ABSTRACT

Title of dissertation: GENETIC AND PHENOTYPIC DIFFERENTIATION AS A CONSEQUENCE OF HOST PLANT USE BY LEPIDOPTERAN HERBIVORES

J. Gwen Shlichta, Doctor in Philosophy, 2011

Dissertation directed by: Professor Pedro Barbosa, Department of Entomology

In this dissertation, I focused on the role of plant hosts as a driving force leading to phenotypic and genotypic changes in insect herbivores. There are three main questions addressed: (1) Do generalist species' populations have broad diet breadth or do they represent a mosaic of sub-populations, each having narrow diet breadths? (2) how does host plant affect the immune response of polyphagous herbivores, and (3) does host plant, or some aspect of host plant such as allelochemicals, alter the interaction between herbivore defense and parasitoid counter-defense?

Do generalist species' populations have broad diet breadth or do they represent a mosaic of sub-populations?

In Chapter 1, I determined, using amplified fragment length polymorphisms (AFLPs), whether host plant-associated genetic differentiation (HAD) was exhibited by a suite of polyphagous tree feeding Lepidoptera. The objective of this research was to test HAD in a suite of polyphagous species that exhibit traits expected to be important in the formation of genetically divergent sub-populations.

How does host plant affect the immune response of polyphagous species?

In Chapter 2, the objective was to examine the effect of host plant species on the immune defenses of polyphagous lepidopteran herbivores, specifically the intensity of encapsulation measured as percent melanization, of three common forest Lepidoptera species.

In Chapter 3, I discussed and assessed the potential role of immune responses in insect outbreaks. I present a brief background on immune responses, discuss the methods used to experimentally measure the components associated with immune response and how immune response varies. Lastly I draw on the studies available and present several potential hypotheses to stimulate further research.

Does host plant, or some aspect of host plant such as allelochemicals, alter the interaction between herbivore and parasitoid?

In the final chapter, I explored the ecological consequences of viral-allelochemical interactions. The objective of this study was to use a model system,

Manduca sexta and *Cotesia congregata*, to directly test the effect of the allelochemical nicotine and the presence or absence of polydnavirus (PDV) on the larval immune responses. PDV allows the parasitoid egg to escape encapsulation (an herbivore defense against parasitism).

GENETIC AND PHENOTYPIC DIFFERENTIATION AS A CONSEQUENCE OF HOST PLANT USE BY LEPIDOPTERAN HERBIVORES

By

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Dissertation submitted to the Faculty of the Graduate School of the
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DEDICATION

Pedro Barbosa

and your incredible career.

Although it brings me sadness to think you will have no other students after me,

I know that your wisdom and kindness echoes in all your students and everyone we mentor will continue to benefit from all you have taught us.

. . . . .

My F1 (aka *the parasite*)

You began your life as I wrote this dissertation and endured some very stressful early months. May the completion of my PhD research and the beginning of my career bring with it many exciting adventures for you and maybe a little love for the insect world.

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CHAPTER 1:

True generalists: Polyphagous lepidopteran herbivores show no host-plant associated differentiation

Abstract

A large proportion of insect species specialize on their hosts and even those classified as generalists (or polyphagous) often may be locally monophagous. Use of genetic methods has helped to elucidate degrees of host-associated differentiation among polyphagous herbivore insects. Further, the degree of host specificity within a species may provide insights into the possible existence of cryptic species complexes within or between hosts.

Low mobility in larval or adult stages presumably promotes differentiation of populations because less mobile species should have limited gene flow, thus enhancing rates of local adaptation. In this study I focused on species with a varying range of mobility (a species with flightless females (*Orgyia leucostigma* J.E. Smith, Lymantriidae), a leaf tier (*Machimia tentoriferella C*lemens, Oecophoridae), and a free-feeding species (*Hypagyrtis unipunctata* Haworth, Geometridae). I tested for host plant-associated genetic differentiation (HAD) using amplified fragment length polymorphisms (AFLPs). AFLPs were used to examine population differentiation and the structure of populations to assess if HAD is present in polyphagous lepidopteran herbivores on the host tree species. The objective of this research was to test host plant-associated differentiation in a suite of polyphagous species that exhibit traits expected to be important in the formation of genetically divergent sub-populations.

Larvae were collected weekly from the five sites throughout central Maryland. Larval DNA was extracted and analyzed using amplified fragment length polymorphism (AFLPs). Both analysis of molecular variance (AMOVA) and Nei-Li distance-based methods were used to examine populations of each species for HAD. The Bayesian clustering method implemented in STRUCTURE was used to assess the degree of population structure.

I found no evidence of HAD in the three species of polyphagous lepidopteran herbivores examined in this study. All analyses from AFLP markers indicate that these three polyphagous forest Lepidoptera are not genetically differentiated by host plant species. This suggests that these populations are panmictic and are not specializing on any particular host plant species across or within site. My results also show that these polyphagous species may not be ecologically driven to specialize on a particular host plant species.

Introduction

The current consensus is that the majority of herbivorous insects have a narrow diet breadth (Bernays and Chapman 1994, Futuyma 1991, Thompson 1994, Thompson 2005, but see Novotny et al. 2010), although the diet breadth of many polyphagous species is poorly known (Wagner 2005). A large proportion of insect species specialize on their hosts (Bernays and Chapman 1994, Futuyma 1991, Jaenike 1990a; Thompson 1994, Thompson 2005) and even those classified as generalists often may be locally monophagous (Fox and Morrow 1981, Scriber 2010). That is, to some degree these latter species exhibit host plant specialization over parts of their range due to constraints or host plant availability (Bossart 2003, Parry and Goyer 2004, Price 1980). Such species also

have been termed functional monophages, apparent monophages, or local specialists. A single polyphagous species might therefore consist of a number of host-associated subpopulations (Dres and Mallet 2002).

The rich diversity of phytophagous insects has led to the hypothesis that the specialization of insects to the plants they feed upon is a driving force behind insect diversification (Ehrlich and Raven 1964, Farrell 1998, Mitter et al. 1988). Further, Ehrlich and Raven (1964) proposed that the constant arms race of adaptation and counter adaptation was integral in plant, and subsequently, insect radiation. This hypothesis specifically highlights the importance of the role host plants play as one of the forces in the ecological specialization and diversification of phytophagous insects. Adaptation that may lead to speciation is an ongoing process, e.g., when herbivorous insects adapt to a novel chemical defense in a plant lineage (Berenbaum 1983, Becerra et al. 2009). This process may begin with phenotypic differentiation at the local level (i.e., local adaptation or site-associated differentiation) and may eventually lead to genetically differentiated populations (Dres and Mallet 2002, Tikkanen et al. 2006). When an herbivore experiences a fitness advantage on a particular host plant and genetic differentiation occurs, it is termed host-associated differentiation (HAD). Later in the differentiation process host races and perhaps new species may evolve (Feder et al. 1993, Feder 1995, Funk and Nosil 2008)).

Use of genetic methods has helped to elucidate degrees of host-associated differentiation among herbivorous insects. For example, DNA barcoding has revealed that some insect species previously classified as generalists are actually two or more cryptic species, many of which are associated with different hosts (Hajibabaei et al. 2006,

Hebert et al. 2004, Smith et al. 2006, Smith et al. 2007). Research on cryptic species or host-associated populations has focused primarily in tropical habitats (Hebert et al. 2004, Smith et al. 2006, Burns et al. 2008), thus little is known about the population structure of generalist herbivores in temperate forests. This topic has become of increasing interest because of the recent debate on whether there really is increasing dietary specialization towards the tropics (Novotny et al. 2006, Dyer et al. 2007). Information about whether generalists typically show evidence of fine-scale host or site-associated differentiation would provide insight into the frequency of local and host specialization and provide data critical of relevance to the relationship between dietary specialization and latitude. Thus, genetic tools to examine population structure of generalist insect species may elucidate the degree of host specificity within a species and provide insights into the possible existence of cryptic species complexes within or between hosts (Smith et al. 2007).

Life-history traits in insect herbivores that may influence HAD

Certain biological and life-history traits may enhance the potential of an herbivore to phenotypically and/or genetically differentiate when on different plant hosts (Edmunds and Alstad 1978, Van Zandt and Mopper 1998). Results from a meta-analysis of local adaptation of insects indicated that certain life history traits are associated with phenotypic (and potentially genotypic) differentiation, specifically endophagy and parthenogenesis (Van Zandt and Mopper 1998). Endophagous species are potentially more prone to differentiate on their host plant due to their intimate and constant interaction with the chemical, physical and phenological traits of the host plant (Feder et al. 1993, Mopper 1996, Mopper et al. 2000, Nason et al. 2002, Rossi and Stiling 1998, Van Zandt and Mopper 1998). Parthenogenesis allows a species to develop host plant

adapted populations more rapidly through asexual reproduction once favorable mutations arise and for this reason parthenogenetic species have been postulated as prone to HAD (Mitter et al. 1979, Via 1999, Dickey and Medina 2010). Similarly, insects with limited mobility may be more likely to be locally adapted to their host plants (Boecklen and Mopper 1998, Edmunds and Alstad 1978). Low mobility in larval or adult stages presumably promotes differentiation of populations because less mobile species should have limited gene flow, thus enhancing rates of local adaptation (Edmunds and Alstad 1978, Tikkanen et al. 2006).

Top-down pressures of predators and parasitoids/parasites impact herbivores differentially according to host plant (Barbosa et al. 2001, Lill et al. 2002). Parasitism of forest Lepidoptera differs by host tree at forested sites in Maryland, USA (Barbosa et al. 2001) suggesting that parasitism may act as a selective force driving herbivores to differentiate. Enemy-reduced space may drive host shifts or cause conspecific populations to maintain single host plant species fidelity (Abrahamson et al. 1994) Enemy-reduced space associated with parasitism has facilitated HAD in several systems (Feder 1995, Thomas et al. 2003, Dorchin et. al. 2009). Furthermore, herbivores that feed on long-lived tree hosts may face an evolutionary incentive to specialize due to the persistence of individual host plants over time (Edmunds and Alstad 1978).

Testing HAD in an assemblage of lepidopteran species

Lepidopteran species have been the focus of extensive studies on host-plant associated differentiation (Ehrlich and Raven 1964, Thompson 1994, Lill et al. 2002, Medina 2005, Singer et al. 2004, Dickey and Medina 2010) for several reasons. First,

lepidopteran species are ideal candidates for studies on host association due to their relatively close ecological interaction with their host plant, particularly with respect to plant chemistry (Barbosa 1988, Courtney 1988, Bernays and Chapman 1994, Ehrlich and Murphy 1988, Ehrlich and Raven 1964). Second, in eastern forests lepidopteran larvae feed on the same trees within a short seasonal time period (April to August) and either remain on a single tree for their entire larval period, although a few species may disperse from a tree as first or last larval stadia. Multiple lepidopteran species feed on the same host plant and can be relatively easily collected for comparisons between species using the same resource (Barbosa 2002, Wagner 2005). Third, extensive studies and background information on abundance, host plant usage, behavior, and parasitoids of Lepidoptera in eastern forests are available (Butler 1992, Butler and Strazanac 2000, Barbosa et al. 2003, Summerville et al. 2003, Covell 2005, Wagner 2005).

In this study I focused on numerically dominant, polyphagous species in an assemblage of forest Lepidoptera at five sites in central Maryland. Although ostensibly Lepidoptera do not seem to fit in the categories of herbivores likely to exhibit HAD, I selected species that did exhibit traits assumed to be important in HAD. To assist in the selection of lepidopteran species for this study, I referenced a database compiled by Barbosa and colleagues in a seven-year study of 78 species in 7 families of Lepidoptera and their parasitoids (Barbosa et al. 2001, Barbosa et al. 2003, Barbosa et al. 2004). This database provides the percent parasitism of each species at our research study sites and estimates of the abundance of the lepidopteran larvae (collected from *Acer negundo* and *Salix nigra*) (Barbosa and Caldas 2004).

HAD, a type of ecological speciation, will be measured using the criterion established by Stireman and colleagues (2005); i.e., the demonstration of significant genetic differentiation between host-associated groups of individuals at one or more sites. Several studies have provided supporting evidence for the HAD hypothesis in insect orders, including Diptera, Coleoptera, Orthoptera, and Hemiptera (Dres and Mallet 2002, Stireman et al. 2005, Sword et al. 2005, Vialatte et al. 2005, Lozier et al. 2007, Dickey and Medina 2010). I tested for host plant-associated genetic differentiation using amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995). AFLPs have been used to examine population differentiation and the structure of populations to assess if HAD is present in polyphagous lepidopteran herbivores on the host tree species (Sword et al. 2005, Falush et al. 2007).

The objective of this research was to test host plant-associated differentiation in a suite of polyphagous species that exhibit traits expected to be important in the formation of genetically divergent sub-populations. Therefore, I included species with a varying range of mobility (a species with flightless females (*Orgyia leucostigma* J.E. Smith, Lymantriidae), a leaf tier (*Machimia tentoriferella C*lemens, Oecophoridae) which obviously has limited mobility, and a free-feeding species (*Hypagyrtis unipunctata* Haworth, Geometridae). *Hypagyrtis unipunctata* is a free-feeding species, and therefore one would expect populations of this species to be panmictic. It is expected that less mobile species would be more likely to exhibit HAD. With the limited mobility of *O. leucostigma* and *M. tentoriferella*, I predicted that these species would be more likely to exhibit host-associated differentiation than the free feeder, either across sites or on host plants within sites.

This study focuses on a suite of generalist species, rather than examining specialists. It is the first study to test for HAD using polyphagous species that are neither endophagous nor parthenogenetic. Examining HAD in a several species within a system allows for a better perspective on whether host plant-associated adaptation is common in temperate forest systems (Stireman et al. 2005, Dickey and Medina 2010).

Methods

Study organisms

I sampled larvae of three numerically dominant species, with broad host tree ranges including but not limited to the tree species used for this study. Each species represents a different lepidopteran family in eastern North American forests.

Hypagyrtis unipunctata Haworth (Geometridae), the one-spotted variant, is a very common and abundant species in the forests of Maryland (Covell 2005). Caterpillars feed on species in numerous tree families, including species in the genera *Acer, Alnus, Carya, Fraxinus, Platanus, Prunus, Quercus, Salix, Ulmus*, as well as many other species (Covell 2005, Wagner 2005). They usually have two to three generations in Maryland each summer (Covell 2005).

Machimia tentoriferella Clemens (Oecophoridae), the gold-striped leaf tier, is easily recognizable due to its unusual silken tent-like shelter it constructs on the leaves of many shrubs and trees (Covell 2005). Larvae have been found on species in the genera *Acer*, *Carya, Fagus, Prunus*, *Quercus*, and *Ulmus* (unpublished data, Covell 2005). Throughout the summer season, the larvae will repeatedly abandon their shelters and

construct a new shelter on a nearby leaf but will feed and pupate on the same tree individual during its entire development (personal observation).

Orgyia leucostigma J.E. Smith (Lymantridae), the white-marked tussock moth, is one of the most ubiquitous generalist caterpillars in eastern North America forests and has two generations in Maryland (Barbosa et al. 2003, Covell 2005, Wagner 2005). It is an extremely polyphagous species feeding on approximately 140 host plants species, including both deciduous and evergreen hosts, and it occasionally is a pest of Christmas tree farms (Covell 2005). Females are wingless and lay eggs in large white to yellowish masses on trees soon after emerging from their pupa. Neonate larvae tend to feed upon trees that are near to the original egg mass from which they emerge (personal observation) but, as in other species (Capinera and Barbosa 1976) are capable of dispersing via ballooning.

Study Host Plants and Sites

Larvae were collected from a total of 6 host tree species. *Acer negundo* (box elder) and *Salix nigra* (black willow) were included in this study because they were the two species used in a long-term study on lepidopteran larvae that demonstrated a significant higher percent parasitism on *Acer negundo* relative to *Salix nigra* (Barbosa et al. 2001, Barbosa and Caldas 2004). In addition, I included four other tree species to encompass a range of host tree species on which the larvae fed, (i.e. *Acer rubrum* (red maple), *Quercus alba* (white oak), *Quercus rubra* (red oak), and *Prunus serotina* (black cherry)) and which differ in secondary chemistry, tree architecture, phenology, forest

microhabitat and family and phylogeny. All these factors may play a role in defining distinct selection pressures on these polyphagous caterpillars.

