly shorter than cultured conidia ($F_{1,556} = 1021$, $P<0.001$). Further decomposition of only the wild-sourced conidia concluded that goldenrod-stem conidia were significantly longer than those from the midge (i.e., eggs or female mycangia) sources ($F_{1,145} = 23.5$, $P<0.001$). Additional decomposition showed that mycangia conidia obtained from adults at the HS site were also slightly shorter than egg conidia obtained from the BCWMA and KWP sites ($F_{1,56} = 11.9$, $P = 0.001$).

Each of four experiments designed to induce the production of galls on the leaves
of *S. altissima* hosts failed; not a single gall was initiated. However, galls induced by natural populations of *A. carbonifera* rapidly appeared in high numbers on a replicated set of 10 *S. altissima* accessions in an un-caged field plot immediately adjacent to the caged field plot (i.e., 435 irregular, 335 crescent, 91 cushion, and 42 flat morphotypes).

2.5 DISCUSSION

2.5.1 Time required for appearance of fungus

The experiment to determine the time for fungus to appear on the top of the leaf took into account the fact that in most cases the oviposition event was not actually observed. This was attempted by incorporating the age of the leaf the clutch was found on as a covariate. If the eggs were initially laid on zero-aged leaves, then the time for fungus to appear on the top of the leaf for a given morph should be the intercept regardless of the age of the leaf the eggs were found on. This linear model indicated that there was a significant interaction between the age of the leaf oviposited on and the time for fungus to appear on the top of the leaf as well as significant main effects of leaf age and morphotype. However, the intercepts and means of this ANCOVA model were only marginally different. Furthermore, one irregular female and one flat female were actually observed ovipositing on different aged leaves (i.e., ages 0 and 3); therefore, my assumption that eggs were always initially oviposited on zero-aged leaves did not hold. These observations make the interpretation of the biological significance of this minor statistical interaction difficult. Nevertheless, with all the morphs pooled, the overall mean time for fungus to appear on the top of the leaf was 7.4 days, which is a good representation of all three morphotypes with an accuracy of ± 1 day.
2.5.2 The nature of the Asteromyia-Botryosphaeria symbiosis

The results of these studies indicate that this insect-fungal system is closely knit, with strong interdependence of the fungus and the midge. This association appears to be obligate for the gall midge and with respect to the successful production of galls on S. altissima. The midge depends on the fungus for food (Janson et al., 2009) and protection from parasitoids (Weis, 1982b), and the fungus benefits from dispersal and requires the midge for hyphal proliferation within the context of the gall. Although the fungus likely persists independently in environments outside the context of the midge galls and could have additional modes of dispersal, within the context of these galls, midge dispersal is necessary. As the gall-fungus only rarely produces sporulating pycnidia, the benefits (or the costs, for that matter) to the fungus of associating with the midges remain unclear. It is possible that the midge is simply parasitizing the fungus with no reciprocal benefit. However, teasing out the costs and benefits of apparently mutualistic associations can be tricky (e.g., Herre & West, 1997); the true nature of this interaction will become clearer with a better understanding of the life histories of both players. This system is not unlike other insect-fungal associations involving Ambrosia beetles, fungus-gardening ants, and other flies. Recent work on other galling insects including cecidomyiids has found that they actively manipulate plant defensive chemistry (Tooker & De Moraes, 2007, 2008; Tooker et al., 2008). This manipulation may be a factor in allowing the fungus to proliferate and form the protective gall-like structure.

Although several larvae died when transferred from galls to agar plates, this experiment still revealed the ability of larvae to grow on a fungus-only diet, strongly supporting the hypothesis that the fungus is the larva’s primary food source within the
gall. Even in young galls, I have observed that mycelium quickly envelops the larva making direct access to the plant tissue difficult. Furthermore, sterol analysis of fungi, plants, and midge larvae indicates that the midge larvae obtain their sterols mainly from the fungus (Janson et al., 2009). The observation that fungal structures resembling galls formed on the agar plates, suggests that the larva is primarily responsible for the general gall structure. Feeding damage and/or salivary-gland secretions are likely responsible for the formation of the hard, black, carbonaceous material (stroma) that formed on agar plates in areas of larval grazing. This material was similar to what surrounds and protects the developing larva from desiccation and parasitism in a natural gall. Larew et al. (1987) describe a cecidomyiid from a lineage evolutionarily basal to Asteromyia (Bisset & Borkent, 1988) that feeds only on fungus and forms similar galls in the absence of a host plant, suggesting that the ability to form fungal galls is a plesiomorphic trait. Haridass (1987) was able to rear Neolasioptera cephalandrae Mani larvae to adults, solely on gall-isolated fungus growing on Petri dishes, although they observed high mortality as well.

It is clear from my consistent observations of specific conidia associated with female ovipositors and eggs that A. carbonifera females intentionally obtain fungal conidia, store them in mycangia, transport them, and deposit them on their eggs. The fungus in these Asteromyia galls has been identified as B. dothidea (Bisset & Borkent, 1988; Janson et al., in press), a member of the family Botryosphaeriaceae. Other fungi assigned to this genus and even species are primary and secondary plant pathogens on many important ornamental and horticultural crops. For example, grapes, avocados, oaks, apples, pears, olives, Prunus species, poplars, pines, ashes, elms, and various berries are known to harbor or present disease symptoms associated with Botryosphaeria spp.
(Bonfiglioli & McGregor, 2006). *Botryosphaeria ribis* is thought to form cankers on the stems of goldenrods (Horst, 2008). Many studies have provided evidence that ascospores of this family are primarily wind dispersed, whereas conidia are primarily water dispersed (Sutton, 1981; Pusey, 1989; Ko & Sun, 1995; Ahimera et al., 2004). To my knowledge, only two studies have provided evidence for this active transportation of fungi by gall midges (Borkent & Bissett, 1985; Adair et al., 2009). No studies have investigated the direct role that cecidomyiids may play in vectoring *Botryosphaeria* spp. to other plants.

2.5.3 **Gall initiation, conidia collection, and eclosion behavior**

*Gall initiation.* Although I was unsuccessful at initiating galls under semi-artificial conditions, I did discover pycnidia producing fungus on the stems of *S. altissima*. However, two experiments including exposure of midges to these infected stems did not result in the initiation of galls, suggesting that these stems are not their conidia source.

   It is possible that midge behavior was constrained in the enclosures. Many insects and vertebrates have behaviors that are fixed action patterns (Matthews & Matthews, 1978). A fixed action pattern is a sequence of stereotypical behaviors that is relatively indivisible. This indivisibility can be so strong as to prevent subsequent behaviors until the sequence is completed. Furthermore, a number of these fixed action patterns can be under hierarchical control, so that the occurrence of one may be required for initiation of another (Matthews & Matthews, 1978). It is possible that my field cages may have prevented behavior, such as medium range dispersal, that may be necessary before conidia collection can occur. However, the field setting and use of large cages in these
experiments strongly suggests that the failure of gall initiation is most likely attributable to a lack of the appropriate source of conidia.

Eclosion behavior and mating. My observations indicated that females did not obtain conidia from their galls as they eclosed. Observations of several female mycangia from each of the four morphotypes after they had spent at least a day in containers containing tens of galls never revealed conidia within their mycangia (n = 35). This appears to be the case in other Ambrosia gall-forming cecidomyiids (Adair et al., 2009). My field observations also failed to reveal any evidence of behavior associated with conidia collection during or after eclosion. Furthermore, the exuvia effectively shields the ovipositor of the eclosing female, making conidia collection from within the gall unlikely. Perhaps most importantly, no pycnidia were ever observed within or on the outside of mature galls during eclosion.

Many cecidomyiid species have sexually dimorphic antennae with the males being more plumose (Gagné, 1989). In some of these species sex-specific pheromones have been identified (Heath et al., 2005). These species tend to mate almost immediately after eclosion (McKay & Hatchett, 1984; Pivnick & Labbe, 1992; van Lenteren et al., 2002; Heath et al., 2005; Suckling et al., 2007). The antennae of A. carbonifera are not sexually dimorphic and attempts to attract males or females with virgin conspecifics or actively sporulating B. dothidea fungus have failed (JJ Heath, unpubl.). On several occasions, males and females of the same morphotype were seen emerging from galls in the field at the same time and very close to one another (i.e., within 5-30 cm), but mating was never observed. One may postulate that this is a result of inbreeding avoidance, but
females have single-sex families. Therefore, these observations suggest that males and females of *A. carbonifera* may aggregate at the conidia collection site or that adults become more attractive after they have collected fungal conidia. Although *A. carbonifera* are diurnally synchronized in emergence, populations are not strongly synchronized seasonally (B Wells, pers. comm.). In my field eclosion observations, over 100 galls were marked, but less than 5% of these produced eclosing adults on any one day. Therefore, the probability of having a male, female, and a sporulating fungal structure present simultaneously may be quite rare. Future gall initiation studies will concentrate on one gall morphotype to increase the probability of the co-occurrence of these factors.

*Conidia morphology.* The morphology of conidia from different sources demonstrates that *B. dothidea* produces pleomorphic conidia and that this is affected by the growth media. The fact that egg-conidia isolates produce long slender conidia when grown as a saprophyte (i.e., on oatmeal agar or autoclaved goldenrod stems) even though the isolates originated from a population of short conidia (i.e., egg-conidia isolates), suggests that the natural source of conidia is not of a saprophytic nature. If the females obtain conidia from a saprophytic source one would expect these conidia to be long and slender, rather than the observed ovoid shape. The morphology of the conidia derived from pycnidia occurring on *S. altissima* stems in the field was more ovoid and overlapped more with those found in mycangia and on eggs, suggesting these as a possible source. However, the conidia source may be from some other plant growing in the same environment as goldenrod, such as blackberries. *Botryosphaeria dothidea* is known to attack blackberries and produce numerous pycnidia on their stems (Maas & Uecker, 1984) that overlap
slightly in size with those found in the mycangia. It is also possible that the midges somehow select smaller conidia mechanically or via odors; though the benefit of this selectivity is unclear. I plan to generate isolates of the *S. altissima* stem fungus and conduct genetic analysis to determine its relationship to *B. dothidea*. Although this may be a possible source, the distribution of these pycnidia in the field is patchy and efforts to find them early in the season, when *A. carbonifera* galls were prevalent, have failed. They are much more abundant later in the season, when the goldenrod has started to form flower buds.

Although, mycangia and egg conidia were slightly different in morphology, this difference pales in comparison to the phenotypic plasticity of the fungus indicated by comparing the morphology of egg-derived conidia to the morphology of conidia derived from egg-conidia isolates in culture. Furthermore, the mycangial conidia and egg conidia came from different field sites, which may be responsible for the small but significant difference in morphology. The conidia I found in the mycangia of *A. carbonifera* females were very similar to those described by Bisset & Borkent (1988, Figure 2.2) for a variety of Ambrosia cecidomyiids that all use *Botryosphaeria* spp. to form their galls. This consistency in conidia morphology across Ambrosia gall midge taxa and a wide geographical range, suggests that conidia morphology is key to understanding where these midges obtain their conidia. However, the phenotypic plasticity associated with substrate makes it imperative that wild sources of conidia be measured. Once a source with similar morphology is found, genetic profiling and behavioral experiments will be necessary to verify its role in this system. Detailed genetic profiling might also be used to differentiate between a single fungal source and random collection by comparing the
genetic variation of single-conidia isolates isolated from the same mycangia to those isolated from a single fungal reproductive structure.

I can now say that *A. carbonifera* is one of a number of complex Ambrosia gall midge mutualisms that hold great promise to provide insight into the contribution that mutualistic relationships make to adaptive radiation and ecological speciation. Practically speaking, mating behavior and conidia collection remain areas of future work. Without this knowledge manipulative studies to assess mating, host plant, and gall morph fidelity will be difficult and direct studies involving reproductive isolation and hybrid fitness will be nearly impossible. These issues notwithstanding, *A. carbonifera* and other Ambrosia gall midges remain tantalizing model systems for studying a range of evolutionary phenomena.
2.6 REFERENCES


Buchner P (1965) Endosymbiosis of animals with plant microorganisms. Wiley Interscience, New York, NY, USA.


Fermaud M & Lemenn R (1989) Association of *Botrytis cinerea* with grape berry moth larvae. Phytopathology 79: 651-656.


Weis AE (1982a) Use of a symbiotic fungus by the gall maker *Asteromyia carbonifera* to


Table 2.1 List of study sites with abbreviations, names, and coordinates\(^1\)

<table>
<thead>
<tr>
<th>Site abbreviation</th>
<th>Site name (all USA)</th>
<th>Latitude (N)</th>
<th>Longitude (W)</th>
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<td>39°45’59.09”</td>
<td>84°00’15.95”</td>
</tr>
<tr>
<td>HS</td>
<td>Huffman Metropark, Dayton, OH</td>
<td>39°48’28.30”</td>
<td>84°05’33.94”</td>
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<tr>
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<td>39°45’57.97”</td>
<td>84°00’40.96”</td>
</tr>
<tr>
<td>VS</td>
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<td>39°46’01.45”</td>
<td>84°00’56.51”</td>
</tr>
<tr>
<td>WSU-1</td>
<td>WSU Common Garden, Dayton, OH</td>
<td>39°47’14.96”</td>
<td>84°03’08.59”</td>
</tr>
<tr>
<td>WSU-2</td>
<td>WSU Services Site, Dayton, OH</td>
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<td>84°03’10.85”</td>
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<tr>
<td>SC-1</td>
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<td>77°50’04.13”</td>
</tr>
<tr>
<td>SC-2</td>
<td>Stewart Drive, State College, PA</td>
<td>40°49’50.16”</td>
<td>77°47’44.52”</td>
</tr>
</tbody>
</table>

\(^1\)Coordinates obtained from Google Earth (Version 4.3).
Figure 2.1 *Asteromyia carbonifera* (A) adult, (B) eggs, and (C) larvae within a dissected gall. Adults and mature larvae are about 1-2 mm in length. Scale bar on (B) is 200 µm and eggs are typically 180-240 µm long.
Figure 2.2 Dorsal view of a malaise-trap-captured female *Asteromyia carbonifera* showing mycangia filled with fungal conidia. Conidia were stained with lactophenol cotton blue. Inset is an enlargement of one of the mycangia (scale bar = 20 µm). Normally, a large proportion of the conidia would be distributed toward the anterior as well, but pressure from the slide mount forces the conidia toward the posterior in what appears to be tubes or specialized folds of tissue that lead the conidia to their point of deposition on the passing egg.
Figure 2.3  Two germinating fungal conidia on an *Asteromyia carbonifera* egg collected from the new growth of a *Solidago altissima* plant. Inset shows an enlargement of the conidia. Conidia stained with lactophenol cotton blue.
Figure 2.4 (A) Mean (± SEM) net fungal growth rate (mm$^2$ per day per larva) after 10 days of incubation; organized by treatment (bars) and experiment (individual graphs). Bars are labeled with treatment abbreviations: negative control (c), mock removal (m), and complete larva removal (r). Mock larvae were either ‘touched’ or ‘not touched’ with the forceps in the indicated experiments. The values of small means are indicated above the bars. (B) Representative before and after photographs ordered by experiment.
Figure 2.5 *Asteromyia carbonifera* pseudo-gall developing on (A) malt extract agar. (B) Same as in A, but it has been dissected. (C) The same as B, but at a higher magnification to show the healthy prepupa inside.
Figure 2.6 *Asteromyia carbonifera* larval length (mm) after 2 weeks of growth on malt extract agar plates treated with Nystatin and with (closed circles) or without (open circles) gall fungus added. The fine dotted line has a slope of 1 and an intercept of 0 and denotes no change in length.
Figure 2.7 Ethograms of Asteromyia carbonifera behavior under natural field conditions. Galls marked and observed for (A) Solidago rugosa morphotypes and (B) Solidago
*altissima* irregular morphotypes. Behavior was observed from eclosion to fly-off. The thickness of the arrows is proportional to the relative frequency of the particular transition from one behavior to the next. Numbers along arrows are the absolute number of transitions observed.
Figure 2.8 Length and width (µm) of individual conidia sampled from different sources. The top four panels show conidia obtained from either (B, F) gall or (A, E) egg-conidia fungal isolates, grown on either oatmeal (A, B) agar or (E, F) fresh-cut autoclaved *Solidago altissima* stems atop water agar plates. These four sources were grown under the same conditions at the same time. The other sources are (C) *Asteromyia carbonifera* female mycangia, (D) pycnidia on field-collected *S. altissima* stems, and (G) field-collected *A. carbonifera* eggs. (H) The final panel is a composite overlay of all the graphs to facilitate comparisons. The insets are representative photographs of some of the conidia from each source. The scale bar (20 × 5 µm) in (A) applies to all the insets. Conidia stained with lactophenol cotton blue.
3 CARNIVORES AND CAROTENOIDs ARE ASSOCIATED WITH AN ADAPTIVE BEHAVIOURAL PHENOTYPE IN A RADIATION OF GALL MIDGES.

3.1 ABSTRACT

1. Adaptive divergence in sympathy is supposed to be inhibited by the homogenizing role of gene flow. However, studies continue to uncover examples of sympatric divergence. Here, I characterize two divergent phenotypes in a complex of four syntopic gall midge morphotypes [nominally Asteromyia carbonifera (Osten Saken), Diptera: Cecidomyiidae: Alycaulini]. The first is a behavioral phenotype governing within-host tissue preference and the second is a trait governing accessory-gland carotenoid quality and quantity.

2. One gall morphotype (crescents) lay most of their eggs on mature tissue while the other three gall morphotypes oviposit only on young emerging leaves. Ecological maintenance of this divergent trait appears to be driven by enemy-reduced space. That is, nearly 40% of the crescent morphotype galls that develop high on the plant are attacked by the egg parasitoid Platygaster solidaginis (Ashmed), while those low on the plant are relatively protected.

3. All morphotypes contain carotenoids in their accessory glands, but the quality and quantity of these pigments differs significantly between the morphotypes and is positively associated with the probability of parasitism by P. solidaginis.

4. Larval salivary glands also contain carotenoids and the plant hormone abscisic acid, which in plants is synthesized from carotenoid precursors and is involved in regulating
plant defenses. This hormone may facilitate gall development and influence gall morphology.

5. Ecological fitness trade-offs between carotenoids, parasitoid attack, and plant resistance may be fostering adaptive divergence in ovipositional phenotypes and sympatric speciation in this complex of gall midge morphotypes.

3.2 INTRODUCTION

Adaptive divergence in sympatry is expected to proceed slowly (if at all) because of the homogenizing role of gene flow (reviewed briefly in Hendry et al., 2001; Servedio et al., 2011), but insect studies continue to reveal evidence of sympatric divergence (Wood, 1993; Dambroski & Feder, 2007; Joy & Crespi, 2007; Dorchin et al., 2009). In these systems, behavioral phenotypes and especially ovipositional phenotypes appear to be the forerunners to deeper physiological changes that may provide reproductive isolating mechanisms. It could be argued that the proximate reason for the evolution of the two host races of the apple maggot fly was host preference via associative learning (Prokopy et al., 1982). This behavioral phenomenon may have led to the subsequent differentiation in diapause phenology that ultimately reduced gene flow between these sympatric host races (Dambroski & Feder, 2007). A similar argument could be made for the host races of Eurosta solidaginis on Solidago altissima and S. gigantea (Waring et al., 1990; Craig et al., 1993; Brown et al., 1996).

Incipient species or host races are ideally suited for studying sympatric speciation because contemporary selective forces are likely to reflect those that drove the initial stages of divergence and continue to maintain discrete populations (Brown et al., 1995;
Host shifts require simultaneous behavioral changes in host preference as well as physiological adaptation to the new host plant. These requirements are thought to hinder the likelihood of host shifts and adaptive divergence (Brown et al., 1995). However, lower predation risk on the new host or tissue is expected to accommodate maladaptation to host-plant resistance (Price et al., 1980; Singer & Stireman, 2005). Gavrilets (2004, p. 396) concludes in his review of evolutionary models of sympatric speciation that models incorporating habitat preference or a “magic trait” (Servedio et al., 2011) are the most likely to result in sympatric speciation. Similar constraints and facilitating factors may operate at even smaller scales; that is, within a host-plant (Joy & Crespi, 2007). Here, I characterize two divergent phenotypes in a gall midge species complex occupying a single host. The first is a behavioral phenotype governing within-host tissue preference and the second is a trait governing accessory-gland carotenoid quality and quantity. These traits covary with each other and with the probability of parasitism.

### 3.2.1 System background

My study system is comprised of a complex of gall midges, *A. carbonifera* on tall goldenrod, *S. altissima* (Crego et al., 1990; Stireman et al., 2008). The galls are the result of a mutualistic relationship between the gall midge and a symbiotic fungus, *Botryosphaeria dothidea*; each partner requires the other for successful galls to form (Janson et al., 2009; Heath & Stireman, 2010). I focus here on a likely paraphyletic group of four gall morphotypes (hereafter, morphs) that occur in spatiotemporal syntopy on tall goldenrod. These morphs are referred to as crescents, cushions, flats, and irregulars
according to their overall morphology, position on the leaf, position within the gall, and degree of loculation (Crego et al., 1990). Current evidence indicates that the midge, not the fungus or the host plant, is responsible for variation in gall phenotypes (Heath & Stireman, 2010; Janson et al., 2010). The morphs are attacked by up to seven different parasitoids and gall structure and clutch size influence parasitism rates (Weis, 1982a; Weis, 1982b; Weis et al., 1983; Weis, 1983; Stireman et al., 2008). Although some gall making insects create sexually dimorphic galls (Cook & Gullan, 2008), have generation-specific morphotypes (Rey, 1992; Miller, 1998; Inbar et al., 2004), or appear to have diverged parapatrically (Mishima & Yukawa, 2007), A. carbonifera morphs are sympatric and genetically differentiated (Crego et al., 1990). The phylogenetic relationships of these four morphs depend heavily on the type of marker used, but amplified fragment length polymorphisms recover at least 4 major groups among these morphs with relatively little geographic genetic structure (Stireman et al., 2008; Stireman et al., 2010), strongly suggesting that they have diverged and are maintained in sympatry.

### 3.2.2 Within-host preference and performance

Intuitively, one might predict that tissue preference and performance would be tightly correlated. However, this prediction depends on the number of trophic levels involved. In a bitrophic herbivore-plant system it is expected, but in a tritrophic system trade-offs may overshadow preference-performance correlations. That is, lower performance on a more defended tissue may be evolutionarily tolerated in order to realize a refuge from natural enemies (Price et al., 1980; Singer & Stireman, 2005). Such trade-offs may drive and maintain host shifts. Furthermore, physiological and behavioral
optimizations for life in these new environments may positively feedback, further driving a wedge between diverging lineages (see Schluter, 2000 on ecological speciation).

The goals of this part of the study are to confirm experimentally the existence of tissue preference (i.e., ovipositional phenotype) among the gall morphs of *A. carbonifera* and evaluate trade-offs between performance and parasitism. These objectives are addressed with the following questions:

1. Do *A. carbonifera* morphs differ in their ovipositional niche?

2. Does this niche provide a refuge from parasitoid attack?

3. Do plant tissues vary in their suitability for different gall morphs?

### 3.2.3 Variation in carotenoids among gall morphs and its consequences

In insects and other organisms, carotenoids are important in vision, development, management of oxidative stress, diapause, photoperiod, mate signaling, resource location, and coloration (see Chapter 4). Carotenoids are bright orange or red, fat-soluble pigments and their presence in insect bodies is visually apparent. They have been found in insect glands (Eichenseer et al., 2002; Sakudoh et al., 2007), but it is generally assumed that animals cannot biosynthesize carotenoids and must obtain them from their diet. However, recent evidence of laterally transferred genes for carotenoid biosynthesis
in some arthropods (Moran & Jarvik, 2010; Altincicek et al., 2011) challenges this dogma.