This study was conducted at five sites in eastern Maryland (Figure 1.1): Patuxent Wildlife Research Refuge (PWRR), Little Bennett Regional Park (LBEN), the Smithsonian Environmental Research Center (SERC), C&O Canal National Park (CNO), and Patapsco Valley State Park (PAT) (Figure 1.1). Sites were selected in the coastal to piedmont regions spanning four counties (Prince Georges, Montgomery, Howard, and Anne Arundel counties) in Maryland, USA. The distances between pairs of sites range from 28.3 km to 78.4 km (Figure 1.1). Two criteria dictated the selection of sampling sites. First, at least three of the selected host plant species had to be present in relatively large numbers (>20 trees of each species) to enable sampling of larvae and provisioning of leaves needed to rear larvae in the lab. Second, sites represented different plant communities (oak-dominated site, maple-dominated site, etc.) and occur in different regions of eastern Maryland (piedmont and coastal plain). The range of conditions increased the likelihood of sampling habitats that might promote HAD, and the diversity of different environmental conditions would enhance my inference beyond those documented at a single site.

Insect Sampling

Larvae were collected weekly from the five sites throughout central Maryland from June to September 2005-2010. Approximately 20 trees of each plant species were selected from each site and used for larval sampling for all five years. At each site larvae were sampled from 10 of the 20 trees and the following week the other 10 were sampled

to minimize over sampling of trees. Two methods of sampling were utilized; larvae were sampled by visually examining leaves for up to 10 minutes per tree, after which each tree was sampled using beat-sheet sampling. Beat-sheet sampling involves holding a canvas sheet below a branch while tapping each branch with a stick. Larvae fall onto the sheet and can be collected into sample cups. Both sampling techniques were used because some lepidopteran species (such as those in the Limacodidae, Arctiidae, Notodontidae, and Lymantriidae) are known to cling to the foliage or branches when the tree is disturbed and must be located visually and removed, while other Lepidopteran species (in the Geometridae and Noctuiidae) are cryptic and difficult to visually locate but will drop when disturbed. Larvae were collected into 118 mL plastic collection cups and transported to the laboratory in a cooler.

In the laboratory, larvae were identified to species and reared to adults to confirm accurate taxonomic identification and avoid potentially confounding genetic analysis due to the presence of parasitoids (and thus their DNA) within caterpillars. Larvae in the laboratory were supplied with a moistened filter paper and fresh foliage from the host tree species from which they were collected every two days until adult moths or parasitoid wasps emerged. If a moth emerged, it was identified, sexed, and then stored at -80° C degrees for subsequent genetic analysis.

DNA Extraction and Molecular Analysis

DNA was extracted from the moths using a DNeasy blood and tissue kit (Qiagen® Corp., Valencia, CA) following the instructions provided by the manufacturer. Wings were removed from moths before extraction to provide vouchers for each species.

The DNA of each sample was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific® Inc.) and then diluted to a concentration of about 50 ng/ul of DNA (Vos et al. 1995, Saunders et al. 2001).

AFLPs are markers used in a DNA fingerprinting technique that involves the amplification of restriction fragments across the nuclear genome. These fragments are selectively amplified and individuals are scored for the presence or absence of that fragment. AFLPs are dominant markers therefore one is unable to distinguish between a dominant homozygote and a heterozygote for the presence of the fragment (Vos et al. 1995). AFLPs are generated with the following protocol; digestion-ligation, pre-selection and selective polymerase chain reaction (PCR) using the reaction cocktail and programs shown in Table 1.3. Using five and six-cutting primers, EcoR1 and Pst1, the following primer pairs were used for selective amplification: Eco-ACT / Pst-AC, Eco-ACT / Pst-AG, Eco-AGA / Pst-AC and Eco-AGA / Pst-AG (Table 1.2). Fragment analysis was run on the ABI 3730 capillary sequencer using ROX500™ dye as a size standard (Applied Biosystem®, Forest City, CA, USA). All plates were run with negative controls and eight individuals replicated within each plate and eight individuals were replicated across every plate to ensure repeatability of all DNA fragments.

Electropherograms were visually inspected and analyzed using Genemarker® v1.51 software (SoftGenetics). Fragments were scored between 49 and 489 base pairs. The data for each species were compiled into a single matrix of individual / fragment presence or absence (1 or 0, respectively) for all possible primer pairs (See Table 1.2 for number of fragments per marker used). All peaks that were also present in the negative

controls were removed from the analysis. I removed any peaks from the analysis that were not identical across all repeated samples (Pompanon et al. 2005).

The SESim statistic (Medina et al 2006) was used to ensure that the number and fragments used were sufficient to detect the presence of host plant-associated genetic differentiation of each larva on the different trees studied. If SESim was larger than 0.05 more molecular markers and/or individuals were added to the sample size.

AFLP Data Analyses

Both analysis of molecular variance (AMOVA) and Nei-Li distance-based methods were used to examine populations of each species for host associated genetic differentiation. The presence/absence character matrix generated from the AFLP data was analyzed in PAUP*v4.0© (Swofford 2001) using an unrooted neighbor-joining algorithm with 1000 bootstrap replicates to compare patterns of host use. Distance was measured as the mean character difference between individuals (i.e. the pairwise number of differences in presence or absence of a given allele). To test for genetic structure associated with host plant, an analysis of molecular variance (AMOVA) was performed using ARLEQUIN v3.5© (Excoffier and Lischer 2010). For each species I analyzed the data among individuals across host-tree species, among individuals nested within host tree species nested within site, and among individuals across sites. To assess the degree of divergence between individuals in all host tree / site combinations, *Fst* values were also calculated.

The Bayesian clustering method implemented in STRUCTURE v2.2 was used to assess the degree of population structure (Pritchard et al. 2007). The most appropriate

model for dominant marker data is the recessive allele model assuming admixture and correlated alleles (Falush et al. 2007). These parameter settings are the most sensitive model for detecting presence of population structure in simulated data (Falush et al. 2003). This model-based clustering method analyzes data for population structure where there are *K* theoretical populations and individuals are assigned to a population or populations (admixture) based on individual genotypes while attempting to maximize Hardy-Weinberg equilibrium and linkage equilibrium. The model was run for each species using a burn in period of 125,000 generations followed by 1,000,000 iterations. Ten replicates were performed at each value of *K*. I tested *K* for one more than the maximum number of host plant species from which larvae were collected. Structure Harvester v0.6.1© was used to predict the most likely number of clusters (*K*) in the data by calculating ΔK, which evaluates differences in likelihood scores across values of K to determine which value of K best fits the data (Evanno et al. 2005). Evanno et al. (2005) demonstrated that the *K* corresponding to the peak in this value predicts the number of populations or K with the highest likelihood (Figure 1.5). CLUMPP 1.1.2© (Jakobsson and Rosenberg 2007) and DISTRUCT 1.1© (Rosenberg 2004) were used to plot the probability of membership in a population or the Q matrix (Q = the coefficient for each individual estimating its membership in a particular population or cluster) produced by STRUCTURE.

Results

Host-associated differentiation

The unrooted neighbor-joining trees generated from Nei-Li distance matrices revealed no clustering by host plant for any of the three species studied (Figures 1.2, 1.3, and 1.4). Absence of host-associated clusters suggests that host plant does not play a role in structuring the genetics of these species' populations. Similarly, the AMOVA results did not show evidence of host plant-associated genetic differentiation (Table 1.4). Further, *Fst* values were close to zero, indicating very little divergence among individuals on the different host plants, across sites, or within sites on different host plants. In all cases the largest portion of the variance (above 95%) was found within sites feeding on the same host plant species and not between sites on different host plants (Table 1.4).

Population Structure

STRUCTURE analysis assigned individuals of *H unipunctata* to five or seven populations with the highest probability indicated by the peak of delta *K* at *K*=5 (Figure 1.5A). STRUCTURE results show that individuals share a relatively equal proportion of alleles across all host plant species (Figure 1.6). Thus, these analysis clearly indicate that *H. unipunctata* demonstrate no population structure associated with host plant species. The equal proportion of each individual's genotype assigned to each subpopulation suggests that this species is panmictic in the study areas.

STRUCTURE analysis assigned individuals of *M. tentoriferella* to two populations with the highest probability at *K*=2 populations (Figure 1.5B). The analysis indicates that individuals did not cluster according to their host plants (Figure 1.7).

STRUCTURE analysis assigned individuals of *O. leucostigma* to three and five populations with the highest probability at *K*=3. Using the Evanno method, the highest increase in ΔK peaked at a population size of three (Figure 1.5C). There was no evidence for HAD (Figure 1.8), that is, no genetic based grouping based on host plant and thus evidence of true polyphagy in these herbivore species. Since this clustering indicated the possibility of some clustering by site or host plant species within site, I performed further analysis using the same STRUCTURE methods to test for site-associated differentiation and found no clustering by site or host plant within site (not shown).

Discussion

I found no evidence of HAD in the three species of polyphagous lepidopteran herbivores examined in this study. All analyses from AFLP markers indicate that these three polyphagous forest Lepidoptera are not genetically differentiated by host plant species. This suggests that these populations are panmictic and are not specializing on any particular host plant species across or within site. These results add to mounting evidence of the absence of HAD in non-parthenogenetic species that feed externally, such as lepidopteran larvae (Kerdelhue et al. 2006, Martinelli et al. 2007, Dickey and Medina 2010, Kohnen et al. 2011).

My results also show that these polyphagous species may not be ecologically driven to specialize on a particular host plant species. Although there is evidence of host plants driving ecological specialization in many phytophagous insects (Berlocher and Feder 2002, Dres and Mallet 2002, Stireman et al. 2005, Funk and Nosil 2008), the lack

of HAD in generalist herbivores, even at a local scale, in this habitat, may indicate the importance of maintaining genetic variation to adapt in a heterogeneous environment.

Polyphagy is not common in herbivorous insects; less than twenty percent of phytophagous insect species are polyphagous with the largest proportion of polyphagous species in the orders Lepidoptera, Orthoptera, and Coleoptera (Bernays and Chapman 1994). Polyphagous herbivores are thought to adapt to the host plant species that are locally abundant and suitable for growth (Tikkanen and Roinin 1991). Although host plant abundance in a forest may remain relatively constant from year to year, host plant quality (based upon herbivore performance, i.e. development time, pupal mass, fecundity, etc.) may influence the diet selection by generalist herbivores (Stephen and Krebs 1986). Indeed, even about extremely polyphagous herbivores like the gypsy moth there is considerable variation in the nutritional value of many of its host plants (Roslin et al. 2008). However, top-down pressure from natural enemies, such as parasitoids, as well as competition from other herbivores affects diet choice (Price et al. 1980, Bernays 1989, Barbosa et al. 2001, Lill et al. 2002, Singer and Stireman 2003). Differential parasitism on at least two of the host plants, *Acer negundo* and *Salix nigra*, in this study (Barbosa et al. 2001) and parasitism data from larval collections for this study (Shlichta, et al. in prep.) indicate the potential for differential selection pressures on the host plants . Moreover, sources of mortality for herbivores are unpredictable and likely to shift throughout the larval growth period from weather and plant factors to parasitism and predation as well throughout the season (Cornell and Hawkins 1997). Adaptation of an herbivore to one host limits the possibilities for using other hosts and can lead to behavioral and physiological restrictions on host use.

Our limited understanding of the traits and conditions necessary for HAD necessitates exploration of this hypothesis in species with diverse traits. Examining HAD in communities of phytophagous insects as was first proposed by Stireman et al. (2005) provides important insight on the ecological conditions that may promote or inhibit HAD and ultimately specialization of herbivores. Research by Dickey and Medina (2010) is the only other study that tests HAD from a community perspective to understand the commonality of HAD among phytophagous insects in the same host-plant species pair. Unlike this study and the studies on *Solidago* (Abrahamson and Weis 1997, Abrahamson et. al. 2003), Dickey and Medina (2010) focused on parthenogenetic species. They documented HAD in the parthenogenic yellow pecan aphid, *Monelliopsis pecanis* Bissel, but the quasi endophagous pecan bud moth, *Gretchena boliana* Granovsky, was found to be panmictic. *Gretchena boliana* had a similar population structure to *H. unipunctata* with individual genotypes assigned to each population with equal probability, indicating no population structure associated with host plant species (Figure 1.6). The results of the Dickey and Medina (2010) study and my results examining three species of Lepidoptera suggest that quasi-endophagy and limited mobility may not be traits that necessarily predictive of HAD. Moreover, as more phytophagous species are tested for HAD, we may find that HAD is not as widespread across herbivorous insect species.

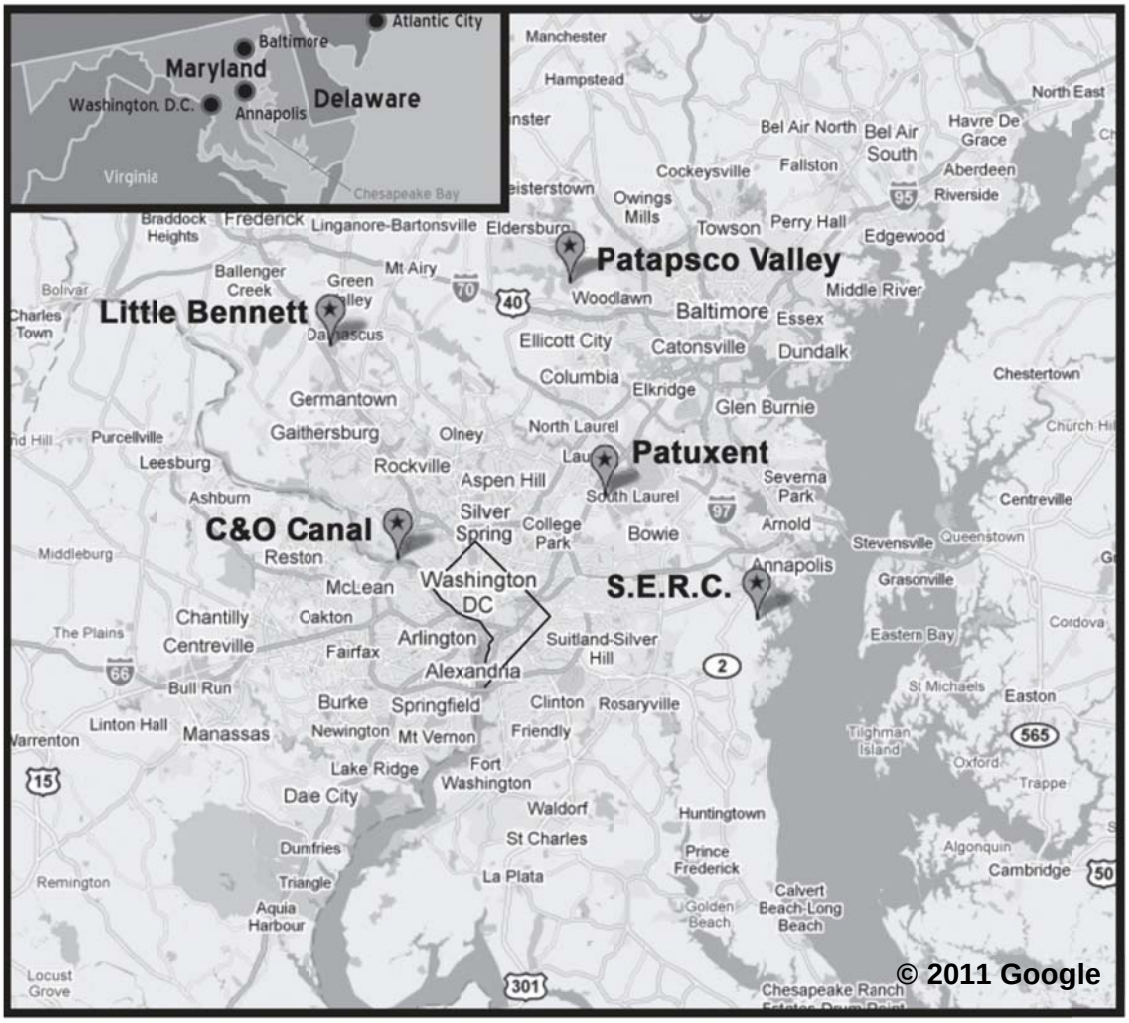

Figure 1.1. Location of larval sampling sites in Maryland, USA. (Map from GoogleMaps ©2011 Google. Adapted for this document by J. Riordan)

Table 1.1. Geographic locations of sampling sites in Maryland, USA and samples sizes of Lepidoptera species on different host plant species.