In my system, carotenoids are found in the adult female accessory glands (AG) and are deposited on eggs along with symbiotic fungal spores (Heath & Stireman, 2010). In addition to the AGs, a particularly dense localization of carotenoids is also found in the filamentary region of the larval salivary glands (SG), which is the primary source of chemical agents that induce gall formation in cecidomyiids (Raman et al., 2005). The accumulation of carotenoids in these glands, representing the biochemical interface between midge and host-plant, suggests that carotenoids play a functional role in gall development and performance, and may underlie divergence among gall morphs in A. carbonifera. I therefore hypothesized that carotenoid quantity and quality profiles represent a possible axis of cryptic phenotypic variation among gall morphs.

In addition to examining variation in carotenoids among morphs, I explored some of the possible roles of these compounds in midge-host and midge-enemy interactions. The basal region of the SGs appear to lack carotenoids, suggesting that they are broken down into unpigmented compounds as they pass from the filament region to the basal region and eventually into the plant via the larval mouthparts. In plants, carotenoids are the precursors to abscisic acid (ABA), which is a hormone important in the regulation of plant defenses (Mauch-Mani & Mauch, 2005) and stomatal aperture (Assmann, 2004) and can be found in the larvae of some herbivores (Tooker & De Moraes, 2011). This led me to hypothesize the presence of ABA in the SGs where it may play a role in gall development. Furthermore, because fungi and plants are known to degrade carotenoids
to volatile apocarotenoids (Zorn et al., 2003; Lewinsohn et al., 2005) and hymenopterans are well known to use apocarotenoids as semiochemicals (see Chapter 4), I hypothesized that AG carotenoids would be positively associated with the risk of attack by the egg parasitoid *P. solidaginis*. To address these hypotheses I asked the following questions:

(1) Do accessory gland carotenoids vary among the morphs?

(2) Is the carotenoid break-down product and plant hormone, ABA, present in midge salivary glands?

(3) Do accessory gland carotenoids covary with the probability of parasitoid attack?

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Testing for differences in ovipositional phenotype

I tested the hypothesis that morphs divide up the plant resource by leaf age by measuring gall diameter, gall height, and plant height of naturally occurring galls in a common garden of 100 *S. altissima* subplots (WSU site, Table 3.1). I established the common garden in June of 2008 with plants grown in the greenhouse that spring from rhizomes collected haphazardly from nearby wild populations. I transplanted the plants to the field in a 10-row by 10-column grid spaced 2 m on center in a randomized complete block design; each 2-row by 10-column block contained 2 replicates of each of the ten clones (presumably different genotypes). I watered the plants after transplanting, but other than periodical weeding and mowing around the subplots, they were left to establish on their own. Starting in May 2009 I checked the subplots daily for new galls which were marked and measured at a rate of about 1-2 rows of the common garden per
day. After about 1-2 weeks a complete census of the galls in the garden was achieved and the process began again, including additional measurements of the previously marked galls and measurements of any newly occurring galls. This was continued until August at which time several hundred galls had been tracked and measured (crescents, n = 1579; irregulars, n = 210; cushions, n = 307; and flats, n = 188). Morph assignment was confirmed after the marked galls were mature (i.e., when the rate of gall-diameter-increase was < 10% per week). I could not determine gall age at the time of discovery and so the height of oviposition was uncertain. I inferred where the eggs were laid by plotting the relative gall height on the plant as a function of relative gall diameter on the day of discovery and took the intercept to indicate where oviposition occurred. I used a linear model to test if the intercepts were significantly different (R Development Core Team, 2010).

To determine whether the difference in gall heights was caused by delayed larval hatching or by actual oviposition on mature tissue, a common garden of 200 potted S. altissima plants representing 20 different clones was established. The plants were grown in 2.5-L pots of soilless media (Pro-mix BX/Mycorise® Pro, Premier Horticulture, Ltd., Quakertown, PA, USA) from rhizome cuttings in the greenhouse and then placed in the field within a deer exclusion plot and watered regularly. I arranged pairs of clones in a randomized complete block design within a 10-row by 10-column grid with pairs spaced 0.5 m on center; each 2-row by 10-column block contained 1 pair of each of 20 different clones (presumably different genotypes). All plants were free of galls at the start of the experiment in July 2009. One plant in each pair had the top 20-cm of young leaves
including the terminal bud covered with screening (n = 5 pairs per clone). The screening was moved up as the plants grew. Galls were marked weekly as they appeared and were assigned to a morph when they were mature. The experiment lasted two months. If eggs were actually laid on mature tissue, then only galls of that morph should have appeared on plants with protected young tissue.

3.3.2 Assessing variation in parasitoid attack

To test whether parasitoids might be driving the divergence in ovipositional phenotype, parasitoid attack was measured at four prairie sites dominated by *S. altissima* in and around Dayton, Ohio (Table 3.1). I collected mature galls and either dissected them under a stereomicroscope or reared them in cotton-stopped vials in constant humidity chambers (~90% RH achieved with a saturated NaCl solution). For the WSU site, logistic regression over the height of the plant was performed on the binary variable of presence or absence of a specific parasitoid for the crescent morph. For all four sites, attack by *P. solidaginis* was analyzed with a chi-squared test and Pearson’s standardized residuals plotted by gall morph. Sample sizes for each site for crescents, cushions, flats, and irregulars were: BCW, n = 100, 113, 93, and 115; SSP, n = 142, 135, 78, and 151; GMP, n = 88, 106, 70, and 97; and WSU, n = 1403, 287, 173, and 204 galls; respectively. All analyses were conducted in R (R Development Core Team, 2010).
3.3.3 Testing suitability of the oviposition site

To test whether cushions, flats, and irregulars could develop on mature leaves, eggs were collected from the terminal bud and adjacent young leaves of field populations of *S. altissima* and transplanted to the leaves of potted *S. altissima* clones. With the aid of a stereomicroscope, transplant eggs were placed in the middle of the underside of the leaf between the midvein and the margin of either young, newly emerged leaves or mature, fully expanded leaves. Gall morph, diameter, and midge stage were recorded after 5 weeks of development in the greenhouse. Final gall diameters were analyzed with a one-way ANOVA followed by planned orthogonal decomposition. Logistic regression was used to test whether survival to adults or pupae was affected by tissue age, morph, or their interaction (R Development Core Team, 2010).

Crescent eggs are extremely difficult to find in the field, but very young crescent galls are relatively easy to locate and crescent morphs almost always develop on the very edge of the leaf. Several crescent eggs are often deposited along the edge of a single leaf (presumably by the same female) and all young *A. carbonifera* galls retain an orange spot of accessory fluid for about the first week after oviposition. Therefore, once a young crescent gall is located the entire edge of the leaf can be checked for unhatched eggs, neonates, or extremely young galls. By marking and tracking the development of these eggs, neonates, and young galls over time, the failure rate of crescents was estimated over the height of the plant. Galls rarely failed once they reached a diameter of about 2 mm; therefore, I am confident in this method of assessing plant resistance to crescent morphs.
Logistic regression was used to test the effect of gall height on the failure rate of crescent galls (R Development Core Team, 2010).

3.3.4 Characterizing carotenoid profiles in accessory glands

To examine variation in carotenoid quality and quantity among morphs, carotenoids were extracted in hexane from the AGs (Figure 3.6C) of A. carbonifera morphs (n = 34) and one A. modesta female. The AGs were dissected from adult females in 0.9% NaCl and blotted dry on a piece of aluminum foil with a camel hair brush before being transferred to hexane and crushed with a flamed glass pipette. The UV/visible spectrum was obtained for a pooled A. carbonifera extract on an HP 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Thin layer chromatography was conducted on silica gel plates (2.5 × 7.5 cm, 200 um, F-254, 60A, Selecto Scientific Inc., Suwanee, GA, USA) and developed with a 3:1:1 ratio of petroleum ether, acetone, and chloroform. All the plates were scanned with the same flatbed scanner under identical conditions and the optical densities (OD) of 17 spots determined using ImageJ (NIH) and the methods described in Valverde et al. (2007). Optical densities were converted to nanograms using a β,β-carotene (22040, Sigma-Aldrich, St. Louis, MO, USA) standard curve (F₁,₅ = 236.4, r² = 0.98, P << 0.001). The ODs measured were all within the linear range of the standard curve. Multivariate analysis of variance (MANOVA) and canonical discriminant analysis (CDA) was used to analyze the carotenoid profiles. Carotenoids c3, c5, c7, and c8 were strongly correlated; therefore, I used their mean (i.e., carotenoid cA) in the MANOVA. Ninety-five per cent confidence ellipses of the mean CDA scores were calculated for each morph (crescents, n
= 6; cushions, n = 11; flats, n = 6; and irregulars, n = 11) using the equations in Owen and Chmielewski (1985). The SGs of A. carbonifera larvae also contain orange pigments (Figure 3.6B) for which a UV/visible spectrum was also obtained as above.

3.3.5 **Associating carotenoid quantity and parasitoid attack**

Midges attacked by *P. solidaginis* do not survive to the adult stage where the AG carotenoids can be measured. Therefore, to assess the association between *P. solidaginis* attack and AG carotenoid quantity of each morph, I regressed the mean total carotenoids by morph on the mean Pearson residual of *Platygaster* attack (i.e., the results from Figure 3.4). To address concerns about the reduction in variation that taking the means might have on the analysis, I conducted both a conventional linear regression and a randomization test. The randomization was based on drawing 4 random samples from each normal distribution with mean and standard deviation estimated from my data and calculating an $r^2$ value 10,000 times. A histogram of these values was generated and the probability of obtaining an $r^2$ greater or equal to the one obtained by conventional analysis was calculated. A two-way ANOVA with interaction was also conducted to assess the association between total AG carotenoids by gall morph and the galls’ relative height at maturity on the plant. Analyses were conducted in R (R Development Core Team, 2010).

3.3.6 **Determining the presence of abscisic acid (ABA) in the SGs**

In plants, ABA is formed from the oxidative cleavage of carotenoids and is a potent hormone controlling the size of the stomatal aperture. ABA is also up-regulated during periods of drought and is known to negatively interact with biotic stress signaling.
In the absence of the midge larva, the mutualistic fungus cannot grow (Heath & Stireman, 2010); therefore, it is likely that the larva somehow manipulates plant chemistry to allow fungal growth. Based on the presence of carotenoids in the SGs, I tested the hypothesis that ABA is present in the SGs of these midges using two independent methods: a stomatal aperture bioassay and an enzyme-linked immunosorbent assay (ELISA).

The methods of Tucker & Mansfield (1971) and Ogunkanmi et al. (1973) were modified slightly for the stomatal aperture assay using *Tradescantia zebrina* Heynh. ex Bosse (Commelinaceae). This method is highly sensitive to ABA, unaffected by the presence of other plant hormones, and reproducible. Young galls of unknown morph were collected from the field and the SGs dissected from the larvae under a stereomicroscope in 10-mM citrate buffer, pH = 5.5. Epidermal peels were taken in the morning from the bottom of dark-adapted leaves, divided into 4 equally sized pieces (ca. 0.5 mm$^2$), and floated on 4 mL of SG extract in citrate buffer. Extracts were prepared by crushing an aliquot of SGs with a plastic pestle in an Eppendorf tube of citrate buffer. The tube was centrifuged and the supernatant drawn off and serially diluted. Four concentrations were tested: 0, 0.1, 1, and 10 midge equivalents per 4 mL of citrate buffer. Each midge larva contains a pair of SGs; therefore, one midge-equivalent (meq) is equal to 2 glands. After the peels were prepared the vials were incubated in a glass-pan water bath (24–26 deg C) over a bank of fluorescent lights (PAR, 80 µmols photons/m$^2$/s) for 2-3 hours. Throughout the incubation time each vial was bubbled with a stream of CO$_2$-free air created by filtering room air through two 1-L Erlenmeyer flasks each with a saturated aqueous solution of Ca(OH)$_2$ (i.e., 2 g/L of pickling lime). Each replication of
the concentration series ($n = 8$ per concentration) was tested on peels obtained from the same leaf at the same time. A mixed effects model with replication treated as a random effect was used to analyze the stomatal response. The stomatal response was measured as the mean stomatal aperture of a random sample (ca. $n = 30$) of stomata on the peels from each concentration. Analysis was conducted in R (R Development Core Team, 2010).

The ELISA kit (PDK 09347/0096, Agdia, Inc., Elkhart, IN, USA) was also used to directly assay the quantity of ABA in SG extracts. Salivary glands were dissected in 0.9% aqueous NaCl (with 0.2% Tween 20) and extracts were prepared as above in 0.5 mL of an 8:2 ratio of methanol:water (v/v). The manufacturer’s directions were subsequently followed. A linear model generated in R (R Development Core Team, 2010) was used to examine the relationship between the number of SGs in the extracts and the amount of (+)ABA measured by the ELISA. Only the flat morph was analyzed because only this morph had levels of (+)ABA significantly above the detection limit.

### 3.4 RESULTS

#### 3.4.1 Ovipositional phenotype

The intercept estimates (Figure 3.1; crescent, $0.58 \pm 0.01$; cushion, $0.93 \pm 0.02$; flat, $0.88 \pm 0.03$; and irregular, $0.93 \pm 0.03$; intercept ± SE) indicated that crescents develop lower on the plant than the other three morphs: The main effect of morph was highly significant in a two-way ANOVA ($F_{3, 2276} = 618$, $P << 0.001$) as was the covariate, relative gall diameter ($F_{1, 2276} = 263$, $P << 0.001$). Because there was a significant interaction between morph and gall diameter ($F_{3, 2276} = 4.63$, $P = 0.003$) I was suspicious
about whether the differences were the result of delayed larval hatching or an ovipositional phenotype. However, the manipulative experiment indicated that only crescent morphs oviposited on plants with screened young leaves, while all gall morphs oviposited on unprotected plants (Figure 3.2).

### 3.4.2 Parasitoid attack

Among the mortality factors for crescents, *P. solidaginis* was the most significant in logistic regressions of these variables over the height of the plant (Figure 3.3; deviance = 38.8; d.f. = 1, 1370; $P << 0.001$). For crescent galls located high on the plant the probability of *Platygaster* attack was nearly 40% (Figure 3.3A). Importantly, across the morphs and sites, *P. solidaginis* consistently attacked the crescent morph more than expected by chance (Figure 3.4). And the chi-squared analyses were significant for every site (BCW, $\chi^2 = 16.0$, d.f. = 3, $P = 0.001$; SSP, $\chi^2 = 11.5$, d.f. = 3, $P = 0.009$; GMP, $\chi^2 = 26.5$, d.f. = 3, $P << 0.001$; WSU, $\chi^2 = 19.0$, d.f. = 3, $P < 0.001$).

### 3.4.3 Egg transplants and crescent failure rates

Crescent morphs failed slightly more often when they developed on mature leaves (Figure 3.3F), but this result only approached significance (deviance = 3.4; d.f. = 1, 1525; $P = 0.06$). However, when other gall morphs were forced to develop on mature leaves they failed to produce full size galls as a whole (Figure 3.5, $F_{8, 179} = 15.6$; d.f. = $P << 0.001$) and for each gall morph individually (cushion-young vs. cushion-mature, $P << 0.001$; flat-young vs. flat-mature, $P << 0.001$; and irregular-young vs. irregular-mature, $P = 0.035$). Likewise, these three morphs exhibited low survival to pupae or adults on mature tissue ($\leq 8\%$, deviance = 28.2, d.f. = 1, 179; $P << 0.001$), but there was no effect
of morph (deviance = 3.47, d.f. = 2, 179, \( P = 0.178 \)) or the interaction of morph and tissue age on their survival probability (deviance = 0.06, d.f. = 2, 179, \( P = 0.972 \)). In the field, these three gall morphs tend to be multilocular, but in this experiment only a single egg was used to initiate galls. As predicted, the morphs still differed in their mature sizes; cushions and flats were larger than irregulars (Figure 3.5, \( P < 0.001 \)) and flats were larger than cushions (Figure 3.5, \( P = 0.009 \)).

### 3.4.4 Carotenoid profiles

The orange pigments found in the AGs and SGs (Figure 3.6) of adult female and larval midges were non-polar pigments readily extractable in hexane. The UV/visible spectra of a sample of each in hexane (Figure 3.6D, AGs: I = 430 nm, II = 450 nm, III = 478 nm, \( \%III/II = 27 \); SGs: I = 435 nm, II = 458 nm, III = 485 nm, \( \%III/II = 17 \)) bears a strong resemblance to typical carotenoids, including \( \beta, \beta \)-carotene in hexane (I = 425 nm, II = 450 nm, III = 477 nm, \( \%III/II = 25 \)). Multivariate analysis of 17 scored carotenes and xanthophylls indicate that the quality and quantity of the AG carotenoids differs consistently and significantly by gall morph (Figure 3.7 and Table 3.2; approximate \( F = 48.7; d.f. = 3, 30; Roy = 35.9; num. d.f. = 14; den. d.f. = 19; P << 0.001 \)) regardless of the test employed (i.e., Roy, Pillai, Wilks, or Hotelling-Lawley).

### 3.4.5 Relationship between carotenoids and *Platygaster* attack

In a linear model, the mean total amount of carotenoids in the AGs of each of the morphs significantly predicted their mean susceptibility to *P. solidaginis* (Figure 3.8, \( F_{1,2} = 26.6, r^2 = 0.93, P = 0.036 \)). Furthermore, the interaction term in a linear regression of total AG carotenoids indicated that only the crescent morph carotenoids increase with
their relative height on the plant (Figure 3.9, $F_{3,15} = 4.41$, $P = 0.021$). Because the slopes of the other morphs were not significantly different from zero, only a single linear model for the crescent morph is presented ($F_{1,2} = 29.1$, $r^2 = 0.94$, $P = 0.033$). Therefore, a positive association of AG carotenoid quantity and \textit{Platygaster} attack exist both within (compare Figure 3.3A and Figure 3.9) and across morphs (Figure 3.8).

3.4.6 ABA in the SGs

Two independent experiments confirmed the presence of ABA in the SGs of these midges. As expected, increasing the quantity of glands extracted caused a concomitant effect in the response of both the stomatal bioassay and the ELISA assay. In the ELISA assay, only the flat morphs were analyzed because only they showed values above the detection threshold (Figure 3.10, $F_{1,8} = 14.0$, $r^2 = 0.64$, $P = 0.006$). In the stomatal bioassay (data not shown), stomatal aperture decreased with increasingly concentrated SG extracts ($F_{1,23} = 9.71$, $P = 0.005$).

3.5 DISCUSSION

Two independent experiments indicate that crescent morphs of the gall midge, \textit{Asteromyia carbonifera} oviposit and develop on mature leaves of \textit{Solidago altissima}, while three other sympatric and syntopic morphs do not. Furthermore, when the others are forced to develop on mature tissue they perform poorly. The ability of the crescents to initiate and develop galls on mature tissue is unusual among gall midges and gall-forming taxa in general. It is well known that galling insects generally prefer younger, less differentiated, actively growing tissue (Esprito-Santo et al., 2007). Presumably, young tissues are more amenable to physiological manipulation by the galler. As stated
previously, *A. carbonifera* galls are not formed by deformations of plant tissue, but are rather due to a controlled growth of their fungal symbiont (Heath & Stireman, 2010). This strategy of building the gall structure from fungus may release these midges from the physiological constraints associated with forming typical plant galls. Although the other three morphs performed poorly on mature tissue, some individuals did successfully pupate and emerge from galls initiated there. This suggests that the evolutionary lability of ovipositional behavior may differ across lineages. Field observations have suggested that other members of the genus *Asteromyia* may also have the capacity to develop on mature tissue; that is, this ability may have evolved several times or been shared by a common ancestor.

Among the selective forces that might maintain this ovipositional phenotype, *P. solidaginis* attack was the most significant. That *P. solidaginis* parasitism is a potent selective force maintaining this phenotype is evident in the fact that crescents continue to oviposit low on the plant even though the plant is more resistant to them there. Singer and Stireman (2005) have proposed the tritrophic niche concept, which predicts that insects diverging with respect to enemy-free space will illustrate exactly such a pattern. That is, natural selection will tolerate lower performance with respect to plant resistance in order to realize a net fitness benefit associated with enemy-free space. Given this theory, I would predict that the ancestor of crescents oviposited exclusively on newly emerging leaves and that the abilities to oviposit and develop on mature tissue are derived traits.
I found that AG carotenoids varied qualitatively and quantitatively across gall morphs. Given that flat, irregular, and cushion morphs culture their symbiotic fungus on the same host species and tissue (i.e., young leaves), the observed variation in carotenoid composition cannot be explained simply by host carotenoids. I suspect that this variation is functionally related to gall morphology, but I currently lack a complete mechanistic hypothesis for how this might occur. Carotenoids in the AGs of flats and irregulars were qualitatively similar. Indeed, natural variability in the morphology of these two morphs can also make them sometimes difficult to distinguish. However, only unambiguous morphs were used in this analysis making it unlikely that morph was incorrectly assigned. If AG carotenoid quality and quantity is genetically determined, this similarity may suggest a close relationship or some level of gene flow between these morphs. Indeed, AFLP analysis of these four morphs suggests possible gene flow between the irregular and flat morphs (Stireman et al., 2010).

Across gall morphs, the total quantity of carotenoids was positively associated with *Platygaster* attack. This association was also apparent within the crescent morph as crescents developing higher on the plant had more AG carotenoids and a higher probability of *Platygaster* attack. The source of carotenoids in these midges is not known, but carotenoids do increase in *S. altissima* plant tissue with plant height (J.J. Heath, unpublished data), suggesting that crescents sequester carotenoids in proportion to the concentration in plant tissue. While the within-morph association may be explained by factors that affect both carotenoid profiles and risk of parasitism (e.g., younger plant tissues), the relationship between parasitism and carotenoids across gall morphs suggests
a possible causal link. Carotenoids could directly influence parasitoid behavior as precursors to attractive volatile apocarotenoids (Zorn et al., 2003; Lewinsohn et al., 2005), illustrating the potential complexity of tritrophic interactions in this system.

The presence of ABA in the SGs of the flat morphs was confirmed in this study and its localization in the SGs suggests that it is secreted into plant cells during the early stages of gall development, possibly inhibiting induced plant defenses. Furthermore, the growth of some pathogenic fungi is enhanced by the topical application of ABA (Kettner & Dorffling, 1995). While ABA is involved in the induction of some defenses, ABA can also negatively affect the induction of other defenses through inhibitory pathway interactions (Anderson et al., 2004). This suggests that ABA in the SGs of A. carbonifera may be used to manipulate plant physiology to enhance the growth of its symbiotic fungus.

Increased carotenoid production or sequestration may provide crescent morphs a key innovation that allows them to attack unutilized mature tissue and gain enemy-free space, but it also has the potential to increase their apparency to P. solidaginis when they develop on younger tissues. The mechanism underlying this innovation is still under investigation, but the presence of ABA and possibly other carotenoid-derived hormones in the larval SGs is worthy of further investigation. Regardless of whether carotenoids provide a key innovation, enemy-free space is likely maintaining the crescent ovipositional phenotype.

Although gall morphology itself may be adaptive (Stone & Schonrogge, 2003; Chapter 6), behavioral phenotypes also appear to be important in this system as in other
adaptive radiations such as orb-weaving spiders, Anolis lizards, and possibly Hawaiian Drosophila (West-Eberhard, 2003, p. 573; Blackledge & Gillespie, 2004; Johnson et al., 2010). I have examined a coarse ovipositional phenotype here, but crescents also oviposit on the edge of the leaf whereas the other morphs are laid on the interior and Weis et al. (1983) have shown that the degree of loculation, determined proximately by oviposition behavior, affects parasitism rates in A. carbonifera. Furthermore, larval feeding behavior within the gall may influence gall morphology and parasitism risk. It is not only midge behavior, but also parasitoid behavior that may be important in maintenance of these cryptic species (Weis, 1982a; Weis, 1982b). However, forthcoming analyses of the adaptive value of gall morphology are required before the relative importance of behavioral versus morphological traits can be adequately assessed.

3.6 CONCLUSIONS

In this study, I have assessed the fitness value of a behavioral (i.e., ovipositional) phenotype as well as its association with gall morphology and glandular carotenoid quality and quantity in an incipient radiation of gall midges. This ovipositional phenotype may represent an early diverging trait in the radiation of the A. carbonifera clade. Documenting this ovipositional phenotype within the radiation of Asteromyia and subsequently illustrating its utility in terms of a fitness advantage satisfies two of Schluter’s (2000) requirements for an adaptive radiation, while Stireman et al. (2010) have provided evidence of common ancestry and the rapidity of the radiation. Understanding and describing these phenotypes provides the experimental framework for testing for similar ovipositional and carotenoid phenotypes in other clades of Asteromyia
on different host plants where anecdotal evidence suggests they also occur (see Stireman et al., 2010). In addition, this study provides the preliminary evidence for future manipulative experiments that will directly test putative causal links between carotenoids and parasitism, carotenoids and gall development, and carotenoids and gall morphology. It may be that many of the major interactions in this system are mediated, at least in part, by carotenoids and their derivatives.

I illustrate that behavioral divergence can be favored by enemy-free space even when an organism may be physiologically maladapted to the new environment. The net benefit of escaping natural enemies may allow natural selection to mold a physiological adapted phenotype in the new environment, which may have pleotropic effects that further drive a wedge between diverging lineages. Conversely, behavioral inflexibility may constrain natural selection and hinder the colonization of new niches.
3.7 REFERENCES


between insect herbivores and natural enemies. Annual Review of Ecology and

in egglaying site selection by apple maggot flies. Science 218: 76-77.

R Development Core Team (2010). R: A language and environment for statistical
computing. R Foundation for Statistical Computing, Vienna, Austria, URL
http://www.R-project.org/.

Raman, A., Schaefer, C.W. & Withers, T.M. (2005) Galls and gall-inducing Arthropods:
An overview of their biology, ecology, and evolution. Biology, Ecology, and
Evolution of Gall-Inducing Arthropods (eds A. Raman, C.W. Schaefer & T.M.
Withers), pp. 1-33. Science Publishers, Enfield, NH, USA.

Biology of Insect-Induced Galls (eds J.D. Shorthouse & O. Rohfritsch), pp. 87-
101. Oxford University Press, New York, USA.

Sakudoh, T., Sezutsu, H., Nakashima, T., Kobayashi, I., Fujimoto, H., Uchino, K.,
Banno, Y., Iwano, H., Maekawa, H., Tamura, T., Kataoka, H. & Tsuchida, K.
(2007) Carotenoid silk coloration is controlled by a carotenoid-binding protein, a
product of the yellow blood gene. Proceedings of the National Academy of
Sciences of the United States of America 104: 8941-8946.

York, USA.


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Table 3.1  List of study sites with abbreviations, names, and coordinates\(^1\)

<table>
<thead>
<tr>
<th>Site</th>
<th>Site name (all Ohio, USA)</th>
<th>Latitude (N)</th>
<th>Longitude (W)</th>
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<td>BCWMA</td>
<td>Beavercreek Wildlife Management Area</td>
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<td>84°00'16&quot;</td>
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<td>GMP</td>
<td>Germantown Metropark</td>
<td>39°38'21&quot;</td>
<td>84°24'50&quot;</td>
</tr>
<tr>
<td>SSP</td>
<td>Sycamore State Park</td>
<td>39°48'08&quot;</td>
<td>84°21'39&quot;</td>
</tr>
<tr>
<td>WSU</td>
<td>Wright State University <em>Solidago altissima</em></td>
<td>39°47'15.40&quot;</td>
<td>84°03'10.10&quot;</td>
</tr>
<tr>
<td></td>
<td>Common Garden</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Coordinates obtained from Google Maps (retrieved 1 Nov 2011)
Table 3.2 Results of individual ANOVAs on the effect of morph on accessory-gland carotenoid quantity and quality. All degrees of freedom are the same (d.f. = 3, 30).

<table>
<thead>
<tr>
<th>ID</th>
<th>Mean (SE) carotenoid quantity by morph (ng)</th>
<th>ANOVA statistics</th>
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<tr>
<td></td>
<td>Crescent</td>
<td>Cushion</td>
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<tr>
<td>c1</td>
<td>163 (31)</td>
<td>132 (16)</td>
</tr>
<tr>
<td>c2</td>
<td>193 (43)</td>
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<td>c4</td>
<td>157 (34)</td>
<td>0 (0)</td>
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<td>c5</td>
<td>58 (10)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>c6</td>
<td>15 (6)</td>
<td>0 (0)</td>
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<tr>
<td>c7</td>
<td>22 (7)</td>
<td>0 (0)</td>
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<tr>
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<td>29 (8)</td>
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<td>236 (24)</td>
<td>81 (7)</td>
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<tr>
<td>------</td>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>c14</td>
<td>125 (20)</td>
<td>107 (14)</td>
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<td>c15</td>
<td>564 (75)</td>
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</tr>
<tr>
<td>c16</td>
<td>468 (77)</td>
<td>443 (31)</td>
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<td>c17†</td>
<td>905 (218)</td>
<td>558 (68)</td>
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†carotenoid c17 is the thin layer chromatography origin
Figure 3.1  Relative gall height on the plant at gall discovery as a function of relative gall diameter. Only 50 randomly selected points are plotted per morph to avoid cluttering the plot, but the lines are fit to all the data (i.e., flat n = 188, cushion n = 307, irregular n = 210, crescent n = 1579). The intercept of each of the lines represents an estimate of the height of oviposition.
Figure 3.2 Histograms of gall morphs on *Solidago altissima* plants with either the top 20 cm of foliage protected with fine-mesh screening (“screened”) or not (“unscreened”). Under protected conditions the histograms for the flat, cushion, and irregular morphs are not shown because there were absolutely no galls of these morphs after 2 months in the field.
Figure 3.3 Crescent-gall death probabilities over the height of the plant for different mortality factors. The “T2” parasitoids are now known to represent 4 different parasitoid
species: *Aprostocetus tesserus*, *Aprostocetus homeri*, *Baryscapus fumipennis*, and *Closterocerus solidaginis*. Plant resistance was determined by the obvious presence of the hypersensitive response by the plant, which resulted in dead galls soon after they were discovered (see Heath and Stireman 2010).
Figure 3.4  Pearson’s standardized residuals from a chi-squared analysis showing deviations from the expected value under a random *Platygaster solidaginis* attack scenario for four different sites around Ohio, USA. See Table 3.1 for site information.
Figure 3.5 Results of Asteromyia carbonifera egg transplants from field Solidago altissima plants to greenhouse grown S. altissima. The numbers above the bars are the per cent survival to either healthy pupae or adults. Crescent eggs were not included in this experiment because they are extremely difficult to find, but estimates of their survival on mature and young tissue can be found in Figure 3.3F (i.e., 65 to 75%, respectively). Asterisks correspond to the level of significance in planned orthogonal decomposition of the final gall diameters (*** $P < 0.001$; ** $P < 0.01$, * $P < 0.05$). See the text for results of logistic regression of the per cent survival to healthy pupae or adults.
Figure 3.6 (a) Representative thin layer chromatography plates of the carotenoids in the accessory glands of each of the Asteromyia carbonifera morphs and one A. modesta (Mod). Carotenoid numbers indicate and correspond to the labels in Figure 3.7. The red color is a result of equal contrast image enhancement: all spots were uniformly orange in the original scans. (b) A pair of A. carbonifera larval salivary glands showing carotenoids in the filament region only. (c) Three adult female accessory glands from three different morphotypes. (d) UV/Vis spectrum of a pooled sample of A. carbonifera accessory glands in hexane.
Figure 3.7 Canonical discriminant ordination of the carotenoids found in the accessory glands of adult female *Asteromyia carbonifera* morphs. The 95% confidence ellipses on the means indicate significant differences in carotenoid quality and quantity. The vectors indicate the direction of increase with respect to each carotenoid (c1 – c17) or strongly correlated group of carotenoids (cA = mean of c3, c5, c7, and c8). See online color version.
Figure 3.8 The association between mean total carotenoid content of the accessory glands of each morph and the preference of *Platygaster solidaginis* for that morph (preference taken from Figure 3.4). Error bars around the points represent ± one standard error of the mean (SEM).
Figure 3.9 The relationship between total accessory-gland carotenoids and the relative height of the gall on the plant at maturity. Note that gall heights will appear lower on average compared to those in Figure 3.1 because these were measured when the gall was mature, while Figure 3.1 galls were measured at the time of discovery. One crescent (open triangle) was not included in the regression because it was identified as a strong outlier according to Cook’s distance and leverage estimates. See online color version.
Figure 3.10 The concentration (nM) of abscisic acid in the salivary-gland extracts of the four morphs of A. carbonifera. Only the flat morph was analyzed because only it showed levels significantly above the detection threshold.
4 THE ROLE OF CAROTENOIDS AND THEIR DERIVATIVES IN MEDIATING INTERACTIONS BETWEEN INSECTS AND THEIR ENVIRONMENT

4.1 ABSTRACT

Carotenoids are long conjugated isoprenoid molecules derived mainly from plants and microbial organisms. They are highly diverse, with over 700 identified structures, and are widespread in nature. In addition to their fundamental roles as light-harvesting molecules in photosynthesis, carotenoids serve a variety of functions including visual and coloring pigments, antioxidants, and hormone precursors. Although the functions of carotenoids are relatively well studied in plants and vertebrates, studies are severely lacking in insect systems. There is a particular dearth of knowledge on how carotenoids move among trophic levels, influence insect multitrophic interactions, and affect evolutionary outcomes. This review explores the known and potential roles that carotenoids and their derivatives have in mediating the ecological interaction of insects with their environment. Throughout the review I highlight how the fundamental roles of carotenoids in insect physiology might be linked to ecological and evolutionary processes.
4.2 INTRODUCTION

Carotenoids are life sustaining molecules. They play such a critical role in photosynthesis that all life in an oxygenated environment depends on them (Britton 1995a). Carotenoids are one of the most ubiquitous groups of organic molecules known, but how they function in modulating insect-environment interactions is only beginning to be understood. In general, they are long conjugated chains of carbon with rings on either end, which may contain oxygenated functional groups. Carotenoids are essential in photosynthesis to harvest light energy and protect chlorophyll in times of excess light energy by quenching reactive oxygen species that are produced during photosynthesis and plant stress. In animals, their bright yellow to red color is employed as mating signals and aposematic coloration. Although it is just beginning to be investigated, there is growing evidence that carotenoids are important mediators of ecological interactions in insects. Further research into the roles of carotenoids and their derivatives in insect ecology promises to dramatically expand our comprehension of their varied functions and importance (Blount and McGraw 2008).

focus on the known and potential modulating roles that carotenoids or their derivatives play in insect multitrophic and environmental interactions. These modulating functions may contribute significantly to shaping the evolution of many insect taxa.

I begin with a brief overview of carotenoid structures, nomenclature, and biosynthesis. I then briefly review the diversity and functions of carotenoids in plants and discuss their uptake by insects. I review some of the key functions of carotenoids in insects, illustrating their fundamental importance with particular focus on the varied roles of carotenoids in mediating insect-plant interactions. Finally I consider the roles of carotenoids in mediating multitrophic interactions. Throughout the review I highlight areas in need of more research and attempt to link the fundamental physiological (or internal) roles of carotenoids with their more ecological (or external) functions. The varied and essential functions of carotenoids in insects as well as their diet-dependent uptake may provide a series of model systems well suited to studying niche specialization and the complex and poorly understood relationship between phenotype and genotype (Badyaev 2011 and references therein).

4.3 NOMENCLATURE AND STRUCTURES

There are over 700 different identified carotenoid molecules and many more if all the potential isomers are considered. For instance, there are theoretically 1056 possible (E/Z)-isomers of lycopene and 272 for β, β-carotene (Pfander 1992). Furthermore, carotenoids often have chiral centers, which greatly increase the number of possible isomers. However, naturally occurring carotenoids are generally in the all-E form and certain chiral structures predominate.
Carotenoids are divided into two groups: carotenes and xanthophylls. The carotenes are hydrocarbons and the xanthophylls are their oxygenated derivatives. Carotenoids derive much of their diversity from the addition of a number of different functional groups, which are most commonly attached to the rings or the ends of the molecule, but rarely to the center. Most of the tetraterpenoids (i.e., carotenoids with 40 carbon atoms) are named by adding prefixes to the name “carotene.” These prefixes are Greek letters corresponding to the end groups. Note that both end groups are written to be unambiguous. For example, the carotenoid generally known as β-carotene is unambiguously named as β, β-carotene (Figure 4.1). Those carotenoids with fewer than 40 carbon atoms are called apocarotenoids if the loss of atoms occurs at one end of the molecule; diapocarotenoids if it occurs at both ends; and norcarotenoids if it occurs within the molecule. These shortened structures are also generally referred to as norisoprenoids (Britton 2008).

Carotenoids are orange because of the large light absorbing chromophore in the center of the molecule. This sequence of conjugated double bonds absorbs light at about 450 nm and dissipates the energy as heat. Higher wavelengths of light are reflected, giving them their characteristic orange color. The extended chromophore of lycopene absorbs more of the yellow-orange light and thus reflects red light. Carotenoids vary in their absorption maxima, which is useful for identification.

4.4 CAROTENOID BIOSYNTHESIS AND DIVERSITY IN PLANTS

In plants, carotenoids are synthesized from precursors derived from the methylerthritol-4-phosphate (MEP) pathway in plastids (reviewed by Eisenreich et al.)
Generally, 3 units of isopentenyldiphosphate (IPP) and one unit of dimethyl allyldiphosphate (DMAPP) from the MEP pathway condense to form geranylgeranyldiphosphate (GGPP, C20) in plastids; two units of GGPP condense to form phytoene (Dudareva et al. 2006). Four rounds of phytoene dehydrogenation leads to lycopene, which can undergo cyclizations, dehydrogenations, and oxidations to form numerous carotenoid molecules. In plants and algae, monoterpenes, diterpenes, and carotenoids are synthesized similarly in the plastids (Lichtenthaler 1999). Movement of precursors from the plastids (MEP pathway) to the cytosol (mevalonic acid pathway) also occurs (Bartram et al. 2006, Dudareva et al. 2006) and may contribute to the formation of sesquiterpenes, sterols (triterpenes), and polyterpenes in the cytosol (Lichtenthaler 1999). Functional groups may be added in the cytosol as well (Grunewald et al. 2001).

The diversity of carotenoids in plant leaves (i.e., chloroplasts) is generally low with components of the xanthophyll cycle (i.e., violaxanthin, antheraxanthin, and zeaxanthin), β,β-carotene, lutein, neoxanthin, β,e-carotene, β-cryptoxanthin, and lutein 5,6-epoxide being the most commonly occurring. The carotenoids in bold above are generally found at the highest concentrations in leaves and lactucaxanthin occurs in some species such as lettuce (Britton 1995b, Britton et al. 2004). Lycopene is a precursor to all of these, but the metabolic pull is likely so strong that it does not accumulate in the leaves. However, lycopene is a common component of red fruits such as tomato and pepper where photosynthesis is low or non-existent. Carotenoids often contribute to flower color, but interestingly the majority of flower pigments are phenolics (i.e., anthocyanins). Fruit and flower carotenoids are often xanthophylls and as such they can
be and often are conjugated to fatty acids (Britton 1995b), which require saponification to release the free carotenoid for proper identification. This is frequently the case for insect xanthophylls as well.

4.5 CAROTENOID DERIVATIVES

Flavor chemists have long been aware of the production of volatile chemicals from the degradation of carotenoids (Stevens 1970). Numerous in vitro studies have shown that oxygenase, peroxidase, and possibly lipoxygenase (Walter and Strack 2011) enzymes from microbes and plants have the capacity to cleave carotenoids to form volatile apocarotenoids (Zorn et al. 2003, Simkin et al. 2004, Baldermann et al. 2005, Bouvier et al. 2005, Lewinsohn et al. 2005a, Lewinsohn et al. 2005b, Auldridge et al. 2006, Goff and Klee 2006, Ibdah et al. 2006, Garcia-Limones et al. 2008, Scherzinger and Al-Babili 2008, Vogel et al. 2008). In addition, singlet oxygen is capable of cleaving carotenoids on its own and the products can have hormonal properties (Ramel et al. 2012).

Lewinsohn et al. (2005b) showed that a mutant tomato plant and a watermelon cultivar, both extremely deficient in lycopene, produced much less geranial and neral (which are insect semiochemicals, Table 4.1) compared to high lycopene producers, suggesting that these volatiles were produced via lycopene degradation. This could be explained by a general decrease in the terpenoid pathway, since these compounds are both monoterpenes (C_{10}). However, the reason the mutant tomato did not produce lycopene was because it had a defective phytoene synthase, which would leave the upstream portion of terpenoid synthesis intact. As another example, β-ionone is easily
generated via cleavage of $\beta, \beta$-carotene by the oxygenase CmCCD1 (Figure 4.1) and white flesh melon plants deficient in $\beta, \beta$-carotene substrate, but with expression of CmCCD1, lack the production of $\beta$-ionone (Ibdah et al. 2006). Additionally, the same oxygenase can generate $\alpha$-ionone from the cleavage of a $\delta$-carotene (Figure 4.1), geranylacetone from phytoene, and pseudoionone from lycopene (Ibdah et al. 2006). Singlet oxygen reacts with $\beta, \beta$-carotene to form a range of hormonally active apocarotenoids (Figure 4.1 and Ramel et al. 2012). Studies similar to these are numerous and have illustrated the production of a variety of apocarotenoids, which may be very important as insect semiochemicals (Table 4.1).

4.6 CAROTENOID ACQUISITION BY INSECTS

Although animals can modify carotenoids (e.g., cleave, add functional groups, etc.) they generally cannot synthesize them de novo (Kayser 1982, Walter and Strack 2011). One study reported $\beta, \beta$-carotene being synthesized by cockroaches (Shukolyukov and Saakov 2001); however, the authors acknowledged that the potential contribution of symbiotic microorganisms could not be ruled out. In two recent studies (Moran and Jarvik 2010, Altincicek et al. 2011), genes laterally transferred from a fungus and integrated into arthropod genomes (i.e., aphids and mites) appear responsible for the de novo biosynthesis of torulene and related carotenoids, but how widespread these genes might be in insects is not known.

Insects generally sequester carotenoids in proportion to the concentration found in the diet (Feltwell and Rothschild 1974, Ahmad and Pardini 1990) and this often results in
accumulation of lutein, which is the most dominant carotenoid in angiosperms (Pogson et al. 1996). However, they can also concentrate specific carotenoids in specific tissues with the aid of carotenoid binding proteins and active transport mechanisms (Kiefer et al. 2002, Bhosale and Bernstein 2007, Sakudoh et al. 2007) and this can be under hormonal control (Starnecker 1997). Mobile insects may also selectively feed on plants or plant parts to bolster their carotenoid intake in response to environmental stress or enemy attack (Smilanich et al. 2011). Sterospecific oxidative transformation of dietary carotenoids is common in insects resulting in a diversity of final carotenoid molecules in insect tissues, but these usually have structural backbones that represent their dietary source (Kayser 1982).

4.7 DIVERSITY OF CAROTENOID FUNCTIONS IN INSECTS

Carotenoids play many important roles in insect structure, physiology, and life history. They provide coloration; are involved in vision, diapause, and photoperiodism; serve as antioxidants; mating signals, and precursors to pheromones. In order to understand their importance in ecological interactions, I first review some of their known functions in insects and then concentrate on their specific roles in mediating multitrophic interactions.

4.7.1 Coloration

Many insects use carotenoids to color various portions of their bodies, eggs, or even galls (Feltwell and Rothschild 1974, Davidson et al. 1991, Inbar et al. 2010a, Inbar et al. 2010b, White 2010), but few studies have investigated their adaptive significance (Oberhauser et al. 1996). The abundance of brightly colored, sexually dimorphic
butterflies would suggest that carotenoids are important in mate choice, but no studies have shown that carotenoids are involved in butterfly wing color (Nijhout 1991, Shawkey et al. 2009). In monarchs, male wing color does influence mating success, but the source of the orange wing coloration has not been identified (Davis et al. 2007).

4.7.2 Vision, diapause, and photoperiodism

As in vertebrate systems, carotenoids are important to invertebrates as precursors to visual pigment chromophores such as retinal or 3-hydroxyretinal. The involvement of carotenoids in insect vision has been known for decades, but a recent key connection was made by Von Lintig et al. (2001) who found that blindness in a mutant Drosophila strain is due to a dysfunctional carotenoid cleavage dioxygenase that is responsible for biosynthesizing Vitamin A, the direct precursor to the visual chromophores. Many other invertebrates have also been shown to require carotenoids or diet-derived Vitamin A to biosynthesize visual-pigment chromophores (Stavenga 2006). The clear physiological and likely pleiotropic genetic connection between sequestration of carotenoids for vision and the induction of carotenoid-based color polyphenisms in some lepidopteran larvae warrants further investigation.

While the mechanism of photoperiodic induction of diapause in arthropods has not been fully elucidated, carotenoids are clearly involved. The photoperiod induction of diapause requires carotenoids (or Vitamin A) in spider mites, moths, wasps, and butterflies (Veerman 2001). The photoreceptor associated with photoperiod measurement appears to be an opsin receptor that requires Vitamin A (Veerman and Veenendaal 2003), while entrainment of the circadian rhythm is independent of vitamin
A or carotenoids (Veerman 2001). It is not hard to imagine how the fundamental requirement for carotenoids in vision and diapause may dramatically influence ecological and evolutionary outcomes (Figure 4.2), but to my knowledge no studies have made this potential connection.

4.7.3 Antioxidants

Several studies have demonstrated that carotenoids can act as antioxidants in insects. In mammals, ultraviolet (UV) radiation can enhance oxidative stress (Jurkiewicz and Buettner 1994, Shindo et al. 1994) and both UV radiation and oxidative stress are known to be damaging to arthropods (Ahmad and Pardini 1990, Aarseth and Schram 2002, Suzuki et al. 2009). Through a combination of blocking UV light, which activates prooxidant allelochemicals, and by direct quenching of singlet oxygen generated from prooxidant allelochemicals, carotenoids can protect vital cellular components from damage (Ahmad 1992, Carroll et al. 1997, Carroll and Berenbaum 2006). The abundance and diversity of carotenoids in particular insects may relate to the UV environment in which the species has evolved. Carotenoids can also contribute to the immune response in arthropods, probably by scavenging reactive oxygen species associated with up-regulation of the immune system (Ojala et al. 2005, Babin et al. 2010, Smilanich et al. 2011).

4.7.4 Mate choice and signaling

Volatile apocarotenoids are found as components of short-range courtship pheromones released from structures in male butterflies called hairpencils and as pheromone components of many hymenopterans (Table 4.1). Males of Pieris napi
butterflies produce citral, which is a mixture of the apocarotenoids neral and geranial (Figure 4.2, Table 4.1). Odorless male models are always rejected by females, but both citral-laced models and freshly killed males stimulate female mate-acceptance behavior (Andersson et al. 2007). In butterflies, these volatiles may be produced within the body from sequestered carotenoid precursors, although this has not been documented.