Site	**Location (degrees decimal)**	**Host plant species**	***Hypagyrtis unipunctata* sample size**	***Machimia tentoriferella* sample size**	***Orgyia leucostigma* sample size**
Little Bennett Regional Park	39.263645 N 77.277117 W	*Acer negundo*	*15*	2	4
		Acer rubrum	*3*	16	2
		Quercus alba	*10*	52	3
		Quercus rubra	*0*	2	4
		Salix nigra	*10*	0	12
		Prunus serotina	*0*	13	0
Patapsco Valley Park	39.274842 N 77.134511 W	*Acer negundo*	*1*	0	5
		Acer rubrum	*0*	2	0
		Quercus alba	*0*	21	0
		Salix nigra	*3*	0	0
Patuxent Wildlife Research Refuge	39.060155 N 76.73558 W	*Acer negundo*	*17*	0	12
		Acer rubrum	*1*	1	0
		Quercus alba	*3*	4	0
		Salix nigra	*4*	0	3
Plummer's Island (C & O Canal National Park)	38.96352 N 77.1385 W	*Acer negundo*	*1*	0	1
		Acer rubrum	*0*	0	0
		Quercus alba	*0*	0	0
		Salix nigra	*0*	0	0
Smithsonian Environmental Research Center (SERC)	38.870483 N 76.55036 W	*Acer negundo*	*2*	0	13
		Acer rubrum	*0*	0	1
		Quercus alba	*2*	6	2
		Salix nigra	*0*	0	0
Totals			***72***	**119**	**62**

Table 1.2. Primer, primer pair combinations and number of polymorphic loci used for amplified fragment length polymorphism analysis.

Primer	Sequence 5'to 3'	Number of polymorphic loci		
		Hypagyrtis unipunctata	*Machimia tentoriferella*	*Orgyia leucostigma*
Pre-selective				
Eco-A	GAC TGC GTA CCA ATT C**A**			
Pst-A	GAC TGC GTA CAT GCA G**A**			
Selective				
Eco-ACT	56-FAM/GAC TGC GTA CCA ATT C**ACT**			
Eco-AGA	56-FAM/GAC TGC GTA CCA ATT C**AGA**			
Pst- AC	GAC TGC GTA CAT GCA G**AG**			
Pst-AG	GAC TGC GTA CAT GCA G**AC**			
Primer combinations				
	Eco-ACT/Pst-AC	62	83	93
	Eco-AGA/Pst-AC	84	75	87
	Eco-ACT/Pst-AG	91	101	112
	Eco-AGA/Pst-AG	79	71	88

Table 1.3. The digestion-ligation reaction, and pre-selective, selective polymerase chain reaction (PCR) programs and cocktail used for amplified fragment -length polymorphism analysis.

PCR Reaction	Program	Cocktail (per reaction)
Digestion-Ligation	1: 37° for 5:00:00 2: 80° for 20:00 3: 4° for ever 4: end	per 50ul reaction: 30.25ul dH_20 5.0ul NEB* 10x buffer 3 0.5ul 10mg/ml BSA 5.0ul 10mM ATP 1.0ul 5uM Pst adaptor 1.0ul 5uMM EcoR1 adaptor 1.0ul 10 U/ul Pst (NEB*) 1.0ul 10 U/ul EcoR1 (NEB*) 0.25ul 400 U/ul NEB*Ligase 5.0ul DNA template
Pre-selective	1: 95° for 1:30 2: 95° for 0:30 3: 56° for 1:00 4: 1.0°/sec to 72.0° 5: 72° for 1:00 6: Go to 2, 21 times 7: 72.0° for 5:30 8: 4° for ever 9: end	per 10ul reaction: 0.3ul 25mM $MgCl_2$ 1.0ul 1.25mM dNTPs 1.0ul 10x NEB* buffer (-Mg) 4.6ul dH_20 0.5ul 10uM primer EcoR1-A 0.5ul 10uM primer Pst-A 0.1ul NEB* Taq 2.0ul DNA template
Selective	1: 95° for 1:30 2: 95° for 0:30 3: 65° for 0:30, -1° per cycle 4: 1.0°/sec to 72.0° 5: 72° for 1:00 6: Go to 2, 8 times 7: 95° for 0:30 8: 56° for 0:30 9: 1.0°/sec to 72.0° 10: 72° for 1:00 11: Go to 7, 22 times 12: 72.0° for 5:30 13: 4° for ever 14: end	per 10ul reaction: 0.3ul 25mM $MgCl_2$ 1.0ul 1.25mM dNTPs 1.0ul 10x NEB* buffer (-Mg) 4.6ul dH_20 0.5ul 10uM primer EcoR1-ACT/AGA 0.5ul 10uM primer Pst-AC/AG 0.1ul NEB* Taq 2.0ul DNA template

NEB* = New England Biolabs, USA.

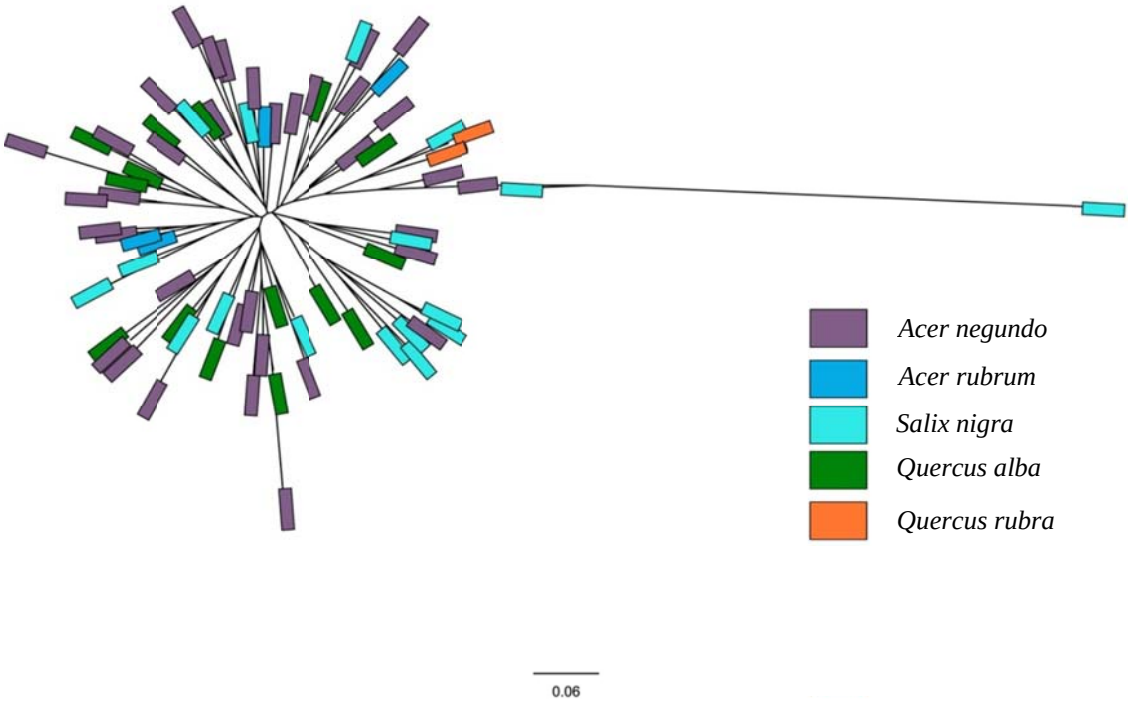

Figure 1.2. Neighbor joining tree of *Hypagyrtis unipunctata*. Color boxes represent an individual collected from the host plant species indicated in the key.

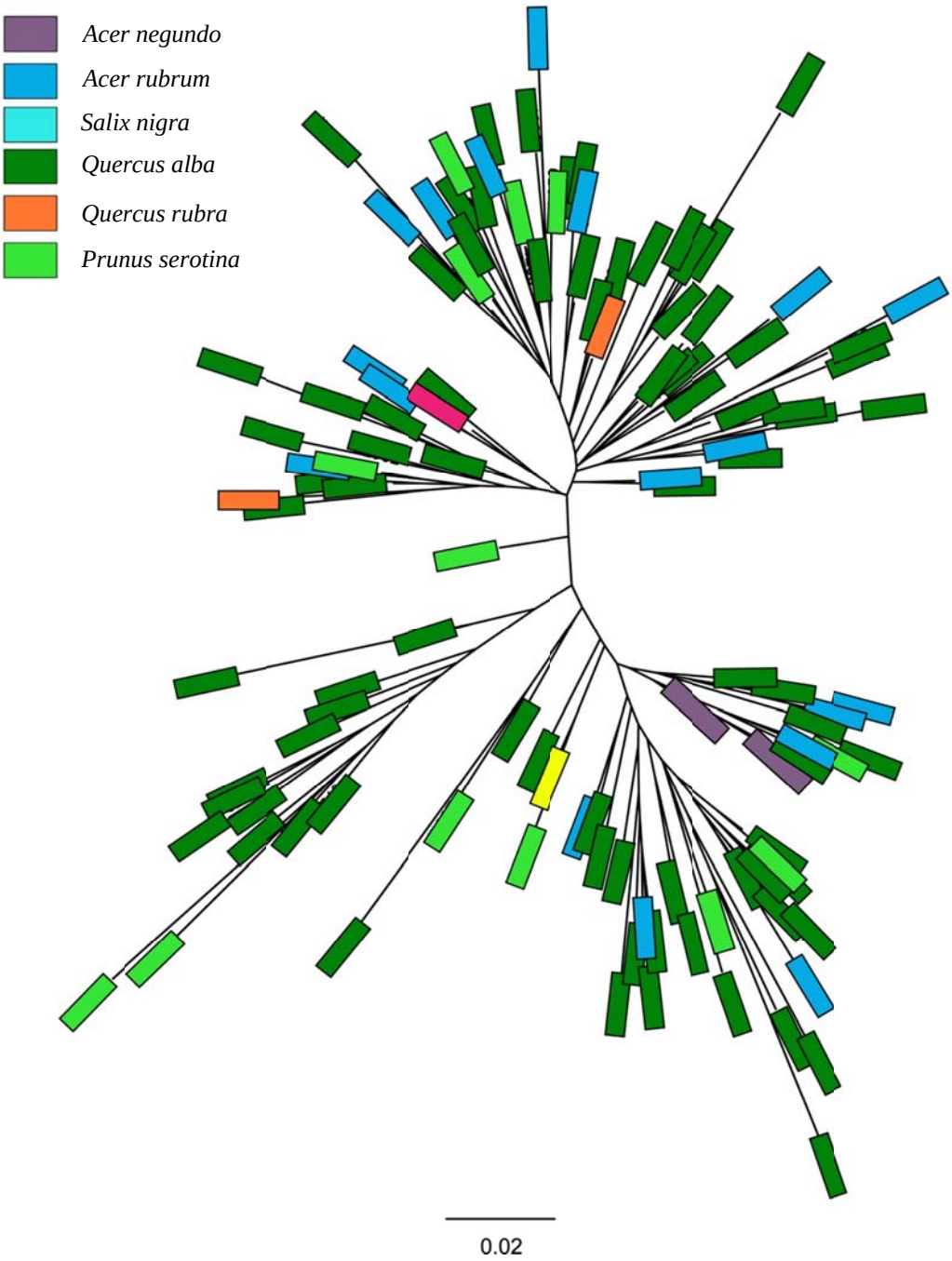

Figure 1.3. Neighbor joining tree of *Machimia tentoriferella*. Color boxes represent an individual collected from the host plant species indicated in the key.

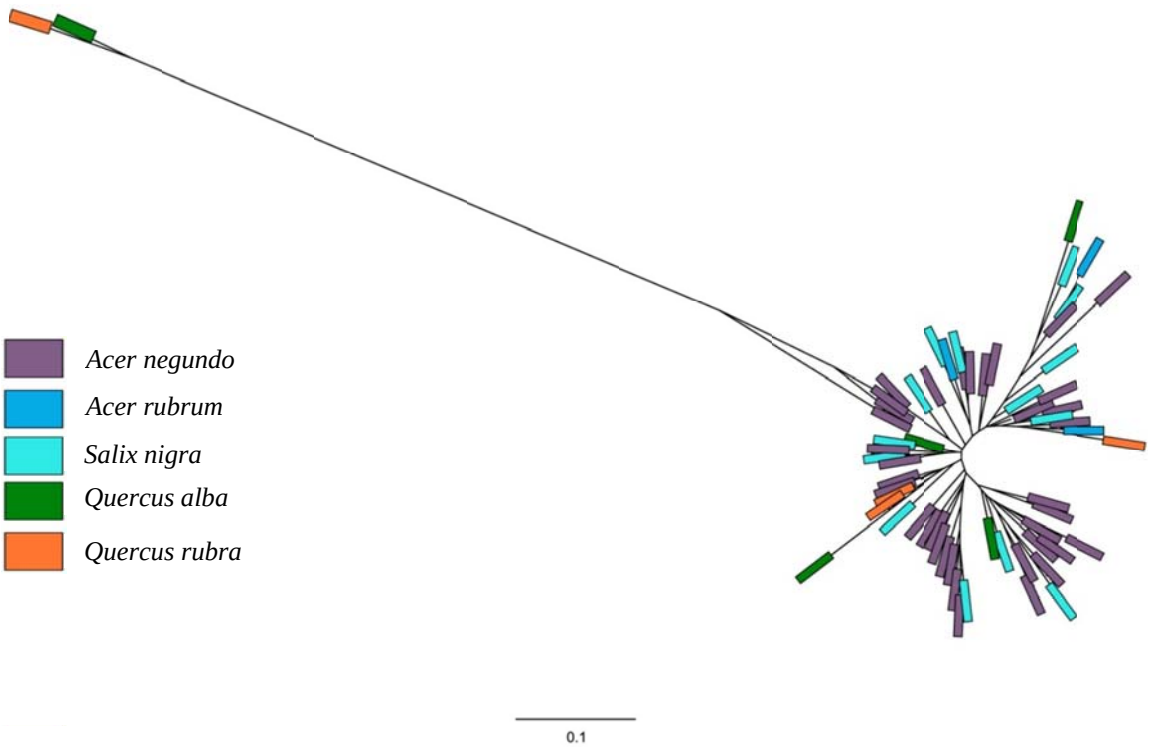

Figure 1.4. Neighbor joining tree of *Orgyia leucostigma*. Color boxes represent an individual collected from the host plant species indicated in the key.

Table 1.4. Analysis of molecular variance (AMOVA) for each species. Variation was partitioned (A) Among individuals across the host-tree species and (B) among individuals nested within site nested within host tree species.

Species	Source of variation	d.f.	SS	Variance	Variance (%)	Fst	P
(A.) Among individuals *across host-tree species*							
Hypagyrtis unipunctata	Among populations	3	40.417	0.12808	1.11		
	Within populations	70	798.232	11.40331	98.89	0.01111	0.03910
	Total	73	838.649	11.53139			
Machimia tentoriferella	Among populations	4	41.717	0.04937	0.50		
	Within populations	111	1080.676	9.73585	99.50	0.00505	0.30694
	Total	115	1122.397	9.78855			
Orgyia leucostigma	Among host tree	4	44.956	0.17778	1.83	0.01826	0.07429
	Within host tree	57	544.850	9.55877	98.17		
	Total	61	589.806	9.73656			
(B.) Among individuals *nested within site nested within host tree species*							
Hypagyrtis unipunctata	Host plants	3	40.417	0.20719	1.80		
	Sites within host	9	96.138	-0.18409	-1.60	0.00200	0.58358
	Within sites	61	702.094	11.50974	99.80		
	Total	73	838.649	11.53284			
Machimia tentoriferella	Host plants	4	41.624	0.03449	0.35		
	Sites within host	5	49.575	0.02118	0.22	0.00570	0.33333
	Within sites	105	1020.349	9.71761	99.43		
	Total	114	1111.548	9.77328			
Orgyia leucostigma	Host plants	4	44.956	0.03336	0.34		
	Sites within host	7	76.037	0.31157	3.21	0.03548	0.02639
	Within sites	50	468.813	9.37626	96.45		
	Total	61	589.806	9.72119			
(C.) Among individuals *across sites*							
Hypagyrtis unipunctata	Among sites	4	46.299	0.00840	0.07		
	Within site	69	792.350	11.48333	99.93	0.00073	0.43695
	Total	73	838.649	11.49173			
Machimia tentoriferella	Among sites	3	27.046	-0.04458	-0.46		
	Within site	113	1102.433	9.75604	100.46	-0.0046	0.62170
	Total	116	1129.479	9.71146			
Orgyia leucostigma	Among sites	4	50.620	0.29222	3.00		
	Within site	57	539.187	9.45942	97.00	0.02997	0.01075
	Total	61	589.806	9.75163			

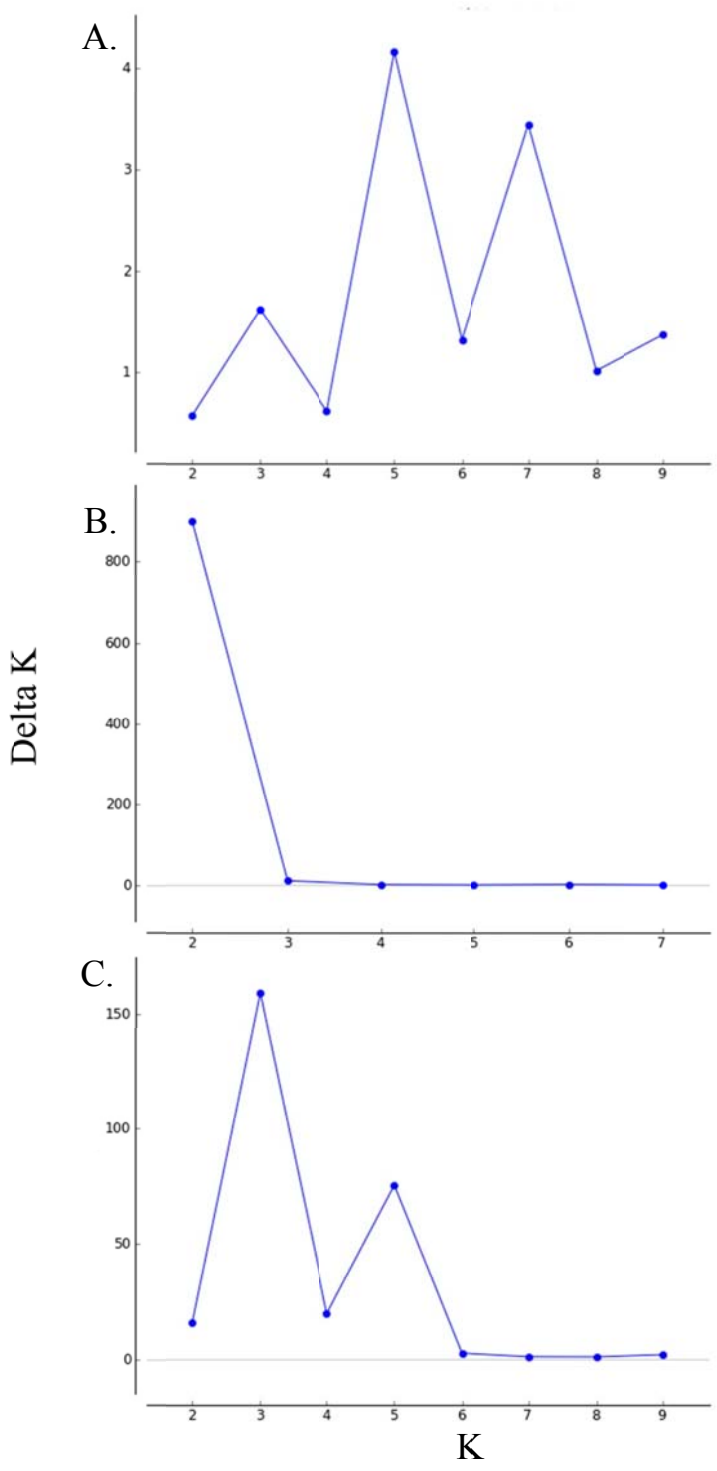

Figure1.5. ΔK calculated using the Evanno method (Evanno et al. 2005) for each species: (A) *Hypagyrtis unipunctata*, (B) *Machimia tentoriferella*, and (C) *Orgyia leucostigma*. Peaks indicate the K with the highest likelihood.

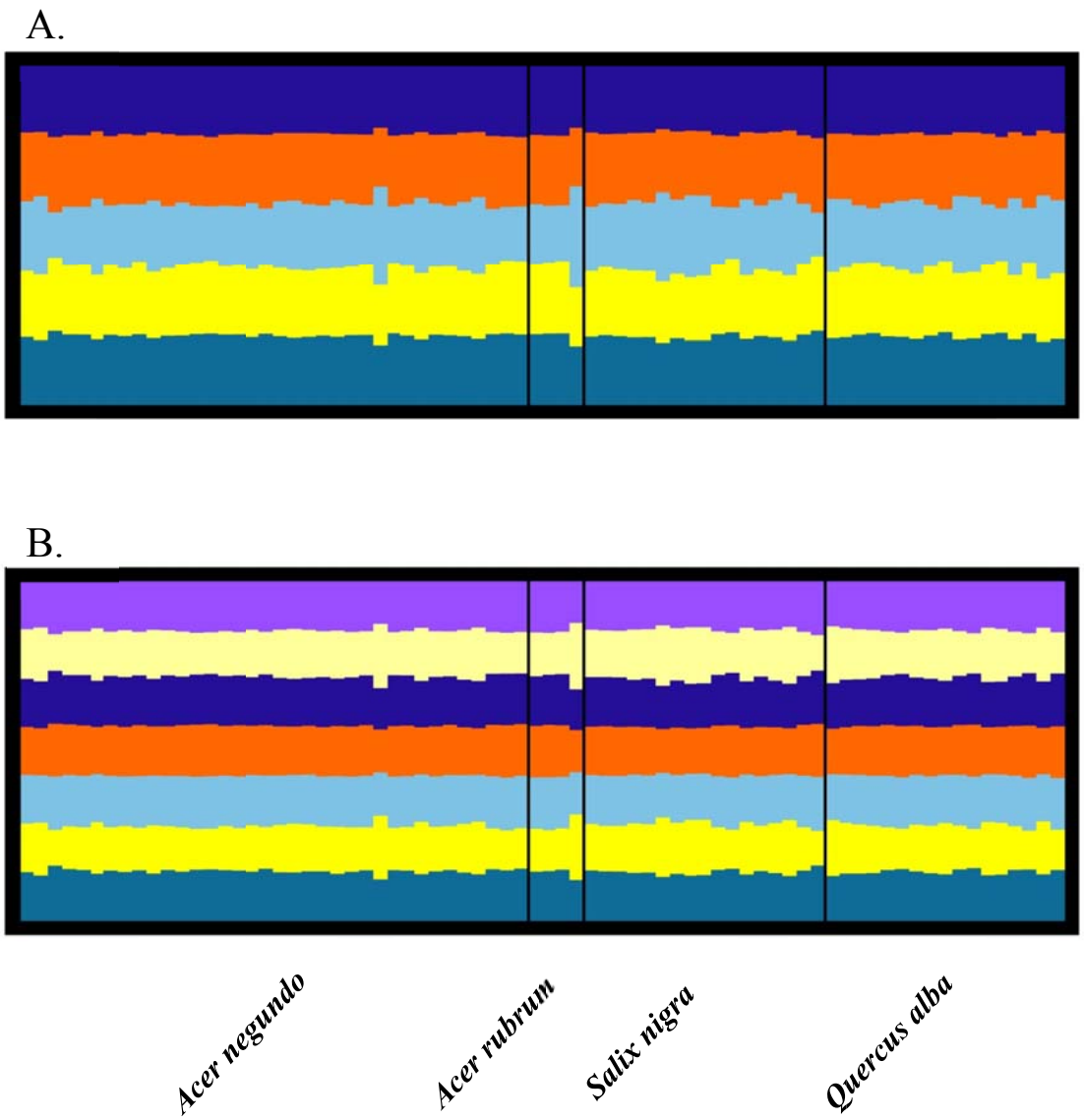

Figure 1.6. Population structure of *Hypagyrtis unipunctata*. The population structure when (A) K = 5 and (B) K = 7). Individual moths sampled are represented as a vertical line. Colors represent individuals that share a proportion of their genome with the population of the same color. Individuals collected from each host plant species are divided by a vertical black line (plant species noted on bottom).

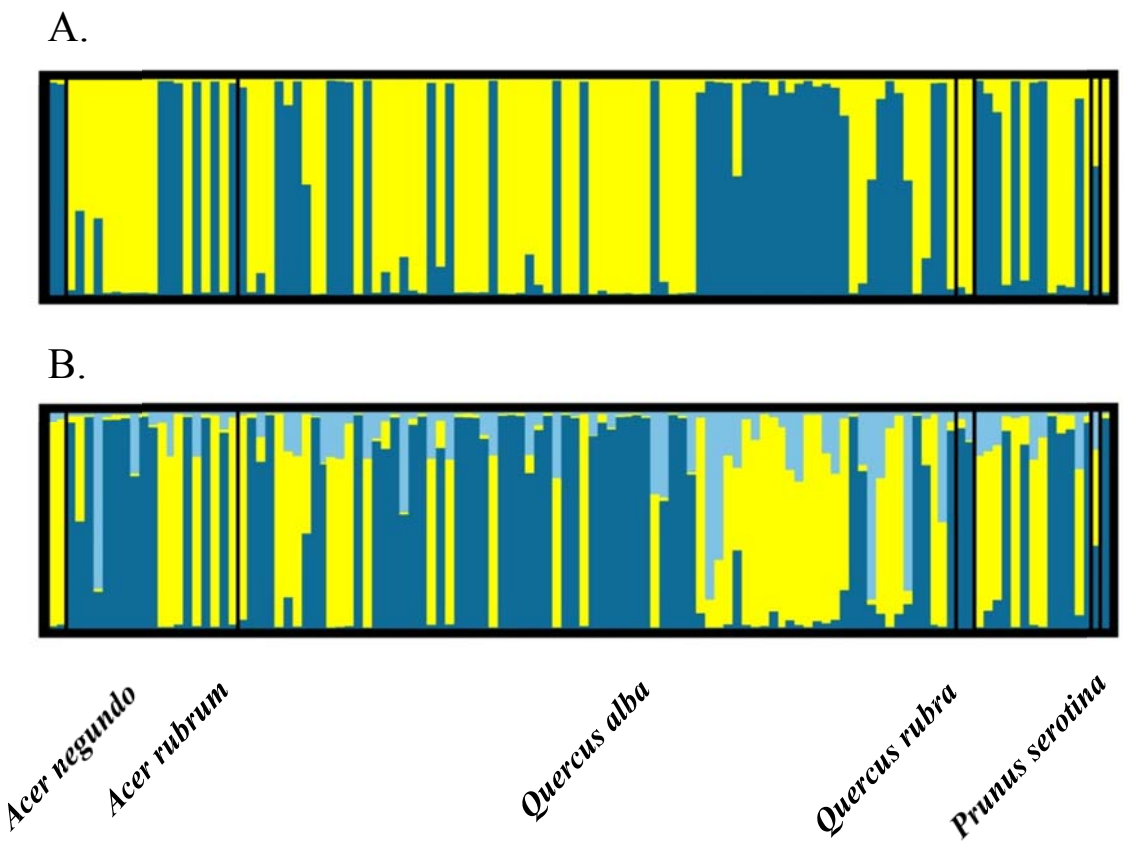

Figure 1.7. Population structure of *Machimia tentoriferella*. The population structure when (A) K = 2 and (B) K = 3. Individual moths sampled are represented as a vertical line. Colors represent individuals that share a proportion of their genome with the population of the same color. Individuals collected from each host plant species are divided by a vertical black line (plant species noted on bottom).

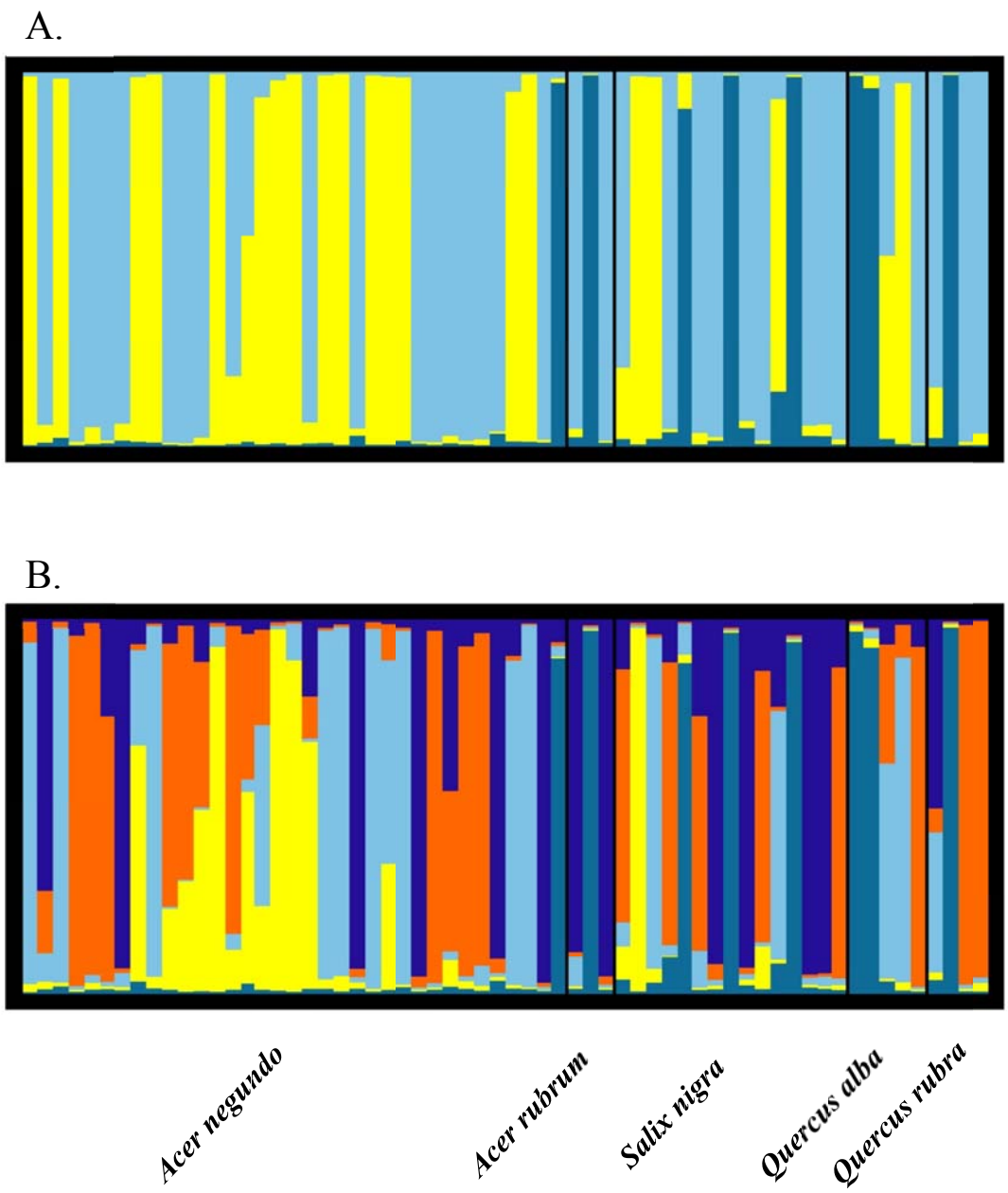

Figure 1.8. Population structure of *Orgyia leucostigma*. The population structure when (A) K = 3 and (B) K = 5. Individual moths sampled are represented as a vertical line. Colors represent individuals that share a proportion of their genome with the population of the same color. Individuals collected from each host plant species are divided by a vertical black line (plant species noted on the bottom).

CHAPTER 2:

Host plant-associated immune response in polyphagous forest Lepidoptera

Abstract

The host plant utilized by an herbivore can influence its susceptibility to parasitism. Thus herbivore host plant selections can play a major role in the outcome of interactions with natural enemies. Recent studies have shown that the immune response is a highly variable trait, both within and between species, and is affected by factors such as age, sex, and diet. Moreover, immune response is a heritable trait. Host plant by genotype interactions have also been found in phytophagous larvae indicating there is a genetic component to immune responses.