4.8 ROLES OF CAROTENOIDS AND THEIR DERIVATIVES IN MEDIATING INSECT-PLANT INTERACTIONS

4.8.1 Mediation of oxidative stress

The antioxidant functions of carotenoids are likely a key feature in modulating insect plant-interactions as many plants produce photosensitized prooxidant compounds such as acetophenones, carboline alkaloids, furanochromes, furanocoumarins, furanoquinoline alkaloids, extended quinones, isoflavonoid phytoalexins, isoquinoline alkaloids, lignans, polyacetylenes, and thiophenes (Berenbaum 1987). Metabolically activated compounds such as quinones and flavonoids are also important prooxidant allelochemicals. These compounds can react with molecular oxygen to produce a range of reactive oxygen species (ROS), including superoxide anion radical (O$_2^•$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (•OH), lipid hydroperoxides, peroxyl radicals, and singlet oxygen (1O$_2$). Of these, •OH and 1O$_2$ are the most reactive and therefore damaging to cellular components such as membranes (Ahmad and Pardini 1990). Carotenoids are most effective against 1O$_2$ either through physical or chemical quenching (Figure 4.1), the latter of which cleaves the carotenoid molecule into smaller volatile apocarotenoids (Stratton et al. 1993, Sommerburg et al. 2003, Ramel et al. 2012), but
they can also scavenge peroxyl radicals (Sommerburg et al. 2003). Carotenoids can also have significant indirect effects through the protection of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), which work together to deactivate $\text{O}_2^\bullet^-$. SOD converts $\text{O}_2^\bullet^-$ to $\text{H}_2\text{O}_2$, which is rapidly converted to $\text{H}_2\text{O}$ and $\text{O}_2$ by CAT before $\text{H}_2\text{O}_2$ forms the more damaging $\text{•OH}$ radical. Singlet oxygen and peroxyl radicals are known to inhibit SOD and CAT activity (Escobar et al. 1996) and thus carotenoids may play key roles in protecting insects and other organisms from both exogenous (e.g., via plant allelochemicals) and endogenous (e.g., via metabolic by-products) sources of ROS (Ahmad 1992).

Carroll et al. (1997) showed that parsnip webworms avoid damaging UVA light when reared on a carotenoid-free diet, but when the diet was amended with lutein (a xanthophyll) or the caterpillars were allowed to feed on their host plant, they did not avoid UVA radiation. UVA avoidance is of particular importance in this system because host plants contain UV-activated phototoxic furanocoumarins. Subsequently, Carroll and Berenbaum (2006) found a significant correlation between larval lutein concentration (i.e., sequestration) and daily UV irradiance in wild populations of webworms collected across an altitudinal/latitudinal UV gradient. More direct studies have also shown the benefits to insects of sequestered carotenoids. For example, $\beta$-carotene amended diets provide significant protection from topical applications of alpha-terthienyl, a phototoxic phytochemical, in *Manduca sexta* larvae (Aucoin et al. 1990, Aucoin et al. 1995). And, an association between sequestration of carotenoids and larval success has recently been
documented in which carotene-sequestering larvae were much more likely to survive feeding on a toxic host plant (Shao et al. 2011).

The antioxidant properties of carotenoids may also augment the immune response in insects and other arthropods by scavenging ROS generated during this process (Cornet et al. 2007), but this may come with ecological costs associated with conspicuous pigmentation. Vander Veen (2005) showed a plastic regulation of carotenoid content in individual copepods in response to predation risk. Copepods that down-regulated their carotenoid content in response to elevated risk of predation were also more susceptible to parasite infection, suggesting a tradeoff between predation avoidance and immunocompetence.

The antioxidant function of carotenoids in arthropods is an area in need of more study, particularly with respect to their roles in influencing interactions between herbivores and their host plants (Ahmad 1992, Felton and Summers 1995). The antioxidant properties of carotenoids may be enhanced by the presence of other antioxidants such as ascorbic acid or tocopherols (Catoni et al. 2008), but studies in arthropods are lacking. The importance of carotenoids in aposematism might be augmented by this potential positive interaction, since other potent antioxidants are colorless. The use of carotenoids as protective antioxidants may be important in shaping insect niches. They may allow herbivores to expand their dietary range to phototoxin-protected plants (or to specialize on such plants for their own defense), persist under conditions with high parasite pressure (Smilanich et al. 2011), or feed in more UV-rich habitats.
Carotenoids may also be important in managing oxidative stress associated with herbivory and pathogen attack in plants. Plants are predicted to benefit from limiting the spread of oxidative stress to localized tissues in order to limit the use of valuable antioxidants and to attract herbivore enemies. The cells closer to the damaged site are more likely to be in an oxidizing state and thus more conducive to the formation of apocarotenoids (Oliveira et al. 2011 and references therein). Attack by phytophagous insects and pathogens is known to cause the induction of several defensive pathways in plants (Maleck and Dietrich 1999). Bi and Felton (1995) showed unequivocally that the oxidative status of soybean plants changes after herbivory and that herbivores are negatively affected by this onslaught of oxygen radicals. Earlier work showed that pathogen infection resulted in a similar reaction (Mehdy 1994). During herbivory, antioxidants such as ascorbic acid, total carotenoids, nonprotein thiols, and catalase decreased systemically in plants (Bi and Felton 1995). The breakdown of carotenoids, chlorophylls, and the production of VOCs may be symptomatic of autotoxicity or the result of strategic management of ROS by plants. Alternatively, these decreases may represent a strategy employed by herbivores and pathogens to overwhelm plant defenses. One would predict that the intensity of this effect should increase with proximity to the damage site, thus explaining the more prevalent decrease of carotenoids and chlorophylls locally as opposed to systemically (see citations in the “Trophic signaling” section below).
4.8.2 Resource signaling - color

The essential physiological roles that carotenoids play in photosynthesis (Britton 1995a, Telfer et al. 2008) such as harvesting light energy and preventing photo-oxidative damage via the xanthophyll cycle (Demmig-Adams and Adams 1996), suggest that changes in host-plant carotenoid content should provide information to foraging herbivorous insects about the quality of the tissue, because photosynthesis ultimately provides carbon sources and energy for nitrogen fixation and/or assimilation. Conversely, if carotenoids correlate strongly with defensive chemicals, insects may be repelled.

W.D. Hamilton proposed that temperate deciduous plants signal their defenses to herbivores via brightly colored fall foliage (Archetti 2000). This idea is consistent with the finding that aphid diversity is greater on host plants with colored fall foliage, suggesting a co-evolutionary history (Hamilton and Brown 2001). However, it may be more likely that colored fall foliage is simply used as a cue rather than evolving as a signal (Schaefer and Rolshausen 2006). This controversial idea (Wilkinson et al. 2002) spurred a series of papers (reviewed in Lev-Yadun and Gould 2007) that proposed alternative ecological explanations for brightly colored fall foliage. All of these hypotheses recognize the fact that the proximate reason for fall color is to protect the plant from photoinhibition during chlorophyll scavenging and nitrogen storage when temperatures drop but light is still relatively intense. Theoretically, carotenoids could signal honestly to herbivores that the carotenoid biosynthetic pathway, which also leads to the biosynthesis of mono- and diterpenoids (Lichtenthaler 1999), is well established or
still functioning. Monoterpenoids (De Moraes et al. 1998) and possibly apocarotenoids (see section on odors) are important attractants for natural enemies, while diterpenoids have demonstrated antiherbivore properties (Gebbinck et al. 2002). Alternatively, it has been argued that green autumn leaves rather than colored foliage should be more attractive to natural enemies and less nutritious to phloem-feeding aphids (Holopainen 2008). Although many studies have confirmed the physiological link between carotenoid quality/quantity and photosynthetic capacity (Telfer et al. 2008), only one study has shown that these pigment differences directly affect the behavior and fitness of insect herbivores. Zheng et al. (2010) used gene silencing techniques to show that plant tissues that lacked carotenoids and became photo-bleached were both less attractive to an ovipositing butterfly and less nutritious for its offspring than tissue from fully competent plants. Future studies in this regard will benefit from focusing on the fitness consequences of intraspecific host-plant variation in carotenoids where stabilizing or directional selection will likely be most obvious and quantifiable. Manipulative experiments designed to disrupt the biosynthesis of specific carotenoid pigment molecules would also be informative.

Many fruits and flowers derive their bright colors from carotenoids. In some cases, these colors may warn potential herbivores (particularly mammals) that the fruit is toxic, such as the red color of capsaicin-containing red peppers; but more often these colors serve to attract seed dispersers and pollinators (Tewksbury and Nabhan 2001). Almost every orange or yellow fruit and many red fruits contain carotenoids. Indeed, the red color of tomatoes is due mainly to the accumulation of the carotenoid lycopene.
The control of flower color by carotenoid content is evident from breeding experiments and transgenic plants. Suzuki et al. (2007) were able to add a gene to the carotenoid biosynthetic pathway of *Lotus japonicus*, thus changing its flower color from a lemon yellow to a bright orange. Ohmiya et al. (2006), changed wild-type white chrysanthemum flowers to yellow via RNA interference of a carotenoid cleavage dioxygenase (CCD) enzyme. These enzymes are widespread in plants and are very important in conferring odor and flavors to fruits and flowers. CCDs cleave carotenoid molecules at double bonds producing molecules with shorter chromophores that do not absorb visible light (see section on odors). The color of fruits and flowers can have dramatic ecological consequences (Whitney and Glover 2007). For instance, Bradshaw and Schemske (2003) illustrated an immense reversal in pollinator preference when they bred color changes into the monkeyflowers *Mimulus lewisii* and *M. cardinalis*. Furthermore, mutant *M. lewisii* with reduced carotenoid-based nectar guides receive fewer successful bumble bee visits (Owen and Bradshaw 2011), but Dyer et al. (2007) warn of the possible confounding effects of not using completely isogenic lines. Regardless of whether isogenic lines are used, the potential for confounding effects seems likely because CCDs can change both color and odor simultaneously.

The most obvious selective force maintaining fruit color would seem to be seed dispersing frugivores such as birds, but evidence for this is surprisingly scarce (Whitney and Stanton 2004). Whitney and Stanton (2004) have shown that pleiotropic effects (or possibly linkage) appear to maintain a carotenoid-derived fruit polymorphism in *Acacia ligulata* via selection by a heteropteran seed predator. Interestingly, carotenoids may also
be important in mate signaling in this system because females develop a yellow color on the legs at sexual maturity. A number of plants incorporate toxic secondary metabolites in their ripe fleshy fruits, an apparent contradiction to the dispersal hypothesis (Cipollini 2000). There seems to be a delicate balance between dispersal, predation, and microbial degradation that must be optimized by plants over space and time (Cipollini 2000, Tewksbury et al. 2008).

### 4.8.3 Resource Signaling - odor

Carotenoids may also play important roles as olfactory cues for insects and as olfactory signals by plants. Volatile apocarotenoid (i.e., derivatives of carotenoids) have a range of functions that can benefit the plant directly, indirectly, or are detrimental. Many apocarotenoids are likely to be pollinator attractants, since they are highly represented in flower volatiles (Table 4.1), including those of orchids (El-Sayed 2011). Interestingly, many also appear to be hymenopteran pheromones (Table 4.1) and some act as courtship pheromones in butterflies. Apocarotenoids are also associated with the flavor and odors of ripe fruit and likely benefit plants by attracting seed dispersers (Cipollini 2000). However, they can also act inadvertently as feeding attractants (Table 4.1).

Apocarotenoids, such as α and β-ionone (Figure 4.1), have been shown to be strong attractants (Williams et al. 2000) or deterrents (Wei et al. 2011 and references therein) of phytophagous insects. α-Ionone may act as an intraspecific aggregation cue produced by actively feeding insects, which by continuously crushing plant cells, may mix cytosolic carotenoid cleavage oxygenases (CCOs) with plastid-localized carotenoids or may directly degrade them with CCOs in their saliva (Heath et al. 2002, Rhainds et al. 2011).
These CCOs are known to be much more active under high light conditions (Scherzinger and Al-Babili 2008) and diurnal phytophagous beetles require high light conditions to find mates (Heath et al. 2001). However, apocarotenoids can also be produced chemically under oxidizing conditions; that is, without the direct aid of enzymes (Stratton et al. 1993, Gessler et al. 2002, Walter and Strack 2011, Ramel et al. 2012). This is interesting as continuously damaged plant cells experience oxidizing conditions and many plants generate singlet oxygen and other ROS with the aid of photosensitized allelochemicals (Ahmad and Pardini 1990).

4.8.4 Manipulation of plant chemistry with carotenoid-derived hormones

Mutualistic associations with microbes have likely played an important role in the phenomenal evolutionary and ecological success of insects (Moran 2002, Janson et al. 2008). Virtually every insect species that has been examined closely has been found to be engaged in some form of microbial mutualism, most frequently in the form of gut-associated bacteria (e.g., Buchner 1965, Douglas 1998). However, insect-fungal symbioses are also widespread across many insect groups including beetles (Scolytinae: Bentz and Six 2006), ants (Formicidae: Mikheyev et al. 2006), moths (Tortricidae: Fermaud and Lemenn 1989), and flies (Borkent and Bissett 1985, Gagné 1989, Schiestl et al. 2006, Heath and Stireman 2010). In these associations, the insect typically benefits from using the fungus as a food source (Bissett and Borkent 1988, Cherrett et al. 1989, Farrell et al. 2001, Janson et al. 2009, Heath and Stireman 2010) in exchange for dispersing the fungus or promoting fungal outcrossing (Schiestl et al. 2006). Given the current understanding of the hormonal regulation of plant defense chemistry, the ability
of microbes to synthesize plant hormones (Table 4.2), and the induction of certain defense pathways in plants by microbes and herbivores, the potential exists for cooperating organisms to short-circuit these pathways toward their mutual advantage. Carotenoid derivatives may provide a means for such manipulation.

There are six groups of plant hormones traditionally recognized. These are auxin, ethylene, cytokinins, gibberellins, abscisic acid (ABA), and brassinosteroids (Kende and Zeevaart 1997). Recently, strigolactones were added as a seventh class (Pichersky 2008). Two of these classes, ABA and strigolactones, are derived in part or in whole from carotenoid precursors and additional uncharacterized carotenoid-derived plant hormones may also exist (Walter and Strack 2011, Ramel et al. 2012).

Strigolactones are biosynthesized via the oxidative breakdown of carotenoid precursors via carotenoid cleavage oxygenases (Matusova et al. 2005, Humphrey and Beale 2006, Humphrey et al. 2006, Lopez-Raez et al. 2008) and have been found in plant species from at least 13 different families (Awad et al. 2006, Bouwmeester et al. 2007, Steinkellner et al. 2007, Yoneyama et al. 2007a, Yoneyama et al. 2007b). Cook et al. (1972) identified strigol as a germination stimulant for the parasitic weeds in the genus Striga and numerous subsequent studies have verified this (reviewed in Scholes and Press 2008). More recently, experiments have shown that the long sought after fungal branching factor associated with successful colonization by arbuscular mycorrhizal fungi is a strigolactone, 5-deoxystrigol (Figure 4.2, Akiyama et al. 2005). Strigolactones are also the causal agent in prohibiting above-ground lateral branching in Arabidopsis, rice,
and pea; illustrating their importance to plant architecture (Gomez-Roldan et al. 2008, Umehara et al. 2008) and quite possibly its manipulation by galling insects.

ABA is synthesized in plants from carotenoid precursors via a carotenoid cleavage oxygenase (Schwartz and Zeevaart 2004, Wasilewska et al. 2008). Interestingly, many fungi also have the ability to produce ABA (Table 4.2), but it appears that they use the mevalonic acid pathway and not carotenoid precursors (Schwartz and Zeevaart 2004). ABA plays a central role in seed development, stomatal regulation, and plant responses to abiotic stressors such as osmotic, drought, and possibly cold stress (Assmann 2004, Finkelstein 2004, Yamaguchi-Shinozaki and Shinozaki 2006); evidence is mounting that ABA is also centrally involved in regulating responses to biotic stressors (Anderson et al. 2004).

It is well established that plants react to biotic stresses such as viruses, bacteria, fungi, and herbivorous insects by up-regulating defensive pathways (Mauch-Mani and Mauch 2005). These induced defenses are regulated by two major biochemical signaling pathways: the salicylic acid (SA) pathway and the jasmonic acid/ethylene (JA) pathway (Spoel et al. 2003). When a plant is attacked, either the SA or JA pathway is induced, but generally not both. This is because the pathways can be reciprocally inhibitory (Kunkel and Brooks 2002, Cipollini et al. 2004, Lorenzo and Solano 2005). Furthermore, ABA can negatively interact with both the SA and JA pathways (reviewed by Lorenzo and Solano 2005, Mauch-Mani and Mauch 2005, Fujita et al. 2006, Asselbergh et al. 2008), thus playing a central role in regulating plant responses to both abiotic and biotic plant stresses. It is known that environmental conditions that induce increases in plant ABA
levels (e.g., drought) enhance disease susceptibility in some pathosystems (e.g., Ma et al. 2001, Mayek-Perez et al. 2002, Koga et al. 2004, Garrett et al. 2006). Positive relationships between drought and disease severity may be related to the negative effect of ABA on biotic stress signaling. However, this response may not be universal as the severity of pathogen attack can also be negatively correlated with drought stress (Achuo et al. 2006, Enright and Cipollini 2011). However, directly increasing plant levels of ABA (either by exogenous application or via mutant plants) has been shown to negatively affect disease resistance in a number of studies (Henfling et al. 1980, Salt et al. 1986, Ward et al. 1989, McDonald and Cahill 1999, Audenaert et al. 2002, Mohr and Cahill 2003, Thaler and Bostock 2004, Mohr and Cahill 2007).

Studies supporting a negative relationship between ABA and plant resistance continue to accumulate. This opens the possibility that microbes or other plant-feeding organisms could manipulate their hosts through hormonal interference; however, documentation that microorganisms produce ABA at the infection site or actively regulate its level in plants are lacking. To my knowledge, only Kettner and Dorffling (1995) have shown unequivocally that fungus-derived ABA can explain variation in plant levels of ABA during the infection process of tomato plants by Botrytis fungi.

One of the few studies to evaluate the effects of ABA on both pathogens and insect herbivores found that ABA-deficient tomato mutants had lower levels of disease caused by Pseudomonas syringae, but supported higher growth rates of Spodoptera exigua larvae than control plants (Thaler and Bostock 2004). The mutants also showed higher levels of the SA-inducible PR4 gene, which likely contributed to the observed
increase in pathogen resistance. The potential for cross-talk among these defensive pathways provides an opportunity for the evolution of mutualistic associations between biotrophic pathogens and insects. For instance, herbivore feeding could induce the JA pathway thus down-regulating the SA pathway or pathogens could induce the SA pathway, down-regulating the JA pathway. Alternatively, herbivore production of ABA could modulate the interaction between the JA and SA pathways as suggested by the results of Thaler and Bostock (2004).

I have recently found carotenoids and ABA in the salivary glands of the Ambrosia galler *Asteromyia carbonifera* in concentrations well above those required for physiological effects on the plant (see Chapter 3). Many cecidomyiids, including *A. carbonifera*, have obligate fungal symbionts. This hormone may be instrumental in allowing growth of their symbiotic fungus, which forms the gall structure and is their primary food source (Janson et al. 2009, Heath and Stireman 2010). As with other systems in which larvae have glandular carotenoids (Eichenseer et al. 2002, Sakudoh et al. 2007) understanding the ecological tradeoffs is key to ascribing a role for these compounds.

### 4.9 CAROTENOIDS IN INSECT-ENEMY AND TRITROPHIC INTERACTIONS

#### 4.9.1 Aposematic and cryptic coloration

Because carotenoids generally must be obtained from the environment and may be limiting, access to carotenoids may influence insect population dynamics and niche evolution via their effects on susceptibility to enemies and presumably mate choice (see
also the section on odors). Carotenoid pigmentation is correlated with levels of toxic defensive compounds and can function as precursors to these compounds, such as the predator-repellent grasshopper ketone (Figure 4.2, Meinwald et al. 1968). Carotenoids can underlie not only yellow, orange, or red coloration, but also green (in some insects) and purple-blue (in other arthropods) when combined with specific carotenoproteins or chlorophyll degradation products such as pterobilin. Carotenoproteins anchor the carotenoids in specific configurations that change the spectral qualities of the chromophore and allow the absorption of light in uncharacteristic regions of the visible spectrum (Britton and Helliwell 2008).

Aphids are known to derive body color from carotenoids and frequently exhibit intraspecific color morphs (Losey et al. 1997). Most insects are thought to obtain carotenoids from their diets; however, aphids are phloem feeders and the carotenoid concentration in phloem is expected to be low (Czeczuga 1976). Several studies have shown the presence of torulene, a red carotenoid, in aphid and katydid color morphs. Torulene is rare in plants and it is hypothesized to be biosynthesized by symbiotic microbes (Weisgraber et al. 1971, Britton et al. 1977, Jenkins et al. 1999), but may be derived from laterally transferred fungal genes in some insects (Moran and Jarvik 2010). Carotenoid-derived colors can have substantial ecological consequences in aphids and butterflies (Gerould 1921). For instance, Losey et al. (1997) found that the maintenance of a red-green color polymorphism in the pea aphid was regulated by differential predation and parasitism. These opposing forces result in equilibrium between the red and green morphs. Frequency dependent selection, mediated by parasitoid learning, can
also result in equilibrium between the aphid color morphs (Langley et al. 2006) and recent studies indicate that increased light can induce a much darker color morph even within a clone (Alkhedir et al. 2010). Whether these aphid color morphs represent some form of crypsis or aposematism has not been determined.

For signals to evolve and persist, they must be honest (Zahavi 1975) or be augmented by a high frequency of honest models in their environment. Current theory and evidence indicates that mate signaling traits should be strongly correlated with individual health and condition (Berglund et al. 1996), but not necessarily genetically linked or even under direct genetic control (Kodric-Brown and Brown 1984, Sandre et al. 2007). And Badyaev et al. (2001) illustrates that carotenoid-based plumage in birds is actually a complex, composite representation of several aspects of individual condition, which suggests the existence of physiological or developmental tradeoffs. The physiological connection between carotenoids and protection from autotoxicity, immunity, biosynthesis of defense (e.g., smaller terpenes), and antioxidant capacity may make them particularly useful for color signaling both within and between species (Blount et al. 2009), but in insects the expected physiological tradeoffs have not been forthcoming (Sandre et al. 2007). Nevertheless, correlations between coloration and defensive traits have been found. The bright red, carotenoid-derived elytral coloration of many ladybird beetles has the potential to signal honestly the level of beetle toxicity to potential predators (Bezzerides et al. 2007). Upon attack by predators, *Harmonia axyridis* ladybird beetles react with reflexive bleeding and release the defensive alkaloid harmonine, the concentration of which is significantly correlated with the proportion of
elytra area that is red (Bezzerides et al. 2007). Aposematic coloration is also correlated with carotenoid concentration in *Leptinotarsa* leaf beetles that appear to sequester carotenoids from their host plants (Poff 1976). Carotenoids have been found in whole-body and hair-tuft extracts of a number of aposematic Lepidoptera species (Sandre et al. 2007, Blount and McGraw 2008), including monarch butterflies where it is responsible for the dramatic yellow striping of caterpillars (Rothschild et al. 1978). Several aposematically colored butterflies exhibit levels of carotenoids that correlate positively with toxic prooxidant compounds, suggesting that carotenoids may protect susceptible tissues from autotoxicity as well as provide warning coloration (Rothschild et al. 1986, Nishida et al. 1994). Interestingly, in butterfly systems involving mimicry, mimics consistently have lower carotenoid concentrations than their models. This pattern does not hold up in the classic monarch-viceroy batesian mimicry (Rothschild et al. 1986) and may have led Ritland and Brower (1991) to re-examine and ultimately reverse our understanding of this classical mimicry system (Rothschild 1991).