Most studies have focused on variation in host plant quantity or quality associated with closely related species of plants. The objective of this study was to examine the effect of host plant species on the immune defenses of polyphagous lepidopteran herbivores, specifically the intensity of encapsulation measured as percent melanization, of three common forest Lepidoptera species, *Orgyia leucostigma* (J.E. Smith) (Lymantriidae), *Halysidota tessellaris* (J.E. Smith) (Arctidade), and *Schizura unicornis* (J.E. Smith) (Notodontidae). Measuring the melanization of small glass beads (simulating a parasitoid egg) injected into larvae has been used as an effective way to quantify the immune response of herbivores.

Five to ten recently molted 4^{th} instar individuals of each species were injected with 10-20 silica beads, allowed to feed for 24 hours, dissected and analyzed for percent melanization.

A significant interaction between host plant and herbivore species was found. Herbivore encapsulation depended on their host plant, but that interaction varied depending on the herbivore species. There was a significant host plant by family interaction which indicates that families of herbivores differed in their host plant-mediated encapsulation response.

This study demonstrated that encapsulation ability is highly dependent on both the host plant diet of the herbivore and herbivore identity. Host plant alone did not significantly affect encapsulation across all herbivore species but did for two out of the three individual herbivore species when examined separately. Further, the host plant by family interaction suggests a genetic component to encapsulation ability.

Introduction

Host plant species influence the susceptibility of phytophagous insects to natural enemies (Benrey & Denno 1997, Raymond et al. 2002, Ojala et al. 2005, Yang et al. 2008), as well as the effectiveness of natural enemies in locating or capturing prey or hosts (Kareiva & Sahakian 1990, Grevstad & Klepetka 1992, Gingras et al. 2003). Both of these perspectives must be considered when trying to gain a better understanding of the influence of plants on the ability of natural enemies to inflict mortality on prey or hosts. Specifically, the host plant utilized by an herbivore can influence its susceptibility to parasitism, i.e. growth, conspicuousness, defense, etc. (Barbosa et al. 2001, Lill et al. 2002). For example, low host plant quality or nutrient value can decrease growth rate, thus increasing the time an herbivore is vulnerable to parasitism (Benrey & Denno 1997). Furthermore, differences in parasitoid success on herbivores feeding on different host plants reflect

trade-offs between herbivore host quality (in large part as a consequence of the nutritional value of the plant for the herbivore) and the degree to which the host plant selection minimizes the likelihood of being found and killed (Hutchings et al. 2001, Hutchings et al. 2006). Thus herbivore host plant selections can play a major role in the outcome of interactions with natural enemies.

Although insects are often seen as helpless victims of parasites, parasitoids, and pathogens, evolutionary processes have favored insect hosts that evolve effective immune responses to defend themselves once they are attacked. The insect immune system responds to foreign bodies that invade the insect hemocoel, from small particles that can be simply removed via phagocytosis, to larger objects, often dealt with via nodulation or encapsulation, including the eggs of hymenopteran or dipteran parasitoids (Nappi 1975, Gotz and Bohman, Godfray 1994, Strand 2008), nematodes (Stoffolano & Yin 1985, Gillespie et al. 1997), and fungi (Vey & Gotz 1986, Mullen & Goldsworthy 2006).

The immune response of insects is divided into a cellular and humoral immune response, although there is a great deal of overlap of the processes (Gotz and Bohman 1985, Lavine & Strand 2002). The cellular response involves the clustering of hemocyctes around the foreign object which eventually leads to the death of the intruder (Lavine & Strand 2002). This process generally begins with the formation of an envelope or capsule around the object consisting of granular cells binding to the invader. Layers upon layers of flattened hemocyctes (plasmatocytes in Lepidoptera) then attach to the target, and finally more granular cells attach and die forming a multicellular capsule (Gotz and Bohman 1985, Pech and Strand 1996, Lavine &

Strand 2002). Once the capsule has formed, the organism within the capsule usually dies due to one or more factors, including asphyxiation, toxicity of quinones produced, or antibacterial peptides associated with the humoral defense (Salt 1970, Gillespie et. al. 1997, Nappi et al. 2000). The specific killing agent or agents has not directly been determined (Lavine & Strand 2002). The humoral response includes production of the enzyme, phenoloxidase, in the hemolymph which catalyzes the oxidation of phenolic compounds resulting in the formation of melanin from the quinones produced (Gillespie et al. 1997 & Strand 2008). Recent studies have shown that the immune response is a highly variable trait, both within and between species, and is affected by factors such as age (Pomfret & Knell 2006, Stoehr 2007, Eleftherianos et al 2008), sex (Zuk et al. 2004, Stoehr 2007), and diet (Ojala et al. 2005).

This variation suggests that immune responses are phenotypically plastic and potentially costly to evolve and maintain. Research has demonstrated that pathogen and parasite resistance is costly to express and maintain and immune function may trade off with other important fitness traits (Sheldon & Verhulst 1996, Kraaijeveld and Godfray 1997). For example, trade-offs between the strength of the immune defense and other fitness traits manifest in male mating success (Rantala et al 2002, Worden & Parker 2005, Sadd et al 2006, Kivleniece et al 2010), growth (Haviola et al. 2007), and wing melanin (Zuk & Stoehr 2002, Rolff & Siva-Jothy 2003).

Moreover, immune response is a heritable trait. Host plant by genotype interactions have also been found in phytophagous larvae (Ojala et al. 2005, Klemola et al. 2007b) indicating there is a genetic component to immune responses. In a half-

sib design, Klemola et al. (2007b) demonstrated that larvae of *Epirrita autumnata* have significant heritable variation in encapsulation ability. Further, Ojala et al (2005) reported a host plant by genotype interaction for encapsulation which also suggests heritable variation in the arctiid moth, *Parasemia plantaginis.*

Exogenous factors may also influence the intensity or effectiveness of the immune response. Some of these factors include, but are not limited to, delayed induced resistance of host plants (Kapari et al. 2006), food quantity and quality (see references below), and abiotic factors such as climate (Selas et al. 2004, Haukioja 2005, Lazzaro & Little 2009). Most studies have focused on variation in host plant quantity (Schmid-Hempel & Schmid-Hempel 1998, Siva-Jothy & Thompson 2002, Lambrechts et al 2004) or quality associated with closely related species of plants (Klemola et al. 2007b, but see Ojala et al. 2005). In one study, individuals that fed on low-quality food had significantly higher encapsulation rate in pupae than individuals that fed on a high-quality food (Klemola et al 2007b). Ojala et al. (2005) examined the immune response of an Arctiid moth, *Parasemia plantaginis,* on five different plant-based diets. They found that host plant species significantly affected the immune defense (reflected in encapsulation score) and was specifically correlated with the amount of antioxidants in the diet (Ojala et al. 2005). The immune response of the autumnal moth, *Epirrita autumnata*, was higher on host plants with experimental defoliation indicating that induced plant response had a positive effect on encapsulation response (Kapari et al 2006).

Most studies that have examined the effects of host plants on insect immune responses have used either artificial diet or the foliage of closely related host-plant

species (but see Ojala et al. 2005 and Diamond & Kingsolver 2011). The reality for polyphagous species is that they feed on a wide variety of plant species, many of which are not closely related (Covell 2005, Wagner 2005). The choice of host plant species can lead to differential parasitism and survival (Le Corff et al. 2000, Barbosa et al. 2001, Lill et al. 2002). Since parasitism is one of the greatest sources of mortality for insect herbivores, especially larvae of Lepidoptera (Hawkins et al. 1997), the ability of an herbivore to mount an immune response against parasitoids may be critical to their survival. No studies have directly examined the effects of plant species in different taxonomic families on the immune responses of more than one polyphagous herbivore. Examination of polyphagous herbivore species feeding on several host plant species may provide a better understanding of the unique role of host plants in the survival of parasitized herbivores that have a variety of potential food resources. Thus, I ask whether specific host plant species are always associated with a higher immune response regardless of the herbivore species within the guild feeding on the plant. Alternatively, the pattern of immune defense may differ among herbivore species independent of host plant species consumed.

The objective of this study was to examine the effect of host plant species on the immune defenses of polyphagous lepidopteran herbivores, specifically the intensity of encapsulation measured as percent melanization, of three common forest Lepidoptera species, *Orgyia leucostigma* (J.E. Smith) (Lymantriidae), *Halysidota tessellaris* (J.E. Smith) (Arctidade), and *Schizura unicornis* (J.E. Smith) (Notodontidae). Measuring the melanization of small glass beads (simulating a parasitoid egg) injected into larvae has been used as an effective way to quantify the

immune response of herbivores (Smilanich et al. 2009a, Smilanich et al 2009b, and Diamond & Kingsolver 2011). This technique allows for the standardization of the larval immune response across numerous species controlling for the variation associated with different parasitoids. The melanization of the injected bead has been correlated to actual field parasitism rates. Smilanich et al. (2009b) demonstrated that caterpillar species that have a high percent melanization have low realized parasitism in the field suggesting immune response is a strong predictor of parasitism risk. Three hypotheses were tested in this study: (1) polyphagous insect herbivores differ from each other in host plant-associated encapsulation response, (2) host plant affects encapsulation ability independent of lepidopteran species, and (3) host plant mediated encapsulation of larvae is dependent on the herbivore genetic family, indicating a possible genotype by environment interaction.

Methods

Study Host Plants and Sites

Larvae and moths were collected from the Central (39°02.70° N, 76°47.31° W) and North Tracts (39°03.64° N, 76°44.24° W) of the Patuxent Wildlife Research Center in Laurel, Maryland (USA) located along the western and northern side of the Patuxent River respectively. The sites were chosen for their abundance of *Acer negundo* (box elder), *Salix nigra* (black willow), and *Quercus alba* (white oak). *Acer negundo* and *S. nigra* trees are in the riparian zones by ponds and *Q. alba* trees are found upland in small stands surrounded primarily by *Fagus spp., Acer rubrum,* and

other species. Twenty to thirty trees of each species were used for larval sampling. Light trapping for moths was done near the same trees used for larval sampling.

Study Organisms

Orgyia lecuostigma (J.E. Smith)

Orgyia leucostigma (Lymantriidae) is one of the most ubiquitous generalist caterpillars in eastern North America forests and it has two generations in Maryland (Barbosa et al. 2003, Covel 2005, Wagner 2005). An extremely polyphagous species feeding on approximately 140 host plants species, including both deciduous and evergreen hosts, *O. leucostigma* occasionally is a pest of Christmas tree farms (Covell 2005). Females are wingless and lay eggs in large white to yellowish masses on trees soon after emerging from their puparium. The larvae of these species are brightly colored and have numerous urticating hairs but still are heavily predated by birds and other invertebrate predators (Medina & Barbosa 2002). *Orgyia leucostigma* is parasitized differentially on *A. negundo* and *S. nigra* (Barbosa et al. 2003). In a data set of free-feeding, Macrolepidopteran larvae collected from 31 May to 20 August over five years (see Barbosa et al. 2000), *Orgyia leucostigma* was parasitized significantly more on *A. negundo* and *S. nigra* with a 35.4/29.7 percent parasitism respectively (Barbosa et al. 2000, Barbosa, unpublished data)

Halysidota tessellaris (J.E. Smith)

Halysidota tessellaris (Arctiidae) is common in Maryland deciduous forests and has two to three generations in the summer. It feeds on a wide variety of species in several plant genera including *Alnus, Betula, Ulmus, Carya, Acer, Quercus,* and

Salix species, as well as some other trees and shrubs (Covell 2005). *H. tessellaris* has an unusual behavior of resting on the tops of leaves near their feeding site, in broad view (Wagner 2005). This species is also covered with urticating hairs and setae which are displaced when held with forceps or touched and cause a mild irritation (personal observation). Barbosa and colleagues (unpublished data) found this species to be numerically subdominant. *H. tessellaris* has similar parasitism levels on *A. negundo* than *S. nigra* with a 13.6/12.9 percent parasitism respectively (Barbosa, unpublished data).

Schizura unicornis (J.E. Smith)

Schizura unicornis (Notodontidae) is another common species in the region with moths found at light traps from May to September (Wagner 2005). In Maryland, they have only one generation per year with mature caterpillars collected on foliage from July to September (J.Lill, personal communication). Larvae feed on the foliage of *Betula*, *Prunus*, *Quercus*, *Fagus*, *Carya*, *Acer*, *Rosaceae*, *Salix* species, as well as many other tree and scrub species (Covell 2005). The larvae are usually gregarious feeders in early instars (J. Lill, personal communication). These species are cryptic, resting and feeding on the edge of the leaf and resembling a brown, withering curl at the tip of a leaf. Although there is limited collection data for this species on box elder, this species was frequently collected on willow in a long term study conducted by Barbosa et al. (2003). Of more than 300 individuals collected from black willow 1991-1997, only 10.1% were parasitized (Barbosa, unpublished data).

Experimental Procedure

Orgyia leucostimgma

Female moths of *O. leucostigma* are flightless and cannot be collected from light traps, therefore larvae were collected from two host tree species, *Salix nigra* (black willow) and *Acer negundo* (box elder), at Patuxent Wildlife Research Refuge (PWRR) in the fall of 2007. *Salix nigra* and *Acer negundo* were used because of contrasting microhabitat, leaf architecture, budburst, and plant chemistry (Barbosa & Caldas 2004). These larvae were reared on their respective plants until they emerged as adults. Females were mated with males in ~500 ml Solo® deli cups until egg masses were laid, at which point the moths were removed and the egg masses were overwintered in an environmental chamber at 4° C in constant darkness and ~50% humidity. In the spring of 2008, eggs masses were removed from the chamber and left at room temperature until larvae hatched. Individuals in this experiment from the same egg mass are full-sibs because the female moths were mated with only one male. About half of larvae hatched from each egg mass (~20-30 individuals) were transferred onto black willow leaves and the other half were transferred onto box elder leaves. Individual larvae were reared in their own ~250ml deli cups and every other day they were given fresh leaves and a new piece of moistened filter paper to provide humidity. Leaves were collected from several trees of each species haphazardly selected in the PWRR site, sterilized in a 10% sodium hypochlorite (bleach solution) for 20 minutes to kill pathogens, rinsed twice for 20 minutes with fresh water, and air dried. These leaves were stored in a 4° C refrigerator until

needed. New leaves were collected, washed, and stored from PRR biweekly. Larvae were reared on this host plant until used for encapsulation studies.

Halysidota tessellaris & *Schizura unicornis*

Female moths were collected using both UV and mercury vapor light traps. These moths were brought back to the laboratory alive and put into ~500 ml deli containers with pieces of wax paper serving as a substrate for egg-laying. Containers were put into a chamber at 25° C programmed for 8:16 light/dark cycle until eggs were laid. The date of egg-laying was recorded and then these eggs were kept in a growth chamber programmed to follow seasonal temperature and light patterns measured at the Fort Meade weather station adjacent to PRR (station ID: MAR489) (Lind & Barbosa 2010, Ecological Archives E091-231-A1). Individuals from these egg masses are half sibs (possibly full-sibs) because the number of males the female mated with is unknown. When eggs hatched, an equal number were place into 500 ml cups each containing the leaves from one of the three host plant species. For these two herbivore species I used *A. negundo* and *S. nigra* but also included another common host species for these larvae, white oak (*Quercus alba*), in this study to get a broader assessment of encapsulation on a variety of plant genera. Larvae were reared using the same procedure as described for *O. leucostigma*.

Immune Response Measurements

I used a technique of injecting silica beads as a proxy for the oviposition of parasitoid eggs as described by Hu et al. (2003) and modified by Smilanich (2009a). This method has been successfully used to quantify immune defense with other

lepidopteran larvae (Lavine and Beckage 1996, Lovallo et al 2002, Rantala & Roff 2007, Smilanich et al. 2009a, Diamond & Kingsolver 2011). Approximately, 10-15 silica beads (DEAE SephadexA25, 40-120 um, Sigma Aldrich®, St Louis, MO, USA), dyed with 0.1% dilution of Congo red dye, were suspended in phosphate buffered saline (PBS) solution and injected into 4th instar larvae using a modified glass pipette or Hamilton 1702RN syringe with an 26 gauge beveled point needle (Hamilton Company®, Reno, NV, USA).

Approximately five to ten recently molted 4th instar individuals were used from each egg mass for encapsulation studies (see Table 2.1 for actual numbers for genetic family and mean individuals per family). For each larva, encapsulation (percent melanization) was quantified, as well as mass at the time of injection. Larvae were cooled at 4° C for 4 minutes and then injected just below the proleg between the 2nd and 3rd abdominal segment. The larva's integument was repaired after injection using Liquid Bandage (CVS Pharmacy®, Woonsocket, RI, USA) to prevent bleeding and infection. Larvae were allowed to feed on their respective food plants for 24 hours. Twenty-four hours post injection their condition was noted, and if they had eaten and frass was present in their cups, they were frozen in a -20° C freezer until time of dissection. Beads were recovered during dissection of larvae in PBS solution and photographed using a Zeiss AxioCamMRc5 digital camera at 80x magnification on a Zeiss dissecting microscope using imaging software (Discovery v.8, AxioCam software®; Carl Zeiss, Oberkochen, Baden-Wurttemburg, Germany). Photographs of beads were evaluated using Adobe Photoshop software® (v.6.0) to quantify changes in red intensity after melanization. The strength of melanization is measured based on

the degree to which the red color is obscured by blackened hemocytes. The Photoshop software measures the red value (r-value) for each bead. A completely non-melanized red bead will have the maximum r-value of 255 (pure red), however, a completely melanized bead with have an r value of 0 (black). For ease of interpretation, I used the following equation to change the r-values to percent melanization for each individual: *1- (r-value/maximum r-value)*, where maximum r-value is 255 (Smilanich et al. 2009a).

Statistical Analysis

Percent melanization was calculated for each individual as the mean of the r-values of all the beads recovered from each individual. All models were checked for normality and homogeneity of variances and data did not need transformations

The data was analyzed using analysis of variance (ANOVA) to compare percent melanization among host plant and herbivore species. I used a mixed linear model (PROC MIXED, SAS 1997), which is designed for analysis of both fixed and random effects with unbalanced data. Herbivore family was treated as a random factor and nested within species, while host plant, herbivore species, and mass at time of injection were fixed factors in the analysis. Larval mass was initially included in the model to test whether encapsulation was correlated to mass at time of injection. If it was not found to have a significant effect, it was removed from the model. In a mixed linear model, random effects are assumed to be normally distributed which I verified graphically. I tested the significance of a random effect is tested using a likelihood-ratio test (Fry 2004).

Each species was also analyzed individually in an ANOVA for the main effect of host plant on percent melanization. Host plant, the interaction of host plant and family, and mass were treated as fixed factors, and family was treated as a random factor.

Results

Polyphagous insect herbivores differ in host plant-associated encapsulation response

In the analysis examining the effect of host plant on the encapsulation of all three polyphagous species, the main effect of host plant species was not significant (Table 2.2). Mass at the time of injection was not found to significantly affect encapsulation and was removed from the model.

Percent encapsulation/r-value varied significantly across herbivore species (Table 2.3). Each herbivore species demonstrated a different intensity of encapsulation (Figure 2.1). The percent melanization of *O. leucostigma* ranged from 41.6-56.6%. The percent melanization for *H. tessellaris* ranged from 49.7-66.2%. Overall *S. unicornis* had the lowest percent melanization on all three host plants from 15.8-35.2% (Table 2.3).

A significant interaction between host plant and herbivore species was found ($F_{3,15}$=8.35, p=0.0001) (Table 2.2). This indicates that the combination of herbivore species identity and the host plant on which they were feeding explain the percent melanization in this study. In other words, herbivore encapsulation depended on their host plant, but that interaction varied depending on the herbivore species.

Individual herbivore species have a host plant-associated encapsulation response

Host plant significantly influenced encapsulation for two out of the three lepidopteran species. *Orgyia leucostigma*'s encapsulation (percent melanization) was significantly higher on *S. nigra* relative to *A. negundo* (Table 2.4). Host plant also had a significant effect on the percent melanization of *S. unicornis* (Table 2.4). Percent melanization of *S.unicornis*, from highest to lowest was *A. negundo, Q. alba,* and *S. nigra* respectively (Figure 2.1). Host plant does not have a significant effect on the percent melanization of *H. tessellaris* (Table 2.4).

Host plant-associated encapsulation is dependent on the herbivore family

No significant main effect of genetic family alone was found (Table 2.2). There was a significant host plant by family interaction ($F_{32, 51}$= 2.34, p= 0.0033) (Table 2) (Figure 2.2). This indicates that families of herbivores differed in their host plant-mediated encapsulation response which suggests a host plant by genotype interaction.

Discussion

Polyphagous insect herbivores differ in host plant-associated encapsulation response

One of the objectives of this study was to better understand whether populations of polyphagous insect herbivores represent a mosaic of host-plant mediated phenotypes, in this case the phenotype is host plant-associated immune

response. Parasitism impacts the survival of many insect herbivores, particularly lepidopteran larvae (Hawkins et al. 1997) and parasitism of herbivore species may vary systematically in insect species feeding on different host plants (Le Corff et al. 2000, Barbosa et al 2001, Lill et al. 2002). Through analysis of parasitoids reared from caterpillars recorded in the Canadian Forest Insect Survey, Lill and colleagues (2002) found that host plant identity influenced the species of parasitoid that attacks the caterpillar, as well as the likelihood of parasitism. A significant interaction between host plant species and herbivore identity was found (Lill et al. 2002). This indicated that individual larvae of the same species may experience differential selection pressure by parasitoids on different host plant species. Larvae that have a stronger encapsulation response are better able to defend themselves against parasitism and are more likely to survive attack, favoring larvae with higher encapsulation ability. If there is a genetic component to encapsulation ability and larvae experience strong parasitism selection on certain host plant species, polyphagous larvae that feed on that host plant species will have a significant advantage. Previous studies clearly demonstrate that host plant or diet affect encapsulation (Ojala et al. 2005, Klemola et al. 2007b, Diamond and Kingsolver 2010).

For this reason, my expectation was that host plant would have a significant effect on encapsulation ability. The result that host plant alone was not significant, but the interaction between host plant species and herbivore species was significant was unexpected in this context. However, studies on the effects of host plant species or diet on encapsulation have usually focused on one plant species (but see Ojala et

al. 2005, Diamond and Kingsolver 2010). It should be no surprise that examining these effects across multiple species would result in each species demonstrating a unique encapsulation response to different host plant species. Further, on examining data on percent parasitism across all caterpillar species on *A. negundo* and *S. nigra* more closely (Barbosa et al., unpublished data), percent parasitism was associated with a combination of the individual caterpillar identity and the host plant choice of the caterpillar. For example, although percent parasitism was significantly higher on *A. negundo* across the whole assemblage, some caterpillar species had higher parasitism on *S. nigra* and the intensity of parasitism was widely variable across species (Barbosa et al. 2001, Barbosa et al., unpublished data). For this reason, it is not host plant alone that it is important but rather the individual caterpillar identity and the host plant on which the caterpillar chooses to feed. This emphasizes how critical it is for studies to address the effect of diet on immune response across multiple species of herbivores to understand the interaction between components of diet and the herbivore immune response.

Studies have demonstrated that nutrition (Rantala et al. 2003, Lee et al. 2006, Povey, 2009) and secondary compounds, such as hydrolysable tannins (Haviola et al. 2007), iridoid glycosides (Smilanich et al. 2009a), and amides (Richards et al. 2010) can have positive or negative impacts on insect immune response. Further, the effect of the same components of a larval diet may differ from one herbivore species to another (Richards et al. 2010). The host plant species in this study are variable in both nutrition and secondary compounds; there are saponins in *Acer negundo,* tannins (condensed and hydrolysable) in *Quercus* alba, and salicortin, cinnamoylsalicortin

and condensed tannins in in *Salix nigra* (Taylor et al. 1994, Glynn et al. 2007). With the exception of one study on tannins (Haviola et al, 2007), we know little about the effects these secondary compounds have on the larval immune response and whether they have a similar effect across herbivore species. Smilanich et al. (2007a) demonstrated that buckeye caterpillars (*Junonia coenia* Hübner) feeding on a diet either high or low in concentrations of iridoid glycosides had a lower percent melanization on the diet with the high concentration of iridoid glycosides. This suggests that some secondary compounds present in the plant species may interfere with the ability of an herbivore to launch an effective immune response.

Due to the potential of herbivore species demonstrating host plant-associated encapsulation, differential parasitism may result from a disparity between actual parasitism and realized parasitism. These findings indicate that encapsulation ability may be a potential mechanism for differential parasitism. Parasitoids may attack species at similar rates on different host plants, but the strength of the larval immune response may depend on their host plant species. Further research into encapsulation ability on different host plants may also provide some insight into differential parasitism or why some species or populations appear to have higher parasitism rate on alternative host plants (Le Corff et al. 2000, Barbosa et al. 2001, Lill et al. 2002). It is likely that the mechanisms driving differential parasitism are not confined to one single causal factor and may indeed vary among sites, assemblages, or within the same system on different host plants. Lill et al. (2002) proposed alternative mechanisms for differential parasitism, such as the potential effect of plant volatiles in attracting parasitoids to the plant, conspicuousness to parasitoids, the density of

larvae on a particular host plant encourages foraging by parasitoids, the variation in the assemblage of other species on different host plant species, and larval defenses against parasitism (Lill et al 2002). One of the potential mechanisms behind differential parasitism that has only recently received some attention is the effects of host plant on the larval immune response to parasitism. Future studies examining polyphagous species across multiple host plants may be able to elucidate this interaction between host plant, herbivore, and parasitoid.

The mass of the larvae at the time of injection did not have a significant effect on larval encapsulation ability. This suggests that encapsulation ability is not a byproduct of the size at the time of the immune challenge although it has been shown in previous studies that encapsulation ability in *Pieris rapae* was higher for fast growing larvae than for slow growing larvae (Benrey & Denno 1997). Recent studies also have demonstrated that there was no association between pre-immune-challenged mass or growth rate and percent melanization (Ojala et al. 2005, Diamond and Kingsolver 2010).

Individual herbivore species have a host plant-associated encapsulation response

Another objective of this study was to examine species individually to understand whether host plant significantly affect their immune response. I found a significant effect of host plant on the immune response of two out of three of the larvae in this study. *O. leucostigma* and *S. unicornis* had host plant-associated encapsulation.

The effect of components of host plant on the herbivore immune response, such as secondary compounds and nutrition, may influence the herbivore interaction with the third trophic level. Smilanich et al. (2009b) demonstrated that the immune response (specifically percent melanization) predicted of parasitism levels in the field, in comparisons of field parasitism data with immune response lab studies on the same species Smilanich et al. 2009b). For the 16 species examined, larvae with the strongest immune response were found to have lower field parasitism rates and larvae with the weakest immune response had the highest parasitism rates. *Orgyia leucostigma* has a high percent parasitism (Barbosa et al 2001, Barbosa et al. unpublished data) and a weaker immune response on *A. negundo*. Further, *O leucostigma* was found to have a low percent parasitism and a strong immune response on *S. nigra*. (Barbosa et al 2001, Barbosa et al. unpublished data). However host plant did not significantly affect immune response of *H. tessellaris* and field parasitism data showed no difference between parasitism rates of this species on *A. negundo* and *S. nigra*. Further the parasitism of *H. tessellaris* was less than half of *O. leucostigma,* parasitism pressure may not be as strong for *H. tessellaris.* There is incomplete information available for larval parasitism of *S. unicornis* on both *A. negundo* and *S. nigra*, so I am unable to make comparisons for this species. More studies are needed in temperate forest, as well as other systems, test the patterns shown by Smilanich et al. (2009b).

Host plant-associated encapsulation is dependent on the herbivore family

I used a full sib (*O. leucostigma*) and a half-sib (*H. tessellaris* and *S. unicornis*) design to investigate the dependence of host plant mediated encapsulation of larvae on genetic relatedness (offspring from a single female). The significant interaction between host plant species and family supports previous studies that demonstrated a host plant by family interaction (Ojala et al 2005). This result suggests a potential genetic basis for the expression of melanization (Lee et al. 2008) and provides further evidence suggesting that encapsulation is a heritable trait (Klemola et al. 2007b). A significant genotype by environment (G x E) interaction between family and host plant species on the encapsulation ability of polyphagous forest lepidopteran species suggests that for each herbivore species a combination of an individual's genetic background and host plant choice play a critical role in determining encapsulation ability.

In summary**,** this study demonstrated that encapsulation ability is highly dependent on both the host plant diet of the herbivore and herbivore identity. Host plant alone did not significantly affect encapsulation across all herbivore species but did for two out of the three individual herbivore species when examined separately. Further, the host plant by family interaction suggests a genetic component to encapsulation ability.

Table 2.1. Number of families and individuals tested for encapsulation. Note: not all families were represented across all host plants.

Herbivore species	No. of families	Mean number of individuals/family
Halysidota tessellaris	18	5
Schizura unicornis	4	4
Orgyia leucostigma	12	5

Table 2.2. Mixed model ANOVA for the effects of host plant (fixed), species (fixed), mass (fixed), and family nested within species (random) on encapsulation (percent melanization).

Source of variation	F	df_1, df_2	p-value
Host plant	1.91	2, 51	0.1583
Species	7.49	2, 51	0.0014*
Host plant x Species	8.35	3, 51	0.0001*
Family (Species)	0.93	15, 51	0.5396
Host plant x Family (Species)	2.34	32, 51	0.0033*

Table 2.3. Encapsulation values, r-value, percent melanization, standard error, and mean mass (at time of injection) for each species on each host plant.

Herbivore species	Host plant species	r-value	Percent melanization	Std Err	Mean mass (g.)
Halysidota tessellaris	BE	95.8	62.4%	19.09	0.223
	W	128.4	49.7%	14.06	0.172
	WO	86.2	66.2%	10.68	0.266
Schizura unicornis	BE	165.2	35.2%	14.77	0.293
	W	214.8	15.8%	17.64	0.369
	WO	172.9	32.2%	18.24	0.338
Orgyia leucostigma	BE	148.8	41.6%	10.30	0.208
	W	110.7	56.6%	12.47	0.193

Table 2.4. Mixed model ANOVA for the effect of host plant on the encapsulation (percent melanization) of individual species with host plant (fixed), mass (fixed), and family (random).

Herbivore species	Source of variation	DF	F value	p-value
Halysidota tessellaris	Host plant	2, 26	0.41	0.6676
	Mass	1, 26	2.05	0.1638
Schizura unicornis	Host plant	2, 20	4.27	0.0286*
	Mass	1, 20	2.80	0.1097
Orgyia leucostigma	Host plant	1, 17	7.67	0.0131*
	Mass	1, 17	1.24	0.2804

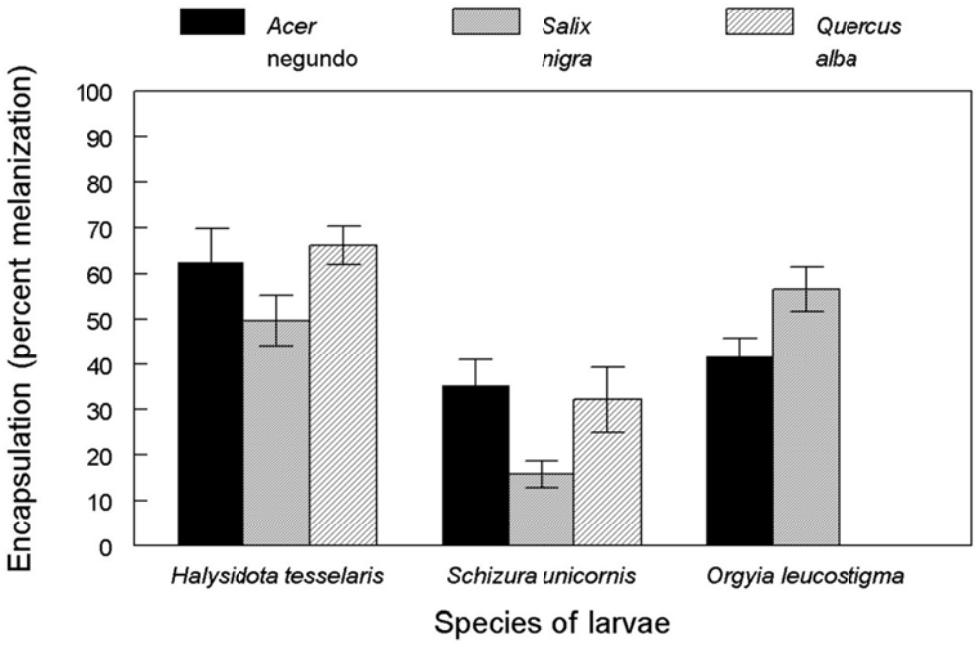

Figure 2.1. Percent melanization of 4th instar larvae fed foliage from *Acer negundo*, *Salix nigra*, and *Quercus alba*. Note: *Orgyia leucostigma* was not tested on *Q. alba* in this study. For ease of interpreting data, I used the following equation to change the r-values to percent melanization: 1- (r-value/maximum r-value).