In insects, blue pigments such as pterobilins can combine with carotenoids to yield cryptic coloration (Rothschild and Mummery 1985). Larvae of the butterfly, *Colias philodice*, and the stick insect *Dixippus morosus* both combine carotenes with unknown blue pigments to achieve a green color (Fox 1976). Many lepidopteran larvae are also colored green by the physical mixing of blue chlorophyll degradation products and carotenoids (Rothschild and Mummery 1985). Furthermore, the intensity of the green color is correlated with carotenoid concentration (Grayson et al. 1991). Interestingly, some of these cryptic larvae have elaborate polyphenisms that allow them to alter their
coloration over the course of larval development to match the color of their environment (Grayson and Edmunds 1989, Noor et al. 2008). This response can be triggered by the quality of the diet (Greene 1996) and/or by the quality of light perceived by the larvae (Grayson and Edmunds 1989, Noor et al. 2008) and appears to be adaptive (Edmunds and Grayson 1991).

These systems seem ideally suited for the investigation of genetic assimilation as some of the species in these groups appear to be relatively fixed for certain color morphs that may have been derived from ancestors with flexible polyphenisms. Genetic assimilation purports to explain how polyphenisms can become fixed in populations and thus facilitate speciation and niche specialization. Although Grayson and Edmunds (1989) allude to this concept, they do not expound on its potential importance. The power of genetic assimilation to facilitate speciation comes from the conditional ratchet effect (reviewed by West-Eberhard 2003) that polyphenisms can have on natural selection. That is, because a particular polyphenism is generally only expressed under conditions in which it is likely to be beneficial, natural selection tends to select for a lower induction threshold for that trait. Therefore, any expressed polyphenisms with an equal or lower induction threshold (i.e., genetically controlled) are more likely to show up in the next generation. Given the right environmental conditions, this process can produce a more-or-less fixed trait (West-Eberhard 2003). Interestingly, threshold evolution has been documented in dung beetles where expression of horned males is ultimately controlled by size and proximately controlled by the terpenoid, juvenile hormone (Moczek and Nijhout 2002).
4.9.2 Tritrophic signaling

One interesting phenomenon observed frequently in studies of plant-arthropod interactions is a decrease in chlorophyll and carotenoids in *uninfested* tissues of plants *infested* with phytophages. This chlorosis occurs locally at the feeding site (Mothes and Seitz 1982, Hildebrand et al. 1986b, Ni et al. 2002, Heng-Moss et al. 2003), but also within undamaged tissue remote from the feeding site (Schuster et al. 1990, McAuslane et al. 2004). Numerous studies have also demonstrated ultrastructural changes in chloroplasts associated with plant cells near feeding sites, which seems to correspond to a decrease in the quantity of pigments in these cells (Oliveira et al. 2011) as well as starch grains. This is especially true of pathogen-infected cells (Camp 1981, Rey 1992) and the nutritive cells induced by galling insects (Birch et al. 1992, Bronner 1992, Rey 1992, Westphal 1992, Oliveira et al. 2011), but also of cells near caterpillar feeding sites (Maffei et al. 2004). The ultimate fate of the chlorophyll and carotenoids associated with chlorosis and major changes in chloroplasts has not been studied. However, the higher activity of prooxidant enzymes in these cells (Hildebrand et al. 1986a) may result in significant releases of volatile apocarotenoids (Salt et al. 1986, Figure 4.2).

Given that many hymenopterans respond to volatile apocarotenoids (Table 4.1) and that insect feeding is associated with chlorosis, it is surprising that the recent proliferation of plant studies on systemically inducible volatile organic compounds (VOCs), which can attract parasitoid wasps, (reviewed in Arimura et al. 2005) has failed to identify any obvious volatile apocarotenoids associated with insect feeding. Although systemic decreases in chlorophyll and carotenoid content have been observed in damaged
plants (McAulane et al. 2004), studies indicating local decreases (i.e., near the feeding site) are more prevalent and more intense. It is possible that volatile apocarotenoids are released at the feeding site, but that inducible, systemically released volatile organic compounds obscure detection of locally released apocarotenoids. Indeed, it is not uncommon to find parasitoid studies with lists of unidentified electroantennographically-active compounds that are either masked by larger peaks or are undetectable by gas chromatography (e.g., Gouinguene et al. 2005). Most studies involving systemically induced volatile emission have measured the volatiles from whole plants; few studies have concentrated on the volatiles emitted only from the feeding site. Coleman et al. (1997) showed that the parasitoid *Cotesia glomerata* can distinguish between feeding sites of *Pieris brassicae* larvae on *Brassica oleracea* with stronger attraction to larvae feeding on the top most leaves. They also illustrated that the parasitoids were unable to distinguish between infested and uninfested plants when the larvae were feeding on lower leaves. Tissue age and environment can influence leaf carotenoid concentration and may explain the lack of attractiveness of larvae feeding on lower leaves (Feltwell and Valadon 1974, Rothschild et al. 1986, Norman et al. 1990, Hartel and Grimm 1998). For example, *B. oleracea* core tissue with limited light exposure has up to 30-fold less carotenoid than light-exposed tissues (Bondi and Meyer 1946).

If apocarotenoids are in fact important volatile cues for natural enemies, they are more likely to be discovered by comparing systemically released volatiles with those emanating from local feeding sites. Theoretically, the volatiles emitted from local feeding sites are predicted to be more important in natural enemy attraction than those
released systematically both from a behavioral and a plant physiological perspective. Therefore, a renewed focus on the volatiles specifically associated with the feeding site could provide better natural enemy attractants as well as test the presence of apocarotenoids.

The quantity of volatiles emitted from a local feeding site will be much lower than those released systemically simply because of the reduction in tissue involved. However, from a behavioral perspective this is not likely to present a problem for predators and parasitoids. Insects are known to be highly sensitive to volatile compounds. Workers have shown that a single odor molecule is sufficient to evoke a nerve impulse (Kaissling 1996) and semiochemicals often have extremely high physiological activity at levels so low as to be nearly undetectable by even the most sensitive of chromatographic equipment (e.g., femtogram levels, Heath et al. 2005). Furthermore, volatiles emanating from a point source are far more useful as odor cues than broadly emitted sources. In fact, male moths flying in a homogenous cloud of female pheromone behave as if they were flying in clean air (Baker 1985). This is due to the physiological and behavioral mechanisms of odor source perception and location in insects. Insects do not locate an odor source by flying up a concentration gradient, but rather by responding stereotypically to pulses or filaments of highly concentrated odor (see Baker and Vickers 1997 for a review of optomotor anemotaxis). Therefore, the volatiles emanating from a point source (e.g., the damaged site of an actively feeding herbivore) are likely to be more important in host location than those released systemically. Furthermore, studies have found that galling insects fail to induce the systemic release of volatile organic
compounds that are typically thought to attract predators and parasitoids (Tooker and De Moraes 2007, Tooker and De Moraes 2008, Tooker et al. 2008) and detailed studies have shown a much more intense reduction in pigments near the feeding sites of galling insects (Oliveira et al. 2011 and references therein). Perhaps the high parasitism rates experienced by many galling insects is based instead on visual or other locally emitted volatile cues.

Carotenoids are a huge reserve of precursors for volatile apocarotenoids that may prove to be important in the attraction of natural enemies to actively feeding hosts. That plants have evolved the ability to induce the production of VOCs for the sole purpose of attracting natural enemies is unlikely. I am not suggesting that natural enemies do not respond to these compounds or that plants do not benefit from attracting them, but rather that natural enemy attraction has been an epiphenomenon of plant defense, including autotoxic protection from their onslaught of prooxidant species. Provided that plants enjoy a fitness benefit from attracting natural enemies, they may have fine-tuned the release of volatiles, especially from the feeding site, over evolutionary time, to serve a secondary purpose of attracting carnivores. Furthermore, I predict that some of the most important volatiles are likely to be derived from carotenoid precursors. In fact, in the rapidly radiating complex of the nominal species Asteromyia carbonifera (Stireman et al. 2008, Janson et al. 2010, Stireman et al. 2010) I have found that the quality and quantity of adult accessory gland carotenoids is consistently gall-morphotype specific and correlates nearly perfectly with attack by an egg parasitoid both within and across morphotypes (see Chapter 3).
4.10 CONCLUSIONS

The ubiquity, physiological necessity, and varied functions of carotenoids and their derivatives across the tree of life suggests that they may also play significant roles in insect ecology and evolution, as limiting resources, as defensive or repellent compounds, and as signals or cues. In particular, emerging evidence of the roles of carotenoids as self-protective antioxidants, as precursors to plant hormones such as strigolactones and ABA, and as precursors to volatile apocarotenoids suggests that they may be important mediators of plant-insect, insect-mutualist, and tritrophic interactions. Their potential as volatile chemical cues that parasitoids and other insects could use to find their herbivorous prey within the torrent of intermingling odors in nature is promising, but largely unexplored. The co-option of hormone derivatives of carotenoids suggests a novel way in which insects may manipulate plant growth and defense signaling pathways and conspire in mutualistic associations with microbes and fungi. The many critical roles of carotenoids in coloration, vision, diapause, photoperiodism, as antioxidants, and as defense compounds suggest that they may often be a limiting resource for insects and therefore represent an important dimension of the ecological niches of many insect taxa. This would also suggest that insects should have fine-tuned systems for detecting and assessing carotenoid quality and quantity of their food, but this remains largely unexplored.

Research aimed at understanding the roles that carotenoids and their derivatives play in species interactions should have a multitrophic perspective starting first at the level of the primary producer and working up the food web to higher trophic levels. With this in mind, tracking the flow of major plant carotenoids (with the use of stable isotopes...
or radiolabelling) through herbivores and on to higher trophic levels would provide a framework for further assessing the relative contribution of maternal, dietary, symbiotic, and potential de novo sources of insect carotenoids. At the plant level, the role of carotenoids in protecting plants from their own production of potentially autotoxic, photosensitized allelochemicals or ROS is largely unexplored and depending on the degree to which autotoxicity is a factor, carotenoids may modulate tradeoffs between defense and other plant systems such as growth, photosynthesis, or reproduction. This work would help place the plant within the emerging concept of universal adaptive strategy theory (Grime and Pierce 2012). At the herbivore level, carotenoids may protect against photosensitized plant allelochemicals either directly or via indirect protection of antioxidant enzymes, but work in this area is lacking in insects. As mating signals, carotenoids may provide both color directly and odor via apocarotenoids, but the potential tradeoffs here have not been assessed nor the degree to which carotenoid-based sexual selection is involved in the evolution of insect life-history strategies. Probably the most interesting and practical area for further study is with regard to the third trophic level. Apocarotenoids generated by the plant at the herbivore feeding site have great potential as cues to foraging parasitoids and predators of the presence of herbivores, but to my knowledge this has not been explored nor has the impact of carotenoids on the immune response of the herbivore once it has been parasitized or infected. Finally, whether insect parasitoids, predators, or herbivorous insects select food resources on the basis of carotenoid quality or quantity has not been addressed directly, but provides a clear and open direction for future research.
Understanding the roles of carotenoids in the evolutionary ecology of insects is a potentially rich field that promises to enrich our understanding of the chemical mediation of ecological interactions in nature and may provide excellent model systems for integrating aspects of organism development with current evolutionary theory (Badyaev 2011). In turn, understanding the factors that drive such phenomena as host-plant attractiveness or repellency to herbivores, pathogens, and natural enemies; pollinator and seed-disperser attraction; and regulation of microbial symbionts has numerous applied implications.
4.11 REFERENCES


Aplin RT, Birch MC (1970) Identification of odorous compounds from male Lepidoptera. Experientia 26:1193-&


Birch M (1970) Pre-courtship use of abdominal brushes by nocturnal moth, *Phlogophora meticulosa* (L) (Lepidoptera-Noctuidae). Anim Behav 18:310-


Borkent A, Bissett J (1985) Gall midges (Diptera: Cecidomyiidae) are vectors of their fungal symbionts. Symbiosis 1:185-194


sexual hormone synthesis in zygomycetes is mediated by a trisporic acid regulated beta-carotene oxygenase. Fungal Genet Biol 44:1096-1108


Catoni C, Peters A, Schaefer HM (2008) Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. Anim Behav 76:1107-1119


Fermaud M, Lemenn R (1989) Association of Botrytis cinerea with grape berry moth larvae. Phytopathology 79:651-656


Fox DL (1976) Animal biochromes and structural colors. University of California Press, Los Angeles, USA

view from the points of convergence in the stress signaling networks. Curr Opin Plant Biol 9:436-442


Garrett KA, Dendy SP, Frank EE, Rouse MN, Travers SE (2006) Climate change effects on plant disease: Genomes to ecosystems. Annu Rev Phytopathol 44:489-509


Gerould JH (1921) Blue-green caterpillars: the origin and ecology of a mutation in hemolymph color in *Colias ( Eurymus) philodice*. J Exp Zool 34:385-415


Kettner J, Dorffling K (1995) Biosynthesis and metabolism of abscisic acid in tomato leaves infected with *Botrytis cinerea*. Planta 196:627-634


Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. New Phytol 178:863-874


McDonald KL, Cahill DM (1999) Influence of abscisic acid and the abscisic acid biosynthesis inhibitor, norflurazon, on interactions between Phytophthora sojae and soybean (Glycine max). Eur J Plant Pathol 105:651-658


Schmidt K, Pflugmacher M, Klages S, Maeser A, Mock A, Stahl DJ (2008) Accumulation of the hormone abscisic acid (ABA) at the infection site of the fungus *Cercospora beticola* supports the role of ABA as a repressor of plant defense in sugar beet. Mol Plant Pathol 9:661-673


Tooker JF, De Moraes CM (2007) Feeding by Hessian fly [Mayetiola destructor (Say)] larvae does not induce plant indirect defenses. Ecol Entomol 32:153-161


*Drosophila* mutant ninaB identifies the gene encoding the key enzyme for vitamin A formation in vivo. Proc Natl Acad Sci USA 98:1130-1135


Table 4.1 Some apocarotenoids known to be generated by oxidative cleavage of carotenoids and have biological activity in plants, animals, or microorganisms

<table>
<thead>
<tr>
<th>Name</th>
<th>Functions</th>
<th>Pherobase citations</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>retinal, retinoic acid, retinol</td>
<td>vision, morphogen</td>
<td>none</td>
<td>1</td>
</tr>
<tr>
<td>abscisic acid</td>
<td>plant hormone</td>
<td>none</td>
<td>1</td>
</tr>
<tr>
<td>strigolactones</td>
<td>plant hormone, fungal growth</td>
<td>none</td>
<td>1,2,3</td>
</tr>
<tr>
<td>mycorradicin</td>
<td>antifungal properties</td>
<td>none</td>
<td>1</td>
</tr>
<tr>
<td>blumenin</td>
<td>antifungal properties</td>
<td>none</td>
<td>1</td>
</tr>
<tr>
<td>trisporic acid</td>
<td>fungal pheromone</td>
<td>none</td>
<td>1</td>
</tr>
<tr>
<td>β-cyclocitrinal</td>
<td>fruit flavor, floral volatile</td>
<td>F^{12}</td>
<td>1,13,16</td>
</tr>
<tr>
<td>4-oxoisophorone</td>
<td>fruit flavor, floral volatile</td>
<td>F^{13}</td>
<td>11,13</td>
</tr>
<tr>
<td>pseudoionone</td>
<td>fruit flavor, floral volatile</td>
<td>F^{155}</td>
<td>12,13</td>
</tr>
<tr>
<td>(E)-β-damascenone</td>
<td>fruit flavor, floral volatile</td>
<td>F^{158}</td>
<td>6,13</td>
</tr>
<tr>
<td>β-ionone, α-ionone, 3-hydroxy-β-ionone, 3-hydroxy-α-ionone, 3-hydroxy-5,6-epoxy-β-ionone</td>
<td>fruit flavors, floral volatiles, insect semiochemicals</td>
<td>F[355,203,0,0,0]H[a]_{1,0,0,0,0}^{1,0,0,0,0}</td>
<td>1,4, 5,13,16</td>
</tr>
<tr>
<td>geranylacetone</td>
<td>fruit flavor, floral volatile, insect semiochemicals</td>
<td>F^{186}H[p]<em>{1,0}^{1,0}Ha[h]</em>{1,0}^{1,0}Da[Mk]HE[Ta]_{1,0}^{1,0}</td>
<td>12,13</td>
</tr>
<tr>
<td>(E)-3,7-Dimethyl-2,6-octadienal aka: geraniol or citral a</td>
<td>floral volatile, insect</td>
<td>F^{186}Hp[21,0,0,0,0]Cal[Bp]_{1,0}^{1,0,0,0,0}</td>
<td>13,15</td>
</tr>
<tr>
<td>(Z)-3,7-Dimethyl-2,6-octadienal aka: nerol or citral b</td>
<td>floral volatile, insect</td>
<td>F^{186}Cal[H]_{1,0}^{1,0}Hp[31,0,0,0,0]Bp[1,0,0,0,0,0,0,0]</td>
<td>13,15</td>
</tr>
<tr>
<td>sulcatone, sulcatol</td>
<td>floral volatiles, insect</td>
<td>F^{1990,205}H[p]_{18,0}^{1,0}Da[6,0,0,0,0]</td>
<td>8,9, 10,13</td>
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<tr>
<td>edulinal II</td>
<td>floral volatile, insect</td>
<td>F^{158}Bp[0,1,1,1,1,1]</td>
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<td>dihydroedulalin I</td>
<td>floral volatile, insect</td>
<td>F^{158}Bp[0,1,1,1,1,1]</td>
<td>7,13</td>
</tr>
<tr>
<td>9,10-epoxytetrahydroedulane(^{c})</td>
<td>floral volatile, insect</td>
<td>F^{158}Bp[0,1,1,1,1,1]</td>
<td>7,13</td>
</tr>
<tr>
<td>1,5,5,9-Tetramethyl-10-oxabicyclo[4.4.0]-3-decen-2-one(^{c})</td>
<td>floral volatile, insect</td>
<td>F^{158}Bp[0,1,1,1,1,1]</td>
<td>7,13</td>
</tr>
<tr>
<td>2,2,6,8-Tetramethyl-7-oxabicyclo[4.4.0]-4-decen-3-one(^{c})</td>
<td>floral volatile, insect</td>
<td>F^{158}Bp[0,1,1,1,1,1]</td>
<td>7,13</td>
</tr>
</tbody>
</table>

\(^{a}\) Pherobase (El-Sayed 2011) citations are represented by a letter code with superscripts. The first set of uppercase letters indicates the Arthropod group except for floral volatiles (F): Heteroptera (HET), Hymenoptera (H), Coleopteran (C), Diptera (D), Moths (M), Butterflies (B), Trichoptera (T), Spiders and mites (S), Dictyoptera (Di), Thysanoptera (THY). The lowercase letters that follow the group code stand for behavioral significance: pheromone component (p), attractant (a), kairomone (k), or allomone (al). The superscript order is with respect to the order in the “name” column and indicates the number of species that release the component listed in Pherobase.


\(^{c}\) These compounds are not known to be generated from carotenoid cleavage, but their structures are suggestive.
Table 4.2 An abbreviated list of fungi known to produce carotenoids or abscisic acid

<table>
<thead>
<tr>
<th>Genus</th>
<th>Division</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Carotenoid biosynthesis</th>
<th>ABA biosynthesis</th>
<th>References</th>
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<tbody>
<tr>
<td>Neurospora</td>
<td>A</td>
<td>Ascomycetes</td>
<td>Sordariales</td>
<td>Sordariaceae</td>
<td>yes</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Cercospora</td>
<td>A</td>
<td>Dothideomycetes</td>
<td>Capnodiales</td>
<td>Mycosphaerellaceae</td>
<td>yes</td>
<td>yes</td>
<td>2,3,4</td>
</tr>
<tr>
<td>Septoria</td>
<td>A</td>
<td>Dothideomycetes</td>
<td>Capnodiales</td>
<td>Mycosphaerellaceae</td>
<td>yes</td>
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Division is either Ascomycota (A), Basidiomycota (B), or Zygomycota (Z)

Cells without information are not necessarily negative; the information may have been overlooked by the authors.

Figure 4.1 Three examples of carotenoids degrading to volatile apocarotenoids (α-, β-ionone, and β-cyclocitrinal) via carotenoid cleavage oxygenases (Ibdah et al. 2006), fungal peroxidases (Zorn et al. 2003), or singlet oxygen (Ramel et al. 2012)
Figure 4.2 A stylized, schematic representation of the various known and hypothesized functions of carotenoids in insects that mediate ecological interactions.
5 TESTING DEFENSE ALLOCATION THEORY IN SOLIDAGO ALTISSIMA

5.1 ABSTRACT

Understanding the costs and benefits and ultimately the evolutionary reason for particular patterns of chemical defense allocation in plants is an ongoing theoretical challenge spawning many hypotheses that may have good predictive power in one ecological context, but fail outright in another. Here I use conventional univariate statistical techniques as well as structural equation modeling to test two of the major defense theories, the growth-differentiation balance hypothesis and optimal defense theory, in an emerging model system, Solidago altissima. Although I find some support for both these theories, genotype appears to explain most of the variation in defense allocation. This suggests the need for a more ecological theory of plant defense that stresses intraspecific alternative strategies, tolerance, and ecological costs of defense.

5.2 INTRODUCTION

It is clear that primary producers are constantly threatened by higher trophic levels. This attack by herbivores and pathogens is thought to have shaped, over coevolutionary time, plant defenses (Ehrlich and Raven 1964). Plants are variously resistant to these attacks (Painter 1958) and use an array of strategies including constitutive and induced chemical defenses (Farmer and Ryan 1990), physical defenses such as spines and thorns, and the attraction of mutualistic predators and parasitoids (De Moraes et al. 1998, Kessler and Baldwin 2001). What is less clear is how plants balance the demands of growth and reproduction with those of defense. And, at what level of
damage does defense begin to provide a net benefit; that is, how tolerant are plants to damage? Here I ask some of these questions in an emerging natural model system based on the perennial forb tall goldenrod (Solidago altissima, Asteraceae).

Optimal defense theory (ODT) was an early attempt to reconcile the demands of growth and reproduction with those of defense (McKey 1974, Rhoades 1979). In its simplest form, it predicts that those tissues with the greatest value and vulnerability in terms of fitness (e.g., seeds) should be the most heavily defended. Other theories include the carbon-nutrient balance hypothesis (Bryant et al. 1983) and the growth-rate hypothesis (Coley et al. 1985). But by far the most mature of the theories to describe the allocation of defense mechanisms is the growth-differentiation balance hypothesis (GDBH) (Loomis 1953, Herms and Mattson 1992), which posits a reciprocal tradeoff between growth and defense. It differs from ODT in that tissue value is not the main predictor of defense allocation, but rather the resource allocation constraints imposed by a strategy to either defend or grow (Herms and Mattson 1992). Nevertheless, these four main hypotheses are not mutually exclusive in their predictions and the hypothesis that is best supported in studies of plant defense may often depend on the context and defense trait measured (Barto and Cipollini 2005). Optimal defense theory in its original form makes specific predictions but in general states that any adaptive defense pattern is likely. This makes it quite difficult to falsify (Stamp 2003). Optimal defense theory does a good job of predicting the relative defense levels of highly valued tissues such as flowers (Kessler and Halitschke 2009) and seeds (Zangerl and Rutledge 1996). However, it does less well within tissues with lower direct fitness benefits such as leaves (but see McKey
For instance, levels of xanthotoxin in wild parsnip leaves of various ages do not vary (Zangerl and Rutledge 1996) even though there are a number of compelling reasons why younger leaves should be more valuable and vulnerable especially in herbaceous plants (McKey 1974, McCall and Fordyce 2010). Young tissues give rise to taller more competitive plants and to flowers and eventually seeds in most forbs; that is, they generally represent an investment in future fitness relative to older leaves, and their higher nitrogen content and relatively undifferentiated tissue make them more attractive to a range of sucking (Llewellyn and Qureshi 1978), chewing (Maddox and Root 1987, Maddox and Root 1990), and galling herbivores (Stone and Schonrogge 2003).

The aim of this work is to test optimal defense theory in its simplest form in an emerging model system, *S. altissima* (Asteraceae) where this question has yet to be asked. Based on work in other species, I predict that younger leaves are more valuable to the plant and therefore the most chemically defended. However, this prediction is buffered by the common observation in this system of the younger tissues being more heavily damaged by herbivores and older tissues being more severely damaged by pathogens, thus risk of attack varies by tissue age and attacker.

In order to test these predictions I set out to answer the following questions:

1) Which leaf tissues are most valuable to seed and rhizome production?

2) Which leaf tissues are more chemically defended by protease inhibitors and terpenoids?

3) How well does leaf tissue value predict chemical defense levels?
4) Is there evidence of a tradeoff between growth and defense?

As potential defenses I have chosen protease inhibitors and terpenoids. Serine protease inhibitors are well known plant defenses against lepidopterans and cysteine protease inhibitors (PI) are effective against coleopterans (Jongsma and Bolter 1997). Generally, they are thought to inhibit gut proteases and thus the assimilation of amino acids. This apparently slows the growth and reproduction of herbivorous enemies, but more complex mechanisms have also been proposed (Jongsma and Bolter 1997). Terpenoids are well known to attract natural enemies, deter herbivore feeding, have allelopathic effects, and inhibit the growth of pathogenic and insect-vectored fungi (Langenheim 1994). Furthermore, terpenoids probably represent one of the major defenses in *S. altissima* (Johnson et al. 2007, Johnson et al. 2010); however, little work has been done on PIs in *Solidago*.

In testing these hypotheses, I include plant fitness as an explanatory variable. To my knowledge, this is the first time that fitness has been used as an independent variable in modeling the contribution it makes to predicting defense levels. Normally researchers visually inspect the correspondence of assumed tissue value with the distribution of defense levels in various tissues (Zangerl and Rutledge 1996, Barto and Cipollini 2005). Although this is an effective method of highlighting a potential pattern, it is highly ineffective at actually measuring the level of predictive power. Here, I use tissue value along with other variables to predict defense levels and then quantify the contribution of each of these variables using univariate statistical modeling. I also apply structural
equation modeling to this system to further elucidate any direct or indirect support for ODT or the GDBH.

5.3 METHODS

5.3.1 Common garden analysis of goldenrod accessions

Tall goldenrod is a perennial forb common in mesic to dry prairie environments. It flourishes especially well on old agricultural land or disturbed sites and its proliferation in these sites may have contributed to major population expansions across North America over the last 200 years or so (Abrahamson and Weis 1997). Its main mode of reproduction is through creeping rhizomes that create clonal genets that manifest themselves as discrete clumps of ramets distributed throughout a site.

I established a common garden of *S. altissima* accessions in 2008 with plants grown in soilless media (Pro-mix BX/Mycorise® Pro, Premier Horticulture, Ltd., Quakertown, PA, USA) in the greenhouse that spring from rhizome cuttings (ca. 5 cm) collected haphazardly from nearby wild populations. Rhizome cuttings were collected and planted the first week of April into 6.5-cm pots to sprout and then transplanted to 2.5-L pots the third week of May and fertilized (200 mL each of 20-20-20 Peters Professional at a 50% rate). I transplanted the plants to the field the third week of June in a 10-row by 10-column grid spaced 2 m on center in a randomized complete block design; each 2-row by 10-column block contained 2 replicates of each of the ten clones (see WSU common garden location, Table 3.1, Chapter 3). I watered the plants after transplanting, but other than periodical weeding and mowing around the subplots, they were left to establish on their own. During the summer of 2009 and the fall of 2010, I measured various
parameters for each of the subplots (n = 10 subplots per accession) to assess the evidence of genotypic variation in the ten accessions. These parameters included plant growth rate (i.e., slope of average height over time per subplot); number of ramets at the end of the season; average leaf length to width ratio; average number of leaf teeth per cm of leaf length; probability of being a cane morph (Wise 2009); probability of having a red stem; relative flowering time; probability of deer damage; density of crescent, cushion, flat, and irregular gall morphotypes of Asteromyia carbonifera (Stireman et al. 2008); average A. carbonifera gall growth rates; probability of A. carbonifera gall failure; probability of insect meristem damage; density of Eurosta solidaginis galls; and the density of Rhopalomyia solidaginis galls. A multivariate analysis of variance (MANOVA) was conducted with accession as the predictor and only variables with P values less than 0.10 were included in the subsequent ordination. Ordination of the significant parameters was conducted by canonical discriminant analysis (candisc package, R Software, version 2.14) and 95% confidence ellipses on the mean centers were calculated using established methods (Owen and Chmielewski 1985).

5.3.2 Testing leaf-tissue value

The same ten S. altissima accessions (referred to as 10.4, 10.6, 10.11, 11.1, 11.5, 11.8, 11.9, 11.12, 12.1, and 12.4) were grown in soiless media (Pro-mix BX/Mycorise® Pro, Premier Horticulture, Ltd., Quakertown, PA, USA) in 2.5-L pots in the greenhouse from rhizomes collected from the common garden. These potted plants were then set out in a deer exclusion plot in a randomized design (Figure 5.1), fertilized once with 5 g each of Osmocote® plus (NPK 15-9-12, 3-4 month formulation, The Scotts Company LLC,
Marysville, OH, USA) at the beginning of the season (26 May 2010) and watered periodically. Each subplot contained 6 plants of each accession for a total of 60 plants per subplot. There were 8 subplots divided into two blocks. Within each block the subplots were treated with fungicide, insecticide, both, or neither three times during the season. These treatments were included to create variation in enemy attack that could be used to infer the induction of chemical defense where defense levels varied concordantly. Within each subplot the six clones of each accession had either the upper one-half or lower one-half of their leaves removed at three different times during the season (early, middle, and late). The leaf removal treatments were applied only once to each individual. The timing of the leaf-removal treatments corresponded closely to the early non-branching, middle branching, and late flowering stages of the plants. These are referred to subsequently as the non-branching, branching, and flowering stages. One small control subplot with only two plants of each accession (total of 20 plants) was also established on the edge and did not receive leaf-removal treatments, but was treated with both fungicide and insecticide (Figure 5.1). The removed tissue was weighed, stored in a cooler of dry ice, and transported to a -20 °C freezer twice daily while the removal treatments were being conducted.

Leaf-tissue value was assessed by measuring capitulum and rhizome mass for the various leaf-removal treatments. Capitula were clipped from the plants twice weekly just as the pappi began to mature but before they began to disperse. This continued until all the capitula were eventually harvested. The above ground biomass was harvested in a single day after all the capitula were collected. Rhizomes were separated from the roots
and harvested over three consecutive days. Dry masses of all these materials were determined by drying in an oven at 55 °C until a constant mass had been established.

### 5.3.3 Protein extraction

Approximately 2 g of frozen leaf tissue from the removal treatments was chopped into 1-cm squares under frozen conditions (i.e., outside during the winter) and re-stored at -20 °C. These aliquots were homogenized to a powdered form in a mortar and pestle under liquid nitrogen, transferred to pre-chilled scintillation vials and stored again at -20 °C. The powdered samples were dried in a lyophilizer (Genesis 25, VirTis Company, Gardiner, New York, USA) and re-stored at -20 °C. The samples were not allowed to thaw at any stage of processing; that is, they remained frozen from the day of harvest until extraction. Weighed portions (ca. 50 mg) of powdered, freeze-dried material were vortexed in 1 mL of sodium phosphate extraction buffer [25 mM, pH = 7.0 plus 8.8 mg/mL NaCl, 2 mg/mL phenylthioureia (Sigma P5272), 5 mg/mL sodium diethyldithiocarbamate (Sigma D3506), 2 mM ethylenediaminetetraacetic acid (EDTA, Fisher S80007-1), and 50 mg/mL polyvinylpolypyrrolidone (Sigma P6755)] until thoroughly mixed, centrifuged for 30 min at 16,000 × g and 4 °C, and the supernatant withdrawn and frozen at -20 °C until analysis.

### 5.3.4 Protein assays

Stored extracts were centrifuged for 15 min at 16,000 × g and 4 °C to pellet any residual material before taking samples for total protein, serine, and cysteine protease inhibitor assays. Samples and plates were kept on ice during loading. Total protein assays followed the protocol of Bradford (1976) with bovine serum albumin as a
I measured both serine and cysteine protease inhibitor levels in the removed tissues with established methods adapted for use in a microplate reader system (Abe et al. 1994).

For serine protease activity, 20 µL of 0.1 M Tris-HCl (pH = 8.0), 10 µL of trypsin (0.25 mg/mL), and 20 µL of standard (soybean trypsin inhibitor, Sigma T9003) or sample was added to each well. Blanks had trypsin replaced with Tris-HCl and positive controls had sample replaced with extraction buffer. The plate with cover was shaken in the microplate reader for 3 s and incubated for 5 min at 37 °C. Then 20 µL of BANA (N-benzoyl-DL-arginine-β-naphtylamide, Sigma B4750, in dimethyl sulfoxide, 3.1 mg/mL) was added and the plate incubated at 37 °C for 20 min. Then 100 µL of 2% HCl was added to each well to stop the reaction and the background absorbance was read at 540 nm. Finally, 100 µL of p-DACA (p-dimethylaminocinnamaldehyde, Sigma D4506, 0.6 mg/mL in ethanol) was added, the plate shaken, and incubated at room temperature. Plates were read at 540 nm after 20, 60, and 120 min of incubation at room temperature, but only the asymptotic reading at 120 min was used. Cysteine protease activity was measured similarly except that papain (Sigma P4762 in 25 mM, pH = 7.2, sodium phosphate buffer, 0.25 mg/mL) replaced trypsin and reaction buffer (250 mM sodium phosphate buffer, pH = 6.0, plus 2.5 mM EDTA) replaced the Tris-HCl. Preliminary experiments indicated that the cysteine PI activity was saturated at the sample concentration; therefore, the cysteine PI assays were conducted with a 1:20 dilution of the sample extract. Milli-Q water (18.2 M ohms) was used for all solutions. For both
proteases, activity was expressed as percent inhibition relative to the positive control because of the lack of an affordable cysteine protease inhibitor to use as a standard:

\[ I = 100 - \frac{(X-B)}{C} \times 100 \]

where,

\( X \) = corrected absorbance of the sample reaction or standard, \( B \) = corrected absorbance of background read, and \( C \) = corrected absorbance of the positive control (i.e., no inhibitor or sample extract added). The final values were standardized by the total amount of protein extracted.

5.3.5 Terpenoid extraction and analysis

To approximately 50 mg of weighed freeze-dried leaf tissue was added 7.5 µg of tetralin (internal standard) and extracted in 1.8 mL of methanol for 24 hr at room temperature. The extracts were centrifuged for 2 minutes at 3000 rpm and the supernatant transferred to 1-dram glass vials to which 1.5 mL of HPLC grade hexanes were added and 200 µL of Milli-Q water. After another 24 hr at room temperature the organic layer was run over hexanes-preconditioned, silica-gel columns (0.20 g) and stored at -20 °C until analysis by GC-MS at a collaborators laboratory (A. Kessler, Cornell University, Ithaca, New York, USA).

5.3.6 Univariate statistics

To test the relative value of ODT and the GDBH in predicting the distribution of defense levels, the same starting linear model was used to predict each of the defense measures. The ANOVA model included these predictors in the following order: (1) the
residuals from an increasing sigmoidal model of fitness (i.e., capitula dry mass) as predicted by final above ground biomass as an index of tissue value; (2) the plant growth rate measured as the slope of the regression line connecting the sum of ramet heights at the no-branching stage to the sum of ramet heights at the branching stage; (3) the stage that the tissue was removed; (4) the nested factor age within stage; and (5) the genotype. The pesticide treatments did not affect defense levels in preliminary ANOVAs and were therefore not included. Genotype was treated as a fixed effect because I was specifically interested in the proportion of variation explained and not necessarily the F statistic. Because only the F statistic is affected by this choice and not the sum of squares, whether I use fixed or random effects for genotype is irrelevant. Furthermore, conclusions drawn from only 10 genotypes (regardless of whether one uses random or fixed effects) are unlikely to be broadly applicable to the entire population because thousands if not tens of thousands of different genotypes likely exist in nature.

5.3.7 Structural equation modeling

Structural equation modeling is uniquely suited for analyzing relationships where predictors may conceivably act as both independent and dependent variables. Conventional univariate and multivariate techniques cannot deal appropriately with such data (Grace 2006, Kline 2011). Although I know of no studies that have applied this technique to defense theory, Grace (2006) suggests that it would be informative and appropriate and therefore I have employed it here.

Before it was possible to fully specify the SEM to test the relative contribution of ODT and the GDBH it was first necessary to determine the direction of the path between
galling insects and growth rate because either direction is plausible. That is, galling insects may be more attracted to genotypes with higher growth rates or they may directly influence growth rates via hormonal mechanisms. Two separate analyses were used to accomplish this. The first was an SEM with genotype effects included. In this SEM I specified paths from genotypes to galling insects, above-ground biomass, and growth rate as well as a feed-back loop from galling insects to growth rate to above-ground biomass and back to galling insects. In a second analysis I regressed the mean growth rates of the genotypes grown in the absence of galling insects (i.e., in the greenhouse) on the mean number of galls found on these genotypes per stage in the field. A positive relationship in this regression would suggest attraction by galling insects to genotypes with high growth rates.

To further test the relative fit of ODT and the GDBH to defense allocation, I specified an exploratory SEM. I specified reciprocal paths between growth rate and all five defense measures because the GDBH predicts that growth should compete with defense and vice-versa (Herms and Mattson 1992). I also specified directional paths from purely exogenous variables (i.e., stage, age(stage), and ducking) to all endogenous variables (i.e., the five defense measures, growth rate, final above-ground biomass, number of chewing, sucking, or galling insects, rust fungus, capitula mass, and rhizome mass). In SEM, the exogenous variables are those that are not explained by any paths whereas the endogenous variables have paths impinging on them. Ducking is a genotypic trait wherein the actively growing apex of the plant is bent downward in the shape of a walking cane (see Wise 2009). To control for potential effects of the amount of above
ground biomass on the number of herbivores (i.e., density) a path was specified from above-ground biomass to herbivores. Two separate analyses (Figure 5.12) indicated that galling insects increased plant growth rates; therefore, this path was also specified. Herbivores were also specified to affect rust fungus, capitula mass, and rhizome mass because studies have shown that herbivore attack can have effects on plant fitness that are not directly associated with tissue removal (Barber et al., van Dam and Heil 2011 and references therein). Furthermore, herbivore damage can either increase pathogen damage (Yang et al. 2011) or decrease it (reviewed in Stout et al. 2006) depending on the herbivore and pathogen type and the order of attack. In my system, rust fungal severity increases as the season progresses while herbivores and specifically galling insects occur throughout. All the defense measures were specified to affect all other endogenous variables, but not each other in order to reveal whether these paths were necessary. Rust fungus was specified to affect above-ground biomass, growth rate, capitula mass, and rhizome mass. Aside from all the covariances associated with the dummy coded exogenous variables two additional covariances were specified between sucking and galling insects and between capitula and rhizome mass. The covariance between sucking and galling insects was specified to account for a significant residual covariance in preliminary models and that between capitula and rhizome mass was theoretically specified based on the assumption of a tradeoff between vegetative and sexual reproduction (Grime and Pierce 2012). The analysis was run in OpenMx (Boker et al. 2011), which is an SEM package developed for R statistical software.
5.4 RESULTS

5.4.1 Goldenrod accessions

Canonical discriminant analysis revealed at least 8 morphologically distinguishable genotypes among my ten *S. altissima* accessions as determined by non-overlapping 95% confidence ellipses on the means in at least one perspective (Figure 5.2A-C). Accessions 11.1, 11.12, and 12.1 were not resolved by the first three canonical axes, which described a total of 80.8% of the variation. However, principal components analysis of the terpenoids revealed that accession 12.1 was completely isolated in ordination space (data not shown) indicating the presence then of at least 9 distinguishable genotypes.

5.4.2 Leaf tissue value

Above ground biomass was the most significant predictor of capitula and rhizome mass in preliminary models and therefore the residuals from optimized models were used in subsequent analyses (Figures 5.3 and 5.4). Of course, final above ground biomass (not including capitula mass) was higher in plants not receiving removal treatments, but interestingly there was no significant differential effect of the removal treatments on above ground biomass (Figure 5.5). Removal of the young tissue at the branching stage reduced capitula mass the most, but rhizome mass was not differentially affected by the leaf tissue removal treatments (Figure 5.5). It appears that removing leaf tissue reduced rhizome mass overall (Figure 5.5), but because the non-removal treatment was not randomly mixed in with the main experimental blocks (Figure 5.1), one has to make these comparisons with caution.
5.4.3 Protease inhibition and terpenoids

The young branching stage leaf tissue had the highest defense levels for serine protease inhibition and for sesquiterpenoids (Figure 5.6). Serine protease inhibition peaked at the young branching stage and then declined (Figure 5.6), while cysteine protease inhibition peaked at the mature branching stage and remained high thereafter (Figure 5.6). Young tissue clearly had higher concentrations of terpenoids, regardless of stage (Figures 5.6) and monoterpenoids peaked at the young branching stage and remained high thereafter. Sesquiterpenoids peaked at the young branching stage, but returned to early levels thereafter.

I used the capitula mass residuals from the Figure 5.3 model as a proxy for tissue value and tested the degree to which tissue value was able to predict defense levels. Table 5.1 shows the results of four linear models, one for each defense measure. Overall, those factors associated with genotype, age, or stage explained most of the variation. Tissue value was a relatively poor predictor of defense allocation and the stepAIC function in R dropped it from every model. Growth rate was retained in the model for serine protease inhibition, but only explained 0.9 % of the variation, whereas genotype was the strongest predictor overall (Table 5.1, Figure 5.7).

5.4.4 Structural equation modeling

The SEM is presented in Figure 5.8 with only significant paths shown (P ≤ 0.05), but for simplicity Figures 5.9 to 5.12 present only a subset of the paths in Figure 5.8 to focus attention on specific areas of the model that support either OD (Figures 5.9 and 5.10) or the GDBH (Figure 5.11).
The age within stage factor (Figure 5.9) was coded as zero for mature tissue and one for young tissue; therefore, a positive coefficient indicates one of three things depending on the path. If the path is directed toward a defense measure then it means that defense was significantly higher in young tissue than in mature tissue at that particular stage. If the path is directed toward a fitness measure then it means that removing young tissue increased that fitness measure. If it is directed toward an enemy then it means the enemy was more frequent on plants having had their young tissue removed. Therefore, at both the “no branching” and “branching” stages defenses were higher in young tissue and removing that tissue had a greater impact on fitness than removing old tissue either directly or indirectly through rust fungus (Figure 5.9).

The stage factor (Figure 5.10) was coded relative to the flowering stage; therefore, a positive coefficient indicates one of three things depending on the path. If the path is directed toward a defense measure then it means that defense was significantly higher at that stage than at the flowering stage. If the path is directed toward a fitness measure then it means that removing tissue at that stage increases that fitness measure. If it is directed toward an enemy then it means the enemy was more frequent on plants having had their tissue removed at that stage. Therefore, at the “no branching” stage defenses were either higher or lower than they were at the flowering stage depending on the defense. At the “branching” stage defenses were either higher or not different than the flowering stage. And removing tissue at either stage either increased or decreased fitness (relative to the flowering stage) depending on stage and fitness measure (Figure 5.10).
The SEM includes reciprocal paths from growth rate to all the defense measures, but they are not shown in Figure 5.8 because none of them were significant in either direction. However, there was a moderate significant tradeoff between growth rate and defense, which manifested itself as an indirect effect through galling insects (Figure 5.11). Subsequent SEM and linear regression showed that the direction of the path from galling insects to growth rate was appropriate because the growth rate of the same clones grown in the greenhouse, which lacked galling insects, was not associated with galling insect frequency measured on the same clones in the field (Figure 5.12). In addition, a path analysis incorporating the effects of genotype on growth rate and galling insects still indicated a significant positive relationship between galling insects and growth rate (Figure 5.12).

Some *S. altissima* genotypes express a “ducking” trait where the apex of the growing stem is bent in the shape of a walking cane (see Wise 2009). In the SEM this “ducking” trait, coded as one or zero for presence or absence, had significant effects throughout the model including both direct and indirect positive effects on fitness as well as negative effects on herbivores and pathogens (Figure 5.13).

**5.5 DISCUSSION**

At first glance it would appear that tissue value would be a good predictor of defense levels, because there is a degree of correspondence between the tissues of highest value (i.e., when removed fitness is reduced) and the defense levels of those same tissues. However, closer inspection of this correspondence reveals that this is not generally true. For instance, while fitness was reduced substantially by removal of the youngest tissues...
(no branching stage), defense levels were among the lowest at this stage. Perhaps there is a tradeoff occurring here. At the early stages of development, winning the competitive fight for resources is paramount across phyla (Simberloff 1982). If defense synthesis draws resources away from growth, or growth and defense are constrained, then one would expect lower defense levels during this stage. The growth differentiation balance hypothesis (Herms and Mattson 1992), growth-rate hypothesis (Coley et al. 1985), and CSR theory predict as much (Fraser and Grime 1999). Serine protease inhibition was negatively related to growth rate in the ANOVA model (data not shown), but this explained very little of the variation in serine protease inhibition and no other defense measures had direct negative relationships with growth rate in either the ANOVA or SEM models. This is surprising since terpenes are generally found in a highly reduced form in plants, which makes them among the most costly chemical defenses (Gershenzon 1994). Grime and Pierce (2012) propose the CSR model which classifies plants and virtually all of the tree of life according to three main tradeoffs: that between reproduction and stress tolerance, stress tolerance and competitive ability, and competitive ability and reproduction. The three different strategies generally represent fast-growing perennials (competitors), slow-growing perennials (stress tolerators), and fast-growing annuals (ruderals or fast reproducers). Although S. altissima has not been classified according to this theory it is most likely a competitor, which is predicted to have moderate growth rates and defenses (Fraser and Grime 1999). Perhaps S. altissima exists in the ordination space of CSR theory away from the tradeoff between growth and defense or its major defense against herbivores is tolerance as suggested by the asymptotic nature of the relationship between above-ground biomass and fitness.
measures or it has evolved a strategy that limits this tradeoff. Future research should evaluate its CSR strategy as well as how stressful conditions such as nutrient or water limitations modulate potential tradeoffs between growth and defense as predicted by the GDBH (Herms and Mattson 1992). Finer scale measurements of growth rate and defenses may also reveal a tradeoff that may have been obscured in this study.

Levels of serine protease inhibitors and sesquiterpenoids decreased in the later stages of development as did the fitness values of these tissues, but monoterpenoids and cysteine protease inhibitors remained high. Terpene biosynthesis in plants generally begins as the leaves are expanding. Biosynthesis and storage in various secretory structures continues until the leaves are fully expanded at which point it generally slows or stops. There is little turnover in healthy plants until flowering time at which time some terpenoids are degraded and transported to seeds or rhizomes (Gershenzon 1994). It is likely that the decrease in sesquiterpenoids at the flowering stage and the strong positive effect of sesquiterpenoids on rhizome mass in the SEM is associated with cost-saving catabolism. If this is true, then it suggests that the monoterpenoids may be more important in late-stage leaf defense than sesquiterpenoids, at least in this system.