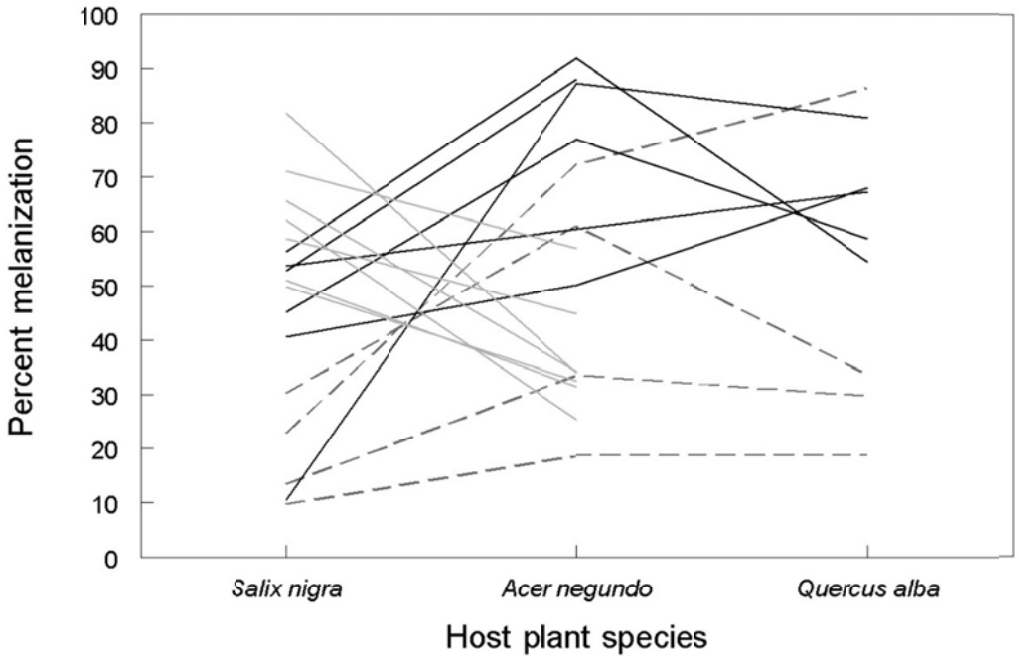

Figure 2.2. Reaction norm for the effect of host plant on percent melanization. The immune responses of each herbivore family on each host plant species are plotted in the same graph so that the immune response exhibited by different families may be compared. Lines represent the mean percent melanization of family, not the percent melanization of individual larvae. Families which had missing data for one of the host plants were not included (except *O. leucostigma* which was only on *A. negundo* and *S. nigra*).

CHAPTER 3:
Immune Responses and Their Potential Role in Insect Outbreaks

Abstract

Parasites, parasitoids, and pathogens play an important role in regulating insect populations. One of the hypotheses for how insects reach outbreak proportions is escaping control of their natural enemies. Changes in the strength of a host's immune response may affect the success of parasites, parasitoids and pathogens in the host population and potentially destabilize insect-pathogen or insect-parasitoid dynamics, and make control of the insect population by disease and parasitoids less likely, which, in turn, might cause outbreaks of the host population. In this chapter I present a brief background on immune responses, discuss the methods used to experimentally measure the components associated with immune response, how immune response varies, and lastly I draw on the studies available and present several potential hypotheses (Table 1) to stimulate further research.

Introduction

In this chapter I focus on the insect immune response as a potential mechanism facilitating escape of insect herbivores from regulation by natural enemies, allowing them to reach outbreak levels. I start by providing background on the insect immune response and discuss the methods used to experimentally measure the components associated with immune response. I go on to discuss how the immune response varies, why it is relevant to understanding the potential mechanisms or conditions associated with outbreaks, and investigate some traits of outbreak species that may be associated with an increased

immune response. Although many studies have intensely examined factors that influence the insect immune response, to date only a handful of studies have examined the potential role of immunocompetence in outbreaks (Kapari et al. 2006, Klemola et al. 2007a, Ruuhola et al. 2007, Yang et al. 2007, Klemola et al 2008, Yang et al. 2008). For this reason I draw on the studies available and present several potential hypotheses (Table 1) to stimulate further research. My goal is to show that changes in the strength of insects' immune responses may affect the success of natural enemies (parasites, parasitoids, and pathogens) in host populations, which in turn may destabilize insect-enemy dynamics and lead to outbreaks.

The roles of parasites, parasitoids, and pathogens in regulating insect populations have been researched extensively. One clear hypothesis that emerges from the outbreak literature is outbreaks result from insect populations escaping the control of their natural enemies (Morris 1963, Berryman 1996, Klemola et al. 2010). Research to explain the mechanisms behind the escape from natural enemies include those that are behavioral (Gross 1993, Gentry and Dyer 2002, Smilanich et al. 2011), morphological (Gentry and Dyer 2002, Barbosa and Caldas 2007), chemical (Dyer 1995, Gentry and Dyer 2002, Nishida 2002), and physiological (Carton et al. 2008, Smilanich et al. 2009a). While each of these mechanisms is an important adaptation for escape from natural enemies, immunity is a critical escape mechanism that has received little attention (but see Godfray 1994, Smilanich et al. 2009b).

A low incidence of parasitism has been found to be characteristic of growing defoliator and other outbreak populations (Berryman et al. 2002). Indeed, the low incidence of parasitism in populations reaching outbreak status has often been attributed

to the inability of parasitoids to “catch up” with the population growth of the outbreak population. Nonetheless, this conclusion has not accounted for the disparity between realized parasitism and actual parasitism. Parasitism rates are often assessed as the number of parasitoids reared from infected hosts (i.e., realized parasitism). This assumes that parasitism rates are singularly dependent on the number of parasitoids and/or the incidence of parasitism, and does not assess those insect hosts that have successfully defended themselves against parasitism (Kapari et al. 2006).

In long-term studies monitoring larval density and parasitism of the autumnal moth, *Epirrita autumnata* Borkhausen (Lepidoptera: Geometridae), on birch in Fennoscandia, *E. autumnata* had delayed density-dependent variation in larval parasitism rates ranging from 0-100% (Ruohomäki 1994, Tanhuanpää et al. 2002). The abundance of some parasitoid species (surveyed from trapping of adults) around the time of outbreaks declined to almost zero within two years after outbreaks (Nuorteva 1971). These results could suggest that the larval immune response to parasitism during periods of high larval density may play a role in decreasing the number of parasitoids post-outbreak. Furthermore, Klemola et al. (2008, 2010) showed decreases in *E. autumnata* density with increases in parasitism and vice versa. Variations in herbivore - pathogen or herbivore - parasitoid interactions may be due, in part, to the susceptibility of the host herbivore to disease or parasitism. For example, the extent of oak defoliation by the gypsy moth, *Lymantria dispar* Linnaeus (Lepidoptera: Lymantriidae), was correlated with increased survival when exposed to nuclear polyhedrosis virus (NPV) (Hunter and Schultz 1993). Hunter and Schultz (1993) suggest that *L. dispar* was inducing changes in the leaf quality of its host plant, thereby increasing gallotannin concentrations that inhibit

NPV. Another possibility in their scenario is that declining leaf quality due to defoliation may positively impact the immune responses of *L. dispar* against NPV.

Insect immune response

The immune response is composed of a humoral and cellular response that work in concert to defend against parasitoids, parasites, and pathogens (reviewed by Carton et al. 2008, Strand 2008, Beckage 2008). The cellular response is directed by specialized hemocytes. In general, when a special type of hemocyte called granular cells, contact a foreign target, they lyse or degranulate, releasing material that promotes attachment of plasmatocytes. Then, multiple layers of plasmatocytes form a capsule around the foreign target, which is then melanized by a humoral response. A suite of proteins, including regulatory proteins, hemocyte response modulators, and melanization enzymes are also involved in the immune response, making it a complex and resource costly process (Schmid-Hempel and Ebert 2003). The humoral response aids in the production of non-self-recognition proteins, enzymatic activity leading to melanization and clotting, and anti-microbial peptides. The combination of asphyxiation from encapsulation and the cytotoxic products produced during melanization is thought to contribute to killing the parasite or pathogen (Strand 2008).

A variety of methods have been used in experiments seeking to understand the variation in the immune responses. A common method for measuring encapsulation and melanization is the injection of beads into the hemolymph or the insertion of a synthetic object through the cuticle and into the insect's hemocoel (Lavine and Beckage 1996, Klemola et al. 2007b). The immune response is quantified by measuring the color

change and/or the thickness of accumulated cells around the object (Beckage 2008). Other measurements rely on quantifying the protein activity of the humoral response by determining the concentration of the enzyme, phenoloxidase (PO), which catalyzes the melanization cascade, or quantifying the activity of antibacterial lysozyme activity (Adamo 2004a). Still other measures include hemocyte counts (Ibrahim and Kim 2006), hemolymph protein concentration (Adamo 2004a), and gene expression (Freitak et al. 2009). Studies measuring the response of multiple immune parameters show that they do not always respond in the same way (Adamo 2004b) and can exhibit trade-offs (i.e., antibacterial efficiency trades-off with PO activity) (Cotter et al. 2004b). For example, not only will a bacterial infection induce a specific component of the immune response that is different from a parasitoid infection, but the response will also be specific to the type of invading bacteria (Riddell et al. 2009). In relation to insect outbreaks, if outbreak species have high immune capacity, then losing natural enemies may result in an outbreak. This idea will be further developed in the hypothesis section of this chapter.

Sources of variation in immune response associated with outbreaks

Host plants—Host plant diet is a major source of variation in the immune response (Smilanich et al. 2009a, Diamond and Kingsolver 2011). Since most aspects of herbivore outbreaks involve interactions with the host plant, it is reasonable to hypothesize that the immune response will play an integral role, from start to finish, in insect outbreak dynamics. The effects of host plants on immune responses can depend on plant chemistry, herbivore health, both herbivore and plant genotype, and the specific immune parameter being measured (i.e. PO activity, encapsulation of an inert object, lysozyme activity, etc.). Certain plant secondary metabolites can alter the effectiveness of immune

responses. For example, ingestion of diets containing carotenoids enhances immune function due to their free-radical scavenging properties (de Roode et al. 2008, Babin et al. 2010). In contrast, ingestion of other secondary metabolites can negatively affect the immune response (Haviola et al. 2007, Smilanich et al. 2009a). Macronutrients can also affect the immune response. Protein and carbohydrates not only boost immune parameters (Lee et al. 2008, Srygley et al. 2009, Cotter et al. 2011), as does plant/diet quality (Yang et al. 2008, Bukovinszky et al. 2009, Diamond and Kingsolver 2011), but also is preferred by immune challenged herbivores (Lee et al. 2006, Povey et al. 2009). In most cases high plant quality (i.e. low secondary metabolite concentration and high nitrogen content) translates to increased immunity (but see Klemola et al. 2007b).

Heritability. Different immune responses have been shown to be heritable, indicating that if immune responses play a role in outbreak formation or decline, there is a genetic component to the potential for a population or species to outbreak (Kraaijeveld and Godfray 1997, Schmid-Hempel and Ebert 2003, Carton et al. 2005). Early work investigating the genetic basis of immune defense mostly focused on *Drosophila melanogaster*, and has since expanded considerably to include many other invertebrate species (Cotter and Wilson 2002, Ojala et al 2005, Moret 2006, Rantala and Roff 2006, 2007, Freitak et al. 2009). Significant heritability for encapsulation rate was found in *E.autumnata* (Klemola et al. 2007b). Knowing the heritability and genetic basis of the immune response helps to understand and illuminate insect outbreaks in an evolutionary context. Later in the chapter I propose and discuss the hypothesis that the heritability of the immune response may play a prominent role in outbreak periodicity.

Traits or conditions associated with outbreak species

Only a small fraction of insect species reach outbreak proportions (Mattson and Addy 1975, Hunter 1995). Most species maintain a relatively low, stable population size and do not become noticeable defoliators (Mason 1987). Although there have been numerous studies trying to find traits or factors to explain why some species outbreak, we are still left with some rather unsatisfying explanations of what contributes to population regulation, from weather and host plant chemistry, to life history traits and natural enemies. However, it remains unclear why some species in the same habitat, under the same environmental conditions are outbreak species and others are not. Furthermore, the questions of why some populations of species outbreak in certain geographical areas, but not others, are still the subject of much debate (Ruohomäki et al. 1997, Bjornstad et al. 2010).

Several studies have used a comparative approach between outbreak and non-outbreak species to examine important characteristics that set them apart (Mason 1987, Wallner 1987, Cappuccino et al. 1995, Hunter 1995, Price 2003). Hunter (1991) examined the Canadian Forest Insect Survey (CFIS) data supplemented with other sources to compare life history traits of outbreak and non-outbreak species of macrolepidoptera. The findings that are most relevant to my focus on outbreaks and immune response include gregarious feeding behavior (Larsson et al. 1993, Cappuccino et al. 1995), overwintering as eggs in clusters or masses, and a wide diet breadth (a mean of 20 plant genera) (Hunter 1991). Although some advantages of gregarious behavior and laying eggs in clusters have been proposed (reviewed in Hunter 1991), I suggest that increased density of herbivores, which may be a result of egg clustering and gregarious

behavior, may additionally lead to an increased immune defense against natural enemies. The importance of these traits will be explored in the next section, where several hypotheses are proposed speculating on how immune responses play an integral role in insect outbreaks (Table 1).

Hypotheses on insect outbreaks and the immune response

Hypothesis 1: Density-dependent prophylaxis (Wilson and Cotter 2009). This hypothesis proposes that hosts living at high densities suffer a greater risk of disease and therefore invest more in immunity.

Insects across a broad range of taxa are able to assess conspecific density, presumably as an indication of a potentially deteriorating quality or quantity of food, or exposure to pathogens or parasites (Carroll and Dingle 1996, Wilson and Cotter 2008). This is most often associated with insects that are able to migrate when conditions deteriorate (Denno et al. 1991, Carroll and Dingle 1996), or are capable of switching from a gregarious to solitary phase (Wilson and Cotter 2008). Given that not all insects are able to migrate when density increases (especially those in the larval stage or with limited mobility), such species may risk increased exposure to pathogens, parasites, and other natural enemies. With increasing density, the risk of transmission of disease increases due to the higher probability of interaction with potentially infected conspecifics (Steinhaus 1958, McCallum et al. 2001). Species that can modify their level of disease resistance to match potential risk of exposure would minimize the costly investment in disease resistance (Kraaijeveld and Godfray 1997, Wilson and Reeson 1998. Moret and Schmid-Hempel 2000). Many of the insects that exhibit density-

dependent prophylaxis (DDP) are outbreak or pest species, suggesting that gregarious outbreak species may be more likely to allocate resources to immune defense as densities increase.

Resistance against parasites or pathogens may be costly to maintain and express (Sheldon and Verhulst 1996), therefore a trade-off may exist between immune function and other traits. For example, Kraaijeveld and Godfray (1997) found a trade-off between competitive ability and immune function in *Drosophila* males. Natural selection should thus favor insects that are able to assess larval density and allocate more resources as needed for resistance against natural enemies (Goulson and Cory 1995, Wilson and Reeson 1998). This phenotypically plastic response of allocation of resources to defense has been demonstrated in numerous insect species (Wilson et al. 2001, Cotter et al. 2004a, Ruiz-Gonzàlez et al. 2009). In a comparative analysis using fifteen studies of temperate Lepidoptera, Hochberg (1991) found a positive correlation between gregarious feeding behavior and age-related resistance to viral infection.