The most striking result is the degree to which genotype predicts defense levels. In terms of the percentage of variation described, genotype was by far the most important predictor overall. I have attributed this to variation in life history traits associated with the genotypes, but it may well be explained by specific ecological strategies employed by different genotypes as was suggested by Maddox and Root (1987, 1990). They showed that different Solidago altissima genotypes appeared specifically adapted to a “suite” of
herbivores. Perhaps some of the success of S. altissima comes from dividing up the enemy space among various genotypic strategies. Pollination constraints may also select against high levels of defense. This would be especially true for an obligate outcrosser such as S. altissima and may explain the negative effects of sesquiterpenes on capitula mass in the SEM. In a wild tomato species, caterpillar damaged plants have higher levels of specific mono- and sesquiterpenes in the flower volatiles that are correlated with lower levels of pollinator activity (Kessler and Halitschke 2009).

Even though terpenoids are among the most costly defenses, they still only require a limited amount of resources to be produced (Gershenzon 1994). Perhaps the majority of these costs are ecological or multiple complicated interactions cancel each other out as suggested by the SEM. Interestingly, one of the genotypes (11.5) in this study that is the most resistant to the fungus-vectoring gall midge Asteromyia carbonifera (data not shown) and had the highest levels of serine protease inhibition was heavily attacked by voles in my experiment. The voles did not appear to eat the foliage, but rather chopped entire stems up into nesting material which they used to build nests in the same potted plants. I have also seen this under natural field conditions, suggesting that it is a common occurrence. Incidentally, this is also why I have points in Figures 5.3 and 5.4 with very little to no above-ground biomass and as a result low fitness.

Because neither the GDBH nor ODT performed well in these experiments, it seems that two more layers of defense theory are needed to effectively explain the variation in plant defense. One would incorporate life history traits mainly associated with strategies evolved to cope with environmental perturbations and stressful conditions.
Another layer of theory is needed to account for ecological idiosyncrasies associated with suites of herbivore pressure, pollination syndromes, mutualistic associations with natural enemies, and perhaps facilitation imposed by herbivores. We should not abandon the developed theories as they explain much of the variation in plant defense, especially with regard to highly valued tissues such as seeds. Instead, we should incorporate these ideas along with new ideas about life history traits and ecological considerations into a more holistic theory of plant defense. This will obviously require careful study of the ecology and life history of specific genotypes, but will be undeniably fruitful.

Practical issues hinder the study of genotypic variation, but with such an overwhelming amount of explanatory power, ecologists studying plant defense theory can no longer afford to ignore it. Future studies should not be dissuaded by the complexities of exploratory SEM and (or) should use sequential sum of squares (Type I), as I have done, with genotype in the model after specific life history traits and ecological peculiarities aimed at explaining genotypic variation in defense levels.
5.6 REFERENCES

Escherichia coli: Investigation of its inhibitory profile and occurrence in corn

interactions with above- and belowground antagonists and mutualists. Ecology
93:1560-1570

Barto E, Cipollini D (2005) Testing the optimal defense theory and the growth-
differentiation balance hypothesis in Arabidopsis thaliana. Oecologia 146:169-178

equation modeling framework. Psychometrika 76: 306-317

Bradford MM (1976) Rapid and sensitive method for quantitation of microgram
quantities of protein utilizing the principle of protein-dye binding. Anal Biochem
72:248-254

relation to vertebrate herbivory. Oikos 40:357-368

Coley PD, Bryant JP, Chapin FS (1985) Resource availability and plant antiherbivore
defense. Science 230:895-899

van Dam NM, Heil M (2011) Multitrophic interactions below and above ground: en route
to the next level. J Ecol 99:77-88


Grace JB (2006) Structural equation modeling and natural systems. Cambridge University Press, New York, USA


Kline RB (2011) Principles and practice of structural equation modeling. The Guilford Press, New York, USA


McCall AC, Fordyce JA (2010) Can optimal defense theory be used to predict the
distribution of plant chemical defenses? J Ecol 98:985-992


Owen JG, Chmielewski MA (1985) On canonical variates analysis and the construction
of confidence ellipses in systematic studies. Syst Zool 34:366-374


GA, Janzen DH (eds) Herbivores: Their interaction with secondary plant
metabolites. Academic Press, New York, USA, pp 3-54

253


Stireman III JO, Devlin H, Carr TG, Abbot P (2010) Evolutionary diversification of the
gall midge genus Asteromyia (Cecidomyiidae) in a multitrophic ecological context.
Mol Phylogenet Evol 54:194-210

Trends in Ecology & Evolution 18:512-522

microorganisms and herbivorous arthropods. Annu Rev Entomol 51:663-689

Wise MJ (2009) To duck or not to duck: resistance advantages and disadvantages of the
candy-cane stem phenotype in tall goldenrod, Solidago altissima. New Phytol
183:900-907

Table 5.1 Statistics for the final models of each chemical defense in *Solidago altissima* genotypes. The same starting linear model was reduced for each defense by applying the stepAIC function in R statistical software with \( k = \ln(n) \), which is the bayesian information criterion (BIC). Although tissue value, which represents optimal defense theory, was included in the starting model it was dropped in all models by the stepAIC function.

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>F</th>
<th>P</th>
<th>%(^1)</th>
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<tr>
<td><strong>Serine protease inhibition (PI)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Growth rate</td>
<td>1</td>
<td>6.0</td>
<td>0.015</td>
<td>0.9</td>
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<tr>
<td>Stage</td>
<td>2</td>
<td>17.7</td>
<td>&lt; 0.001</td>
<td>5.6</td>
</tr>
<tr>
<td>Genotype</td>
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<td>16.2</td>
<td>&lt; 0.001</td>
<td>22.9</td>
</tr>
<tr>
<td>Model</td>
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<td>15.6</td>
<td>&lt; 0.001</td>
<td>29.4 (0.28)</td>
</tr>
<tr>
<td><strong>Cysteine PI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>2</td>
<td>51.0</td>
<td>&lt; 0.001</td>
<td>15.7</td>
</tr>
<tr>
<td>Genotype</td>
<td>9</td>
<td>8.5</td>
<td>&lt; 0.001</td>
<td>11.8</td>
</tr>
<tr>
<td>Age(stage)</td>
<td>3</td>
<td>8.2</td>
<td>&lt; 0.001</td>
<td>3.8</td>
</tr>
<tr>
<td>Model</td>
<td>14, 447</td>
<td>14.5</td>
<td>&lt; 0.001</td>
<td>31.3 (0.29)</td>
</tr>
<tr>
<td><strong>Monoterpenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>2</td>
<td>284.0</td>
<td>&lt; 0.001</td>
<td>25.6</td>
</tr>
<tr>
<td>Genotype</td>
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<td>139.1</td>
<td>&lt; 0.001</td>
<td>31.3</td>
</tr>
<tr>
<td>Age(stage)</td>
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<td>&lt; 0.001</td>
<td>23.5</td>
</tr>
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<td>&lt; 0.001</td>
<td>3.7</td>
</tr>
<tr>
<td>Age(stage) x Genotype</td>
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<td>56.5</td>
<td>&lt; 0.001</td>
<td>89.0 (0.87)</td>
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<td><strong>Sesquiterpenes</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>&lt; 0.001</td>
<td>14.3</td>
</tr>
<tr>
<td>Genotype</td>
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<td>60.2</td>
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<td>35.8</td>
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<tr>
<td>Age(stage)</td>
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<td>17.6</td>
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<tr>
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<td>10, 271</td>
<td>56.9</td>
<td>&lt; 0.001</td>
<td>67.7 (0.67)</td>
</tr>
</tbody>
</table>

\(^1\)Percent of variation explained = factor sum of squares divided by total sum of squares. Values in parentheses are adjusted R\(^2\).
Figure 5.1 Experimental plot layout and diagram of leaf defoliation treatments. Each subplot contained $2 \times 3 \times 10$ plants to allow for defoliation treatments (mature or young on non-branching, branching, or flowering plants over 10 accessions). Flower buds or apical meristems were not removed to allow relatively normal growth. All subplots, except the small one, had defoliation (D) treatments. Various subplots had fungicide (F, thiophanate-methyl, Cleary 3336 F, 5.97 mL/3.78 L), insecticide (I, esfenvalerate, Asana XL, 1.58 mL/3.78 L), or both (F + I) applied over three evenly spaced times during the growing season. There were also two replicates each of nine of these accessions grown in the greenhouse (not illustrated).
Figure 5.2 Canonical discriminant analysis on *Solidago altissima* traits measured on ten accessions grown in a common garden. Vectors indicate the direction of increase in the various traits measured: number of subplot ramets (ramets), plant growth rate (pgr),
density of *Asteromyia carbonifera* morphotype galls (cres, irr, cush, flat); flowering time (flower); average *Asteromyia carbonifera* gall growth rate (ggr); probability of red stem (red_stem); probability of exhibiting the cane morphotype (cane); average number of leaf teeth per cm (teeth); probability of deer damage (deer); leaf length to width ratio (lwratio). The vector origins actually start at (0,0), but are shifted for clarity. Large solid circles indicate ellipse centers. Note that accessions 11.1, 11.12 and 12.1 were not resolved with 95% confidence ellipses on the means in at least one perspective and therefore may represent the same genotype, but ordination of the terpenoid data clearly differentiates 12.1 (data not shown).
Figure 5.3 Covariate used to generate normalized residuals for capitula mass analyses and serve as the tissue value factor for the models in Table 5.1. AIC values and tests of normality are also shown for a linear model. Blue triangles are plants with no tissue removed and solid points are the 11.5 genotype, which in some cases experienced severe rodent damage.
Figure 5.4 Rhizome mass as above ground biomass increases. AIC values, evidence ratios, and probabilities are also shown for linear, single, and double sigmoidal models. The parameter estimates for the single sigmoidal model are shown in the bottom right corner. Blue triangles are plants with no tissue removed and solid points are the 11.5 genotype, which in some cases experienced severe rodent damage.
Figure 5.5 The results of tissue removal on three fitness measures: (a) final above ground dry biomass, (b) capitula dry mass, and (c) rhizome dry mass (g). The notches approximate 95% confidence intervals on the median and the “×” represents the mean. The tissues removed are symbolized as follows: Y = young; M = mature; NB = tissue removed at the no branching stage; B = branching stage; or F = flowering stage. The “control” treatment was intact plants and is represented as the lower control block in Figure 5.1. These are shown only for rough comparison. See Figure 5.8 for significant differences.
Figure 5.6 The results of chemical analysis on removed tissues: (a) per cent serine protease inhibition, (b) per cent cysteine protease inhibition, (c) total monoterpenes in mg per g dry mass extracted, (d) total sesquiterpenes in mg per g dry mass extracted. The notches approximate 95% confidence intervals on the median and the “×” represents the mean. The tissues removed are symbolized as follows: Y = young; M = mature; NB = tissue removed at the no branching stage; B = branching stage; or F = flowering stage. Significant differences are represented in the SEM (see Figure 5.8).
Figure 5.7 The results of chemical analysis on removed tissues by genotype: (a) per cent serine protease inhibition, (b) per cent cysteine protease inhibition, (c) total monoterpenes in mg per g dry mass extracted, (d) total sesquiterpenes in mg per g dry mass extracted. The notches approximate 95% confidence intervals on the median and the “×” represents the mean. Only a subset of the genotypes was analyzed for terpenes. Genotype was highly significant for all defenses (see Table 5.1).
Figure 5.8  Structural equation model for the subset of genotypes (see Figure 5.7) where a complete set of data were available. Path widths are proportional to their corresponding standardized coefficient. Solid and dashed paths indicate positive and negative coefficients, respectively. Only significant (i.e., $P \leq 0.05$) paths are shown, but all specified paths were retained. All covariances and variances were specified for the purely exogenous dummy coded variables but are not shown for clarity. This is also true for endogenous disturbances. Model statistics: $n = 282$; $df = 7, 190$; $\chi^2 = 9.2$; $P = 0.24$; RMSEA = 0.033
Figure 5.9 A portion of the full model (see Figure 5.8) with only the paths from the “age within stage” dummy variables that support optimal defense theory shown for clarity. Note that defense measurement of, or removal of, the mature leaves was coded as zero and therefore the path coefficients (line widths) are relative to mature tissue at that stage. For example, a positive coefficient from “Age branching” to “Mono” indicates that total monoterpenes were higher in young tissue and if that tissue is removed “Capitula mass” is decreased more than if mature tissue is removed (i.e., negative coefficient). Therefore, to a small degree young tissue is more defended and more valuable. Note the cumulative indirect effects through rust fungus.
Figure 5.10 A portion of the full model (see Figure 5.8) with only the paths from the “stage” dummy variables that support optimal defense theory shown for clarity. In this case the coefficients are relative to the flowering stage; therefore, a positive coefficient from “Branching” to “Ses” indicates that total sesquiterpenes were significantly higher at the branching stage than they were at the flowering stage and removal of this tissue decreased rhizome mass more at this stage than removal at the flowering stage. Note the effects tend to cancel each other out.
Figure 5.11 A portion of the full model (see Figure 5.8) with only the paths that support the growth differentiation balance hypothesis shown for clarity. Because galling insects significantly increase growth rates (see also Figure 5.12) and sesquiterpenes decrease galling insects, there is a degree of tradeoff between growth and defense, but only indirectly.
Figure 5.12  Two different analyses on the effect of galling insects on plant growth rate. The left panel is a structural equation model with genotype effects included to control for the potential confounding effects of insect attraction to specific genotypes with high growth rates. Path widths are proportional to their corresponding standardized coefficient. Solid and dashed paths indicate positive and negative coefficients, respectively. Only significant (i.e., P ≤ 0.05) paths are shown. The right panel is a linear regression of mean genotype growth rate in the absence of galling insects (i.e., greenhouse-grown genotypes) on the mean number of galls per stage in unprotected (i.e., field-grown genotypes) clones. The line is the relationship for those genotypes used in the SEM analysis (solid points). Genotype 11.9 (triangle) was used in the SEM analysis but was not grown in the greenhouse; therefore, it has a field growth rate. The open circles are genotypes also grown in the greenhouse, but not include in the SEM analysis. Regardless of which set of points is used, the relationship is not significant.
Figure 5.13 A portion of the full model (see Figure 5.8) with only the paths from the dummy coded “ducking” variable shown for clarity.
6 THE CONUNDRUM OF GALL MORPHOLOGY: DO CARNIVORE PRESSURES SHAPE AND MAINTAIN GALL MORPHOLOGY IN THE ADAPTIVE RADIATION OF ASTEROMYIA CARBONIFERA?

6.1 ABSTRACT

Natural enemies as selective forces maintaining and shaping morphological divergence in adaptive radiations have received very little attention. Until recently, the focus has been on primary resource competition as a major driver of trait divergence in adaptive radiations. Here I evaluate the role of natural enemies and their interaction with host plants in shaping trait phenotypes in an incipient adaptive radiation of gall midges (Diptera: Cecidomyiidae) on tall goldenrod (*Solidago altissima*). I find strong evidence that gall morphology (i.e., gall thickness and gall diameter), an extended phenotype of these gall midges, is under stabilizing, directional, and diversifying selection depending of the gall morphotype considered. Overall, natural enemies select for either thin or thick galls. I conclude that gall morphology is, at least in part, determined by natural selection by parasitoid enemies and that these interactions may help explain the diversity of gall morphologies found in nature.

6.2 INTRODUCTION

The selective forces that drive adaptive radiation and morphological divergence have mainly focused on inter- and intraspecific competition for primary resources (silverswords: Carr 1985, finches: Grant and Grant 2002, cichlids: Hulsey et al. 2006) and the role of secondary resources, such as enemy free space and its mechanistic
associate (i.e. apparent competition), has largely been ignored. Contemporary forces that drive population changes in extant taxa are obviously likely to have played key roles in their adaptive radiation, especially in the youngest radiations (Schluter 2000). However, long-term and short-term studies regarding these factors have found the role of natural enemies wanting with respect to other more important abiotic and biotic environmental factors such as rainfall and host-plant traits (Waring and Price 1989, Price and Hunter 2005, Egan et al. 2011).

The extended phenotypes of galling insects (i.e. the galls) are extremely diverse in morphology (Gagné 1989, Stone and Schonrogge 2003) and gall forming insects typically experience heavy mortality from parasitoids (up to 80% in the current system). In comparison to free-living herbivorous insects, the rates are generally similar or higher for galling insects (Hawkins et al. 1997, Hayward and Stone 2005). This seems to contradict the idea that galls provide a refuge from natural enemies and has spurred vigorous discussions about the ecological selective forces that have favored the repeated evolution of the galling habit (Cornell 1983, Price et al. 1987, Stone and Schonrogge 2003). Some have proposed the “ghost of parasitism past” as an explanation for such diversity in gall morphology (Hayward and Stone 2005 and citations therein). They posit that the diversity of gall form is a genetic leftover from a time when it did provide—maybe only a brief—but potent refuge from parasitism and predation. If this is true, then one would expect the youngest radiations to exhibit the strongest evidence for the shaping of gall morphology or other divergent traits by top-down forces.
Here I address this hypothesis by analyzing selection on gall morphology for seven different natural enemies of a complex of Ambrosia gallers, nominally characterized as *Asteromyia carbonifera*, but which appear to be in the throes of an incipient adaptive radiation (Stireman et al. 2008, 2010, 2012). One would expect to find evidence of stabilizing selection if natural enemies are maintaining gall morphology and evidence of directional or disruptive selection if gall morphology is being shaped by natural enemies.

There are at least three main hypotheses regarding the selective forces that maintain and shape gall morphology. These include the nutrition, micro-environment, and enemy hypotheses. Briefly, the nutrition hypothesis states that the gall provides improved nutrition over other feeding methods, the micro-environment hypothesis argues that the gall protects the inhabitant from abiotic stresses such as desiccation, and the enemy hypothesis suggests that galls protect their dwellers from natural enemies (Hawkins and Gross 1992, Abrahamson and Weis 1997, Hawkins et al. 1997). Various lines of evidence supporting each of these hypotheses have been reviewed by several authors (Cornell 1983, Price et al. 1987, Stone and Schonrogge 2003). It is quite probable that all three of these factors contribute to the maintenance of gall form and its diversification; however, to what degree is not well known (Stireman et al. 2008).

In addition, this work also strives to inform our understanding of the process of ecological and sympatric speciation, as the genetically distinct gall morphotypes studied here (see “Study system” below) occur sympatrically and often syntopically on a single host plant or even host leaf. Therefore, the potential for gene flow between these morphs
is high and it is suspected that strong divergent ecological selection imposed by parasitoids on morphology and behavior maintains divergence despite their sympatry (Gavrilets 2004).

6.2.1 Study system

The vast majority of the family Cecidomyiidae (Diptera) is composed of midges with the ability to gall fungus or host-plant tissue. Several species in this family are known to carry fungal spores in structures called mycangia. A few of these asexual spores are deposited on each egg that is laid (Borkent and Bissett 1985, Bissett and Borkent 1988). One of these so called “ambrosia gall midge” species, Asteromyia carbonifera, is known to have a symbiotic relationship with the fungus Botryosphaeria dothidea. The adults of A. carbonifera lay eggs on the underside of goldenrod (Solidago spp.) leaves, where the larva burrows into the leaf tissue within seven days of oviposition. The fungal spores germinate on the egg and grow into the leaf tissue. Together the midge and fungus form a gall, which in this case is not so much plant tissue differentiation as it is fungal proliferation. Upon dissection of the gall, one finds at least one larval cell buried in fungal mycelia and surrounded by fungal stroma, a hard, dark layer of differentiated fungal mycelium (Heath and Stireman 2010).

Asteromyia carbonifera larvae and fungus can form at least four different gall morphotypes (hereafter, “morphs”) on Solidago altissima. These have been termed 'crescent', 'cushion', 'flat', and 'irregular' based on morphology (Crego et al. 1990). These gall morphotypes vary in their number of chambers, position of larvae inside the gall, position on the leaf, color, thickness, and diameter (Crego et al. 1990, Stireman et al.

It has been shown that *A. carbonifera* consists of a number of genetically distinct populations which are physically identifiable by their gall morphology. What is not known is what has been driving this diversification. It is assumed that the diversification is caused by interactions between *A. carbonifera*, *B. dothidea*, *S. altissima*, and the parasitoids which prey upon them (Stireman et al. 2012). The goal of this study was to test the enemy hypothesis for variation in gall morphology, by determining first if there are differences between the gall morphs in gall thickness and diameter, and then to test the effect of these traits on overall survival with respect to enemy attack. In addition, I was interested in whether plant genotype may indirectly affect parasitism through its effect on gall traits. I initially hypothesized strong associations between parasitism and gall morphology because parasitoids are known, even in this system, to have different attack behaviors and ovipositor lengths (Weis 1982b).
6.3 MATERIAL AND METHODS

6.3.1 Common garden of goldenrod accessions

I established a common garden of *Solidago altissima* accessions in June of 2008 with plants grown in the greenhouse that spring from rhizomes collected haphazardly from nearby wild populations (see Chapter 5). I transplanted the plants to the field in a 10-row by 10-column grid spaced 2 m on center in a randomized complete block design; each 2-row by 10-column block contained 2 replicates of each of the ten clones. I watered the plants after transplanting, but other than periodical weeding and mowing around the subplots, they were left to establish on their own. During the summer of 2009 and the fall of 2010, I measured various parameters for each of the subplots (n = 10 subplots per accession) to assess the evidence of genotypic variation in the ten accessions. Ordination of these variables illustrated that all but three of these accessions are morphologically distinguishable (see Chapter 5, Figure 5.2). However, a separate ordination analysis of leaf terpenoid quality on a subset of these clones grown outside in pots significantly distinguished two of these similar genotypes (data not shown). Therefore, the ten accessions represent at least 9 genotypes of *S. altissima*, with accession 11.1 and 11.12 likely representing the same or closely related genotypes.

6.3.2 Tracking gall development and parasitism assessment

Starting in May 2009 I checked the common garden subplots daily for new galls which were marked and measured at a rate of about 1-2 rows of the common garden per day. After about 1-2 weeks a complete census of the galls in the garden was achieved and the process began again, including additional measurements of the previously marked
galls and measurements of any newly occurring galls. This was continued until August at which time several hundred galls had been tracked and measured (crescents, n = 1579; irregulars, n = 210; cushions, n = 307; and flats, n = 188). The same process was continued in 2010 which yielded data for several thousand galls (crescents, n = 1591; irregulars, n = 2167; cushions, n = 1079; and flats, n = 903). Morph assignment was confirmed visually after the marked galls were mature (i.e., when the rate of gall-diameter-increase was < 10% per week) at which time the leaf they were on was collected. The collected galls were stored at 4 °C for at most a few days until they could be either reared out in individual cotton-stopped vials in plastic chambers (moist peat moss below a perforated Plexiglas tray) or dissected. Because the galls were collected when they were mature, most of the parasitoids and gall midges were in the pupal stage, which made identification relatively easy and rearing typically successful. The thickness of the galls was also measured with digital calipers to 0.01 of a millimeter. After rearing, which was given at least 2 weeks, the galls were dissected and assessed for the number and presence of 7 different parasitoid species (see above) and the number of gall-midge chambers. Survival was calculated as the number of healthy midges divided by the number of chambers. The identity of representative parasitoid specimens collected in 2009 were confirmed by two experts (i.e., John La Salle and Christer Hannson).

6.3.3 Statistical analyses of parasitism

Attack by each of the parasitoids was analyzed with a chi-squared test for independence of row and column variables for the pooled 2010 data. Pearson standardized residuals were plotted by gall morph and parasitoid, $(n_{ij} - \mu_{ij})/\sqrt{\mu_{ij}}$, where n
and $\mu$ are the observed and expected cell frequencies, respectively. Sample sizes (and number parasitized) for crescents, irregulars, flats, and cushions, respectively for each parasitoid were: *Aprostocetus* sp.: 1025(46), 1392(87), 711(72), and 550(42); *A. tesserus*: 1026(100), 1392(135), 711(63), and 552(49); *A. homeri*: 1039(11), 1397(9), 713(4), and 555(4); *B. fumipennis*: 1025(36), 1392(20), 711(29), and 552(17); *C. solidaginis*: 1025(77), 1392(142), 712(212), and 550(50); *T. capite*: 1025(6), 1392(26), 711(85), and 550(76); *P. solidaginis*: 1025(104), 1392(17), 711(24), and 550(21); totals: 1441(781), 2006(1030), 1019(733), 827(517). Totals are not simply the sums because sometimes the parasitoid had already emerged at the time of gall collection. Analyses were conducted with basic core functions in R (R Development Core Team 2010).

6.3.4 **Statistical analyses of the effects of plant genotype**

I first used MANOVA to determine the effect of plant genotype on gall thickness and diameter as I expected these characters to be somewhat correlated. A highly significant MANOVA allowed me to proceed with univariate ANOVAs for each characteristic separately as well as its interaction with gall morphotype. Analyses were conducted with basic functions in R statistical software (R Development Core Team 2010).

6.3.5 **Statistical analyses on the shape of selection**

I used a generalized additive model (cubic spline, R package mgcv, gam function, Wood 2011) to assess the shape of selection imposed by natural enemies on gall morphological characters. These characters were gall thickness and diameter, both measured at the time of gall maturity. This model was proposed and described
mathematically by Schluter (1988) and used subsequently by others (e.g., Egan et al. 2011) for similar analyses. Schluter’s website also describes the use of the spline model with R statistical software as well as other software (http://www.zoology.ubc.ca/~schluter/bio501/Rtips.models.html#gam). It is a flexible nonparametric technique that makes no assumptions about the shape of selection. That is, it will detect stabilizing, disruptive, or directional selection gradients with equal probability. Although many workers choose to also generate selection coefficients via logistic regression analysis (Lande and Arnold 1983, see Egan et al. 2011), I felt that the generalized additive model was sufficient because standard errors were generated. Furthermore, the need for logistic regression coefficients is for subsequent use in simulation modeling, but the gam function can easily produce predicted values for use such simulations.

The generalized additive model was run in two ways because of differences in ovipositional behavior of the midges. Crescents always lay only one egg and thus survival on a per gall basis is a binomial response of presence or absence of parasitism where absence was coded as 1. The other gall morphs form galls with varying numbers of eggs and thus survival was calculated by dividing the number of healthy midges found in the gall by the number of cells. In both cases the logit link argument was specified because both measurements were bounded between one and zero. Occasionally, galls fail to produce a chamber and the midge larva dies early during development. This mortality is assumed to be caused by plant resistance traits and therefore these records were excluded from the analysis.
One practical constraint prevents either of these parasitism response variables from perfectly representing survival. It is difficult to know the actual egg clutch size which initially formed the gall and what proportion of those eggs actually survived to become healthy midges. Experimentally, it would be possible to find and track egg clutches to obtain these data. Alternatively, when galls are found early in development there is always an orange stain on the bottom of the gall from excess carotenoid-containing accessory fluid deposited during oviposition. These stains could be used as an index of clutch size, but galls would need to be found very early in development before the stains degrade or become enveloped by the developing gall fungus. Future experiments could take these clues into account, but in the current experiment sample sizes were judged as paramount and time constraints were prohibitive.

6.4 RESULTS

6.4.1 Parasitoid preference for different gall morphs

Chi-squared analysis of contingency tables indicated significant differences in preference of the parasitoids for different gall morphs (Figure 6.1). These analyses were conducted for each of the parasitoids and all except A. tesserus and A. homeri displayed significant preference with respect to what would be expected under independence (Aprostocetus sp.: $\chi^2 = 22.6$, df = 3, $P << 0.001$; A. tesserus: $\chi^2 = 0.7$, df = 3, $P = 0.88$; A. homeri: $\chi^2 = 1.9$, df = 3, $P = 0.60$; Baryscapus fumipennis: $\chi^2 = 16.0$, df = 3, $P = 0.001$; C. solidaginis: $\chi^2 = 220$, df = 3, $P << 0.001$; T. capite: $\chi^2 = 222$, df = 3, $P << 0.001$; P. solidaginis: $\chi^2 = 113$, df = 3, $P << 0.001$. Preference here is actually an index of preference since it requires the assumption of one hundred percent survival of the
parasitoids, which may not always be the case. Hyper- and (or) superparasitism as well as other sources of mortality may alter the actual preference measure somewhat and only manipulative experiments can distinguish between preference and performance. Nevertheless, the morphs are differentially susceptible to the different parasitoids with crescents being more heavily attacked by the egg parasitoid *P. solidaginis*; the larger and thicker gall morphs being preferred by *T. capite*; flats experiencing much greater attack by *C. solidaginis* and the undescribed *Aprostocetus* sp.; and irregulars apparently avoiding attack by the majority of the parasitoids.

### 6.4.2 Effects of plant genotype on gall morphology

Analysis of variance showed that gall thickness and gall diameter were both significantly affected by plant genotype, but the magnitude of that effect was small relative to the effect of gall morphotype (Figures 6.2 and 6.3). At most, the *S. altissima* genotype explained 7% of the variation in gall thickness and only 3% of the variation in gall diameter even when the interaction is included. However, gall morph explained 49% of variation in gall thickness and 36% of variation in gall diameter (see Figures 6.2 and 6.3 for ANOVA results).

### 6.4.3 Estimating the shape of selection on gall morphology

Using non-parametric generalized additive models the overall shape of selection pressure imposed on gall morphology by parasitoid enemies took on different modes for each gall morphotype (Figures 6.4-6.6). For crescents, the selective pressure was stabilizing for gall thickness and mostly disruptive for gall diameter. However, the pattern of disruptive selection for crescents on gall diameter was complicated by the
possibility of multiple adaptive peaks. For irregulars, it was mostly stabilizing, but ambiguous for diameter. For flats, it was directional for thinner galls and ambiguous for diameter. Finally, for cushions it was directional for thicker and larger diameter galls. Overall, the selective forces imposed by parasitoids were disruptive, favoring either thinner or thicker galls, and directional for smaller diameter galls (Figures 6.4-6.6).

6.5 DISCUSSION

Whether natural enemies (and their mechanistic associate, apparent competition) play a significant role in adaptive radiation and ecological speciation has been heavily debated and some have even demonstrated that predation can decrease the strength of primary resource competition and slow the rate of diversification (Meyer and Kassen 2007). The complex of cryptic species and host races comprising the nominal species A. carbonifera appears to be in the throes of an adaptive radiation (Stireman et al. 2012) and provides an ideal system in which to study the roles of natural enemies in diversification. Researchers have proposed the “ghost of parasitism past” as an explanation for the vast diversity of gall forms found in nature (reviewed in Stone and Schonrogge 2003) and if this hypothesis is true, one would expect to find the strongest evidence in young, incomplete radiations such as that studied here. Analyses indicate that gall morphology is shaped and maintained by natural selection from parasitoids in this incipient adaptive radiation and this may extend to other gall systems in which contemporary selection imposed by parasitoids appears to be absent, lending support for the hypothesis of the “ghost of parasitism past.” Furthermore, the indirect effects of host-plants on gall morphology are weak suggesting that natural enemies are the main driving force behind
divergence in gall morphology in this system. However, because gall morphology appears to have converged a number of times on different host plants (Stireman et al. 2012), the role of primary resource competition cannot be ruled out. Primary resource competition may play a role in driving colonization of new hosts, but it is unlikely to be contributing strongly to divergence in gall morphology as I found very little effect of plant genotype on gall characters. Furthermore, escape to new host plants is likely to provide a significant refuge from natural enemies and the symbiotic fungus, which is the primary resource of the developing larva (Janson et al. 2009, Heath and Stireman 2010), may buffer the physiological constraints associated with colonization of new hosts.

6.5.1 Indirect effects of the host-plant on gall morphology

Plant genotype has mild indirect effects on parasitism rates through its differential effect on gall thickness and diameter, but this factor is weak with respect to the apparent genetic control imposed by the gall morphs on their own morphology. A future analysis aims to describe the direct effects of plant genotype on parasitism rates, which may alter the strength of apparent competition and further our understanding of the complicated processes driving this adaptive radiation. In cynipid wasp gall-makers, individual plants can have strong effects on the shape of selection on gall morphology and have been shown to impose stabilizing selection pressure (Egan et al. 2011). In my system, gall morphology is only weakly affected by plant genotype suggesting that the major force shaping gall traits in this system comes from natural enemies.
6.5.2 The role of natural enemies in adaptive radiations

My analyses demonstrate that parasitoids exert strong selection on gall morphology. I find moderate support for the hypothesis that natural enemies maintain gall morphology (stabilizing selection) and strong support for the hypothesis that natural enemies shape gall morphology (disruptive and directional selection). Interestingly, significant stabilizing selection is only evident for the thickness of crescent galls and among these four gall morphs crescents are clearly the most genetically distinct and their morphology is consistent despite the fact that they continue to diverge genetically (Stireman et al. 2008, 2010, 2012). Perhaps crescents are beginning to stabilize with regard to morphology. Crescents and flats are known to attack other Solidago species, but the dominant host for crescents in Ohio is clearly S. altissima (unpublished data) and is likely the host where a connection between microevolutionary processes and macroevolutionary patterns will be most obvious. Flats may be in the process of host-associated divergence perhaps due to apparent competition on S. altissima because they are also known to colonize S. gigantea, possibly to a greater extent than S. altissima (unpublished data). This may explain the lack of a prominent selection gradient and their very low survival with respect to parasitoids on S. altissima (Singer and Stireman 2005). Overall, parasitoids exert disruptive selection on gall morphology for either thin or thick galls, which may play a significant role in the divergence of gall morphology seen in this system.

Aside from a few compelling studies (Nosil and Crespi 2006, e.g., Craig et al. 2007, Bailey et al. 2009), the role of enemies in driving trait divergences has been largely
ignored. Here I provide strong evidence that natural enemies play a significant role in adaptive radiation and ecological speciation in this system. First, because only successful galls were included in my analyses the effects of host plant resistance on gall survival was eliminated. Therefore any correspondence between gall traits and selection gradients is due only to natural enemies. Second, I tested the possibility that parasitized galls were thicker simply because they were parasitized by finding instances in my dataset of both parasitized and unparasitized galls of the same morph on the same leaf. These galls are likely to come from eggs deposited by the same female; therefore, genetic variability is minimized. These tests indicate that only parasitized crescents are significantly thicker, but only by 0.1 mm. This difference is marginal with respect to the overall variability in crescent-gall thickness and that over which selection by parasitoids act. Third, significant selection gradients with respect to quantitative gall traits are augmented by clear differential attack by parasitoids. That is, certain morphs are attacked more or less heavily by different parasitoids, suggesting that divergence in gall morphology divides up the available enemy free space.

6.5.3 The functional significance of gall morphology

Although I did not explicitly test the relative contribution of the various hypotheses put forth to describe divergence in gall morphology it appears that avoiding natural enemies is a good candidate for the function of divergent gall traits. However, it is still unclear whether enemies are actually responding to gall morphology or some correlated trait such as larval size or number of larvae per gall, and determining this will require manipulative experiments. In *Eurosta solidaginis*, gall size is predicted well by
larval size and avian enemies impose directional selection for smaller galls while parasitoids and inquilines impose directional selection for larger galls (Abrahamson and Weis 1997). A similar phenomenon may be occurring in my system as *T. capite* appears to prefer larger flat and cushion larvae over the smaller crescent and irregular larvae. Likewise, cushion and flat galls generally have multiple larvae per gall and it is not uncommon for *T. capite* larvae to consume more than one larva. Irregulars generally have fewer larvae than either flats or cushions and crescents never have more than one larva per gall.

### 6.5.4 Traversing adaptive landscapes

Adaptive landscapes, first proposed by Sewall Wright (1932) and then adapted by Simpson (1944, 1953) for use with phenotypic traits, provide both a conceptual and mathematical (Schluter 2000) understanding of divergent selection pressures. My fitness functions are essentially adaptive landscapes for individual traits and therefore all the factors associated with interpretation of these landscapes apply.

Both irregular and cushion galls are restricted to *S. altissima* and although there is evidence of directional selection toward thicker, larger diameter galls in both these morphs, it appears that only the cushion morph has the physiological ability to capitalize on this opportunity and (or) the valley in the shape of selection on irregular thickness is too deep to circumvent. Genetic linkage, pleiotropic effects, and a lack of genetic variability can all prevent circumvention of valleys in adaptive landscapes (Schluter 2000). For these reasons the distribution of a single trait may or may not correlate with selection pressure on that trait.
I conclude from this study that gall morphology, at least in part, appears to be maintained by natural selection imposed by parasitoid enemies. Detecting such selection pressures may require surveying large numbers of individuals in order to gather information associated with the full extent of the morphologically diversity in any one lineage, as I have done. Future analyses aim to measure the degree to which gall morphology responds to selection by natural enemies by analyzing selection differentials with multi-year data.
6.6 REFERENCES


Borkent A, Bissett J (1985) Gall midges (Diptera: Cecidomyiidae) are vectors of their fungal symbionts. Symbiosis 1:185-194


Egan SP, Hood GR, Ott JR (2011) Natural selection on gall size: Variable contributions of individual host plants to population-wide patterns. Evolution 65: 3543-3557


Simpson GG (1944) Tempo and mode in evolution. Columbia University Press, New York, NY, USA


Weis AE (1982a) Use of a symbiotic fungus by the gall maker Asteromyia carbonifera to inhibit attack by the parasitoid Torymus capite. Ecology 63:1602-1605


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Figure 6.1 Pearson’s standardized residuals for Chi-squared analysis on parasitism contingency tables. Parasitoid names with asterisks indicate significant departures from the expectation of independence (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).
Figure 6.2 The interaction of *Solidago altissima* genotype and gall morphotype on gall thickness. Note that the labels on the x-axis are the ten different *S. altissima* accessions grown in the common garden. The ANOVA results are presented along with the percent of variation explained by each term.
Figure 6.3  The interaction of *Solidago altissima* genotype and gall morphotype on gall diameter. Note that the labels on the x-axis are the ten different *S. altissima* accessions grown in the common garden. The ANOVA results are presented along with the percent of variation explained by each term.
Figure 6.4 The shape of selection pressure on gall thickness analyzed separately for each gall morphotype. Probability of survival was calculated as the number of healthy midges resulting from the gall divided by the number of gall chambers. The ‘|’ marks represent the calculated survival probability for individuals with a given gall. The black lines represent the fitted cubic spline generalized additive model with its associated standard error indicated by the grey shading. The lower colored lines are density histograms where the area under each of the curves is standardized to one making them comparable across panels (the histogram y-axes are irrelevant and not shown for clarity).
Figure 6.5 The shape of selection pressure on gall diameter analyzed separately for each gall morphotype. Probability of survival was calculated as the number of healthy midges resulting from the gall divided by the number of gall chambers. The “|” marks represent the calculated survival probability for individuals with a given gall. The black lines represent the fitted cubic spline generalized additive model with its associated standard error indicated by the grey shading. The lower colored lines are density histograms where the area under each of the curves is standardized to one making them comparable across panels (the histogram y-axes are irrelevant and not shown for clarity).
Figure 6.6 The overall shape of selection pressure on gall thickness and diameter analyzed with gall morphotype pooled. Probability of survival was calculated as the number of healthy midges resulting from the gall divided by the number of gall chambers. The “|” marks represent the calculated survival probability for individuals with a given gall. The black lines represent the fitted cubic spline generalized additive model with its associated standard error indicated by the grey shading. The lower colored lines are density histograms where the area under each of the curves is standardized to one making them comparable within a panel (the histogram y-axes are irrelevant and not shown for clarity).
7.1 THE KEY INNOVATION

The key innovation that originally allowed colonization and population expansion of *Asteromyia* on *Solidago* is likely the acquisition and maintenance of the fungal mutualist, *B. dothidea* (Chapter 2). This mutualist may have buffered the effects of plant defense and environmental perturbations allowing the lineage to take advantage of untapped resources, which would lead to population increases and subsequent increases in the standing pool of genetic diversity for which other forces such as primary and secondary resource competition could act. This is not only true for *Asteromyia*, but for other genera of radiating cecidomyiids such as *Asphondylia* (Adair et al. 2009) and *Neolasipotera* (pers. obs.) because it appears that they all use the same fungal mutualist.

7.2 THE IMPORTANCE OF BEHAVIORAL DIVERGENCE

Although, Schluter (2000) stresses that radiations where only behavioral divergence is evident cannot be classified as “adaptive” without accompanying physiological or morphological diversification, I disagree. Behavioral divergence has been an important component of the evolution of many groups of insects and ignoring it when it is present may actually decrease our chances of capturing adaptive radiations in the earliest stages. These incipient adaptive radiations are the very ones most likely to provide insight into the causes of adaptive radiation and ecological speciation. Behavioral divergence is associated with the evolution of sociality and nest architecture in bees and wasps and the result of behavioral differences (i.e. nest architecture) is used
to build phylogenies (Wenzel 1993). Furthermore, one might argue that the proximate reason for the development of host races in *Rhagoletis pomonella* was host choice driven by associative learning (Prokopy et al. 1982). And Blackledge (2004) has nicely illustrated an adaptive radiation of Hawaiian web-building spiders driven in part by divergence in web architecture. The ovipositional phenotype that clearly divides the crescent morph and the others on *S. altissima* (Chapter 3) and provides a refuge from parasitoids may represent a major branch in the diversification of *A. carbonifera*, but further research is necessary on other members of these two clades (see Stireman et al. 2012) before this conjecture is substantiated.

7.3 **THE ROLE OF NATURAL ENEMIES**

The majority of adaptive radiations that have been studied have focused on divergence with respect to primary resource competition. That is, competition for food, nutrients, water, light, etc. Few studies have investigated the effects of secondary resource competition; that is, competition for enemy free space or apparent competition. There are multiple ways in which prey may avoid shared enemies and these include colonizing new host-plants or habitats or evolving defensive structures or behaviors. The expected result of the evolution of differential strategies for avoiding apparent competition are expected to divide up the enemy free space in such a way as to maximize the fitness of the surviving members of the radiation. And over time they should come to some relatively stable equilibrium, given that the environment is relatively stable. Although I have not investigated apparent competition directly in this work, I have made the first preliminary steps toward understanding it. I have defined, identified, and
characterized the most important natural enemies in this system (Chapter 3 and 6) and future exclusion and augmentation experiments should test the degree to which apparent competition maintains divergence in this system. Future experiments should also aim to understand the degree to which enemies have coevolved with their hosts. If apparent competition is important in driving adaptive radiations, one would expect the associated enemy community to show little evidence of coevolution. And the degree of coevolution should be positively related to the age of the radiation. The current system appears very young and therefore I would expect to find little evidence of sequential evolution (Forbes et al. 2009, Feder and Forbes 2010).

7.4 THE BENEFITS AND TRADEOFFS ASSOCIATED WITH CAROTENOID S

One of the most interesting findings to stem from this work is the presence of carotenoids in nearly every organ and life stage of these midges (Chapter 3). Carotenoids are known to have various physiological and ecological functions in insects (Chapter 4), but truly understanding how they may influence ecological and evolutionary outcomes involves understanding both their benefits and costs. This work illustrates that their presence in the salivary glands of *A. carbonifera* larvae may serve as precursors to abscisic acid, a plant hormone instrumental in abiotic stress signaling but also with negative effects on biotic stress signaling. Abscisic acid in the salivary glands may block defense responses in the plant and allow the fungal symbiont to proliferate and form the gall structure. Conversely, carotenoids in the accessory glands which are deposited on every egg that is laid may degrade in the presence of plant, insect, or fungal enzymes and cause the release of volatile apocarotenoids which attract natural enemies. It is also
possible that singlet oxygen, a reactive oxygen species associated with photosensitized plant allelochemicals and known to degrade carotenoids to volatile apocarotenoids (Chapter 4), plays a similar role. Future experiments should aim to test the presence and attractiveness of volatile apocarotenoids associated with midge eggs or young neonate larvae as these may provide important beneficial insect attractants.

With respect to ecological speciation and adaptive radiation the important physiological roles that carotenoids may play, the fact that they may be a limited resource, and their bright colors may all come together to allow sexual selection to enforce reproductive isolation in diverging lineages. Carotenoids have the potential to be “magic traits.” These traits are those that simultaneously provide a fitness advantage with respect to natural selection and a reproductive advantage with respect to sexual selection (Servedio et al. 2011). And simulation studies on sympatric speciation suggest that magic traits have the greatest potential to allow such speciation to occur (Gavrilets 2004).

7.5 PLANT GENOTYPE HAS LITTLE DIRECT EFFECT ON GALL MORPHOLOGY

Plant genotype was expected to have strong effects on gall morphology, but this was not the case (Chapter 6). About a third to a half of the diversity in gall morphology (i.e., the extended phenotype) appears to be associated with genetic control by the midges and very little associated with plant genetics. This is surprising as other studies have found a much higher contribution of the plant to gall morphology (Abrahamson and Weis 1997, Egan et al. 2011). This result supports the role of natural enemies in driving
divergence in gall morphology, but does not negate the potential role of the host plant in this process. Different *S. altissima* genotypes are more or less resistant to *A. carbonifera* (data not shown) and this resistance may be associated with sesquiterpenoids (Chapter 5). Plant resistance could cause selection on gall morphology if traits associated with successful host-plant colonization are correlated with traits associated with gall morphology. However, if this was an important factor one would expect to find that certain genotypes support high levels of one morphotype but not the other. This is not the case for *S. altissima* and *A. carbonifera*. Genotypes, although differing in their resistance to *A. carbonifera*, generally support the same relative abundance of the four morphotypes.

### 7.6 APPLIED IMPPLICATIONS FOR BIOCONTROL OF INVASIVE GOLDENROD

Understanding the proximate biochemical processes that allow ambrosia galls to develop on plants has real applied implications for biocontrol of invasive plants. This would seem especially true if one could combine the fungal growth factor with species specific dispersal of the gall fungus. In Europe and Asia *Solidago* species have been introduced and have become invasive. Even in North America *S. altissima* can become a problem in prairie restoration projects. Attempts to restore old agricultural land to prairie is often only successful for the first few years after replanting, at which time *S. altissima* quickly begins to dominate and eventually creates huge monocultures, at least in Ohio (D. Geiger and J. Amon, pers. comm.). I had originally though that introducing *A. carbonifera* to Europe and Asia as a potential biocontrol for invasive goldenrods or
augmenting its presence in prairie restoration projects might be a viable option. However, evidence of galling insects increasing the growth rate of *S. altissima* (Chapter 5, Figure 5.12) suggests that this may make the problem worse. Increased growth rates equate to increased competitive ability. Nevertheless, understanding the details of this biochemical interaction may still provide insights into ways one might encourage the fungus to grow even stronger on the plant and ultimately cause fitness disadvantages and potential control.
7.7 REFERENCES


Egan SP, Hood GR, Ott JR (2011) Natural Selection on Gall Size: Variable Contributions of Individual Host Plants to Population-Wide Patterns. Evolution 65