Several studies indicate that there may be a connection between DDP and species that have been known to be outbreak pests (D'Amico et al. 1996, Rothman and Myers 1996, Wilson and Reeson 1998) (Table 1). For example, egg masses of the northern tent caterpillar, *Malacosoma californicum pluviale* Dyar (Lepidoptera: Lasiocampidae), range in size from 130-375 eggs per mass. Rothman and Myers (1996) exposed caterpillar siblings from a range of egg masses of different sizes to NPV in their 2nd larval instar. They found that individuals that came from larger eggs masses had significantly higher resistance to NPV than those from smaller egg masses. Although these differences associated with larval density may be attributed to resource availability, genetic

differences, or maternal effects, density appears to play a role in intensity of resistance to viral infection across studies (Table 1).

The majority of DDP research indicates that there is a positive association between density and immune response (Wilson & Cotter 2009). There are still many aspects of DDP that warrant further investigation, such as how insects assess density, the trade-offs associated with increasing immune response, the specificity of immune responses, and the mechanism behind allocating resources for immune defense. Although several levels of density have been tested experimentally, whether there is a critical density threshold that facilitates an increase in immune response remains unclear. A large number of the studies examining DDP were conducted in the laboratory (Kunimi and Yamda 1990, Barnes and Siva-Jothy 2000 , Cotter et al. 2004a). These studies may not allow for the most biologically relevant assessment of immune defense. Field studies on outbreak species, such as that of Klemola et al. (2007a) which assessed the immune response of *E. autumnata,* allowed for direct assessments of immune response at different densities. Examining density dependent resistance under more natural conditions will allow us to gain a better understanding of how immune responses influence the population dynamics of outbreak species.

Hypothesis 2: Melanism and disease resistance. Outbreak species have higher cuticular melanin which is positively correlated with immune response.

Phase polymorphism, a phenomenon closely associated with DDP, occurs when species change to darker, more melanized forms under high density conditions (Wilson and Cotter 2009). Changes in color form are often correlated with other traits such as

increased activity, respiration rate (Shibizaki and Ito 1969), desiccation resistance (Parkash et al. 2008), thermotolerance (Parkash et al. 2010), or flight activity (Parker and Gatehouse 1985). Phase polymorphism has been shown in a diverse array of taxa, including many lepidopteran agricultural pests or outbreak species (reviewed by Goulson and Cory 1995). It has been suggested that melanism may be a potential indicator of high levels of immunocompentence in insects (Majerus 1998, Barnes and Siva-Jothy 2000, Wilson et al. 2001). The mechanism behind the association of melanism and immune defense remains unclear, although some studies have suggested possible mechanisms.

Cuticular melanin and the melanin involved in the immune response are sometimes correlated, thus successful outbreak species are more likely to have stages with increased melanin (Mikkola and Rantala 2010). Melanin is a product of the phenoloxidase (PO) cascade and is found in the cuticle, hemolymph, and midgut (Cotter et al. 2008). It plays a critical role in immune defense during recognition of foreign object and encapsulation of large invaders (Wilson et al. 2001, Cotter et al. 2004b). It also improves the cuticle's ability to function as a physical barrier to the penetration of fungus, parasites, and pathogens (St. Leger et al. 1988, Hajek and St. Leger 1994, Wilson et al. 2001). An association between cuticular melanin and the insect immune defense against pathogens and parasites has been demonstrated in numerous species (Hung and Boucias 1992, Beckage 1993, Wilson et al. 2001, Cotter et al. 2004b). Furthermore, melanin is toxic to microorganisms and has potential antimicrobial activity (Ourth and Renis 1993). Melanin can bind to a range of proteins and act as an inhibitor to many of the lytic enzymes produced by microorganisms (Bull 1970, Doering et al. 1999). The PO cascade involves a suite of enzymes that oxidize tyrosine to quinones and their

polymerization product melanin (Nappi and Vass 1993). These same enzymes are also involved in cellular encapsulation, humoral encapsulation, and nodule formation (Hung and Boucias 1992, Beckage and Kanost 1993).

Although many of the studies mentioned showed increased melanism under crowded conditions, it is important to note that melanism is not just a byproduct of increased density. Controlling for the effect of larval density, several studies have examined the association of melanism with disease resistance. At least four studies demonstrated that more melanic individuals had higher resistance against disease (NPV) or entomopathogenic fungi (Kunimi and Yamada 1990, Reeson et al. 1998, Barnes and Siva Jothy 2000). One similar study did not support these findings; Goulson and Cory (1995) found that melanic *Mamestra brassicae* Linnaeus (Lepidoptera: Noctuidae) larvae were less resistant to NPV compared to their less melanic conspecifics. The association of melanism and defense may be a highly specific interaction between herbivore host and natural enemy, such that in some cases the natural enemy may have evolved to overcome the herbivore host's defenses.

Conflicting data has been found on whether melanism is always associated with immune response, suggesting that it may be one of a number of factors that is important in immunocompetence. Using field data to quantify parasitoid attack rates in winter moth larvae, *Operophtera brumata* L. (Lepidoptera: Geometridae), Hagen et al. (2006) found that parasitoids were the greatest source of mortality (26%). However, cuticular melanin was positively associated with parasitoid attack (Hagen et al. 2006). Given that no research has definitively demonstrated that melanism is associated with increased immune response, there is a need for further research examining the role melanin plays in

immunocompetence, especially in outbreak species. The expression of melanism has been shown to be affected by a variety of environmental factors, including temperature and humidity (Goulson 1994), light (Faure 1943), and population density (Goulson and Cory 1995). There can be a high degree of phenotypic variation between individuals and a significant effect of genetic family on melanism, indicating that melanism is heritable (Cotter et al. 2002, 2004a, 2008).

Hypothesis 3: Thermal effects on the immune response. Continued increases in yearly temperature averages will lead to fluctuations in normal outbreak periodicity, due to altered immunity.

This hypothesis centers on climate change and the projected increases in global mean temperatures. According to Mann et al. (2008), average global temperature will increase by 4° C over the next 100 years. Undoubtedly, these temperature changes will impact insect populations and may disrupt population regulation. The immune response will also be impacted by these changes since it is affected by temperature. However, the effects of temperature on the immune response are quite variable depending on the taxa, preventing a simple prediction between temperature and outbreaks. Thomas and Blanford (2003) state that temperature can affect parasite virulence, host resistance, and host recovery. Thus, since multiple variables are affected by temperature, a complex relationship with many possible outcomes exists. For example, heightened temperature can either enhance natural enemy performance (Fellowes et al. 1999, Thomas and Blanford 2003) or the immune response (Fellowes et al. 1999, Wojda and Jakubowicz 2007), while in other cases it depresses natural enemy performance (Blanford et al. 2003, Thomas and Blanford 2003) or the immune response (Suwanchaichinda and Paskewitz

1998). Thus, the outcome of increased temperature will depend on the response of the enemy and the response of the host, especially where temperatures reach the boundary of normality based on their evolutionary history (Thomas and Blanford 2003). One pattern that has emerged from parasitoid-host data shows that increased variability in climate patterns will disrupt host tracking by parasitoids (Stireman et al. 2005), possibly leading to greater frequency in outbreaks. Whether this effect is due to increased immunity at higher temperatures is undetermined, yet holds implications for insect outbreaks. Greater immune defense at high temperatures could mean that parasitoids will not be as effective at controlling and maintaining insect populations.

With selection history in mind, dramatically altering temperature will at the very least lead to destabilization of normal insect-enemy interactions, which may lead to increased outbreak frequency (Parmesan 2006, Ims et al. 2008). Johnson et al. (2010) showed that outbreak periodicity shifts of the larch budmoth (*Zeiraphera diniana* Guenée), which exhibited regular 8-10 yr. outbreaks since A.D. 800, is partially explained by increases in mean winter temperatures. Using a travelling wave model, they showed that increases in temperature at the optimal elevation for larch budmoth population growth leads to destabilization of outbreak periodicity (Johnson et al. 2010). In this case, the net effect of increased temperature is clear, but whether the immune response contributes to the destabilization is unclear.

Hypothesis 4: Host plant quality. Species tend to outbreak on host plants that provide food quality conditions that are favorable for growth and immunity. In most cases, these conditions will involve high nutrient quality and low levels of toxins.

As mentioned earlier, high quality host plants enhance immune parameters, either directly (i.e. increased nitrogen for melanization precursors) or indirectly (i.e. increased body fat). However, the term 'high quality' should be defined in order to allow one to make a meaningful prediction. Here, I follow suit with most insect ecology literature and define quality based upon herbivore performance (i.e. development time, pupal mass, fecundity, etc.) and nutrient profiles. In terms of nutrients, host plants that are high in nitrogen and water content are considered high quality hosts since nitrogen is a limiting resource for insects, and insects risk desiccation from low water content (Scriber and Slansky 1981, Behmer 2009).

Most of the examples given earlier in the chapter deal with the macronutrient, protein, showing that high protein content enhances immunity (Lee et al. 2006, 2008, Povey et al. 2009, but see Cotter et al. 2011). Since the immune response relies heavily on enzymatic reactions that require amino acid precursors, it is fitting that immune-stressed herbivores would not only have enhanced immunity on high protein diets, but prefer these diets to a nutritionally balanced diet. However, regardless of whether an herbivore is immune challenged, decades of research show that herbivores will regulate their nutrient intake, favoring a high protein to carbohydrate ratio (Behmer 2009). With this evidence, it is easy to speculate that a characteristic of outbreak species is the ability to achieve high protein content in their diets, either by consuming host plants with high protein:carbohydrate ratios and/or by superior nutrient regulation. For example, the gregarious tent caterpillar, *Malacosoma disstria* Hübner (Lepidoptera: Lasiocampidae), is an outbreaking species that feeds preferentially on aspen and sugar maple. Research investigating the nutrient regulation of this forest pest shows that it does not regulate its

diet to favor protein (Despland and Noseworthy 2006). Instead, its phenology is such that the beginning of its life cycle is in sync with new leaf flush on its host plants; a time when leaf nitrogen content will be high, which should favor a strong immune response.

Another reason why high quality host plants may favor the occurrence of insect outbreaks may arise from the documented trade-off between immunity and growth. Since the immune response is costly in terms of resources, it is predicted to trade-off with other metabolic functions such as growth and fitness (Zuk and Stoehr 2002, Diamond and Kingsolver 2011). In other words, herbivores that are resource limited are not able to invest in both high growth and high immune capacity. Using path analysis, Diamond and Kingsolver (2011) found that host plant resource quality had a significant indirect positive effect on the encapsulation response via enhanced body condition (measured by growth rates) of *Manduca sexta* L. (Lepidoptera: Sphingidae) larvae. However, individuals with higher immune capacity exhibited slower growth. If outbreaking species are more likely to be found on high quality host plants, then they may invest more in immune capacity than growth, which would favor escape from natural enemies. However, in some cases high quality plants do not enhance immune capacity. Sandre et al. (2011) found that despite host plant dependent resistance of *Orgyia antiqua* (Lepidoptera: Lymantriidae) caterpillars to the entomopathogenic fungus, *Metarhizium anisopliae* Sorokin, this resistance was not correlated with host plant quality or the encapsulation response, indicating a direct effect of host plant on the pathogen.

Not surprisingly, the effects of secondary chemistry on immune parameters are much more variable and dependent upon the action that the compound takes in the herbivore's body. For example, certain secondary metabolites, such as carotenoids, can

increase the effectiveness of the immune response by ameliorating the autoreactivity of the melanization cascade (de Roode et al. 2008, Babin et al. 2010). However, ingestion of hydrolysable tannins, imides, and high concentrations of iridoid glycosides reduced encapsulation and melanization (Haviola et al. 2007, Smilanich et al. 2009a, Richards et al. 2010), while still other secondary metabolites have no effect on the immune capacity (Smilanich et al. 2011). These differing results reflect the enormous diversity of secondary metabolites and the role of evolutionary history between herbivore and host plants. In short, overall host plant quality will most likely be a better predictor of immune capacity than specific secondary metabolites.

Hypothesis 5: The key natural enemy hypothesis. Even though the immune response is an effective defense, certain natural enemies can circumvent the immune response. Outbreaks are predicted to occur when these enemies are less abundant.

Since the immune response is one of the most effective defenses against parasitic enemies (Smilanich et al. 2009b, Godfray 1994), it is evolutionarily fitting that these enemies will have evolved counter adaptations to cope with or suppress the immune response. Indeed, the best example of a counter adaptation to the insect immune response is exhibited by hymenopteran parasitoids in the families Braconidae and Ichneumonidae (Webb and Strand 2005). Species in these two families harbor polydnaviruses, which have become integrated into the wasp's genome and are passed vertically through the germ line to offspring (Strand 2009). The virus replicates in the reproductive tract of the female wasp and is injected into the host during oviposition (Beckage 2008). Once inside the host's hemocoel, the virus infects immune functioning cells, enzymes, and tissues

such as hemocytes, phenoloxidase, and fat body, and thereby suppresses the immune response (Strand 2009). Another example of counter adaptation to the immune response is found in certain species of tachinid flies. Bailey and Zuk (2008) found a positive correlation between the phenoloxidase activity of the field cricket, *Teleogryllus oceanicus* Le Guillou (Orthoptera: Gryllidae), and the melanization of the respiratory funnel in the attacking tachinid fly, *Ormia ochracea*. Since the respiratory funnel is the means by which many tachinid flies receive oxygen, a stronger funnel that is less likely to break is beneficial. In this way, these flies may co-opt the immune response for their own benefit as the funnel is strengthened by the encapsulation process. Other species of tachinids have evolved a behavioral counter adaptation to the immune response. The broad generalist tachinid, *Compsilura concinnata*, hides from the host immune response by developing between the peritrophic membrane and the midgut, where the immune response has limited access (Caron et al. 2008). Similarly, other tachinids reside in certain tissues, such as fat bodies, to avoid the immune response (Salt 1968).

In insect populations, these natural enemies that are capable of suppressing or circumventing the immune response may be key sources of mortality. When populations of these key natural enemies are low, it may lead to outbreak situations. Moreover, insect species with the fewest key natural enemies will be the most likely to outbreak. Recent models by Bjørnstad et al. (2010) and Dwyer et al. (2004) demonstrate that population cycles of outbreaking insects are determined by a complement of natural enemies. In particular, Bjørnstad et al. (2010) show that the periodicity of outbreaks in *L. dispar* populations is governed by both generalist and specialist natural enemies. Generalist predators maintain the population up to a certain carrying capacity at which point an

outbreak occurs. The outbreaking population is brought back to pre-outbreak size by a specialist pathogen that is capable of escaping the immune response. Similarly, Dwyer et al. (2004) use a host-pathogen-predator model to show that generalist predators maintain the population whereas specialist pathogens maintain the cycles. Although these data are not a perfect demonstration of my hypothesis, they support the idea that different types of natural enemies each play a role in outbreak cycles. Both examples hinge on the premise that the natural enemy is capable of escaping host defenses. In general, whether or not key natural enemies are avoiding or overcoming the immune response, and whether they are specialists or generalists, have yet to be tested.

Hypothesis 6: Trans-generational maternal effects and natural selection. Periods of high parasitism may be followed by outbreaks due to (1) trans-generational maternal effects where individuals that survived parasitism attack have a heightened immune response and produce offspring that are immune-primed, or (2) individuals surviving the attack are genetically selected for higher resistance, and progeny of these individuals will also have higher resistance via inheritance.

Data sets monitoring caterpillar density/numbers and parasitism support this hypothesis (Karban and Valpine 2010, Schott et al. 2010). These data sets show peak periods in natural enemy populations followed by peaks or outbreaks in insect populations. In addition, host-pathogen models predict this same trend where periods of high parasitism are followed by outbreaks (Elderd et al. 2008). Although the empirical data sets focus on caterpillars and parasitoids, the models are general for outbreaking species. An exception to this trend is seen in the data set with *E. autumnata* (Klemola et al. 2007a, 2008, 2010). This dataset started in the 1970s and shows periods of high

parasitism matching periods of low caterpillar density. In addition, Klemola et al. (2008) found no relationship between *E. autumnata* immunity and parasitism status, suggesting an alternative mechanism regulating population outbreaks for this caterpillar. Thus, my hypothesis may be most relevant for populations that rely heavily on the immune response to resist pathogens and parasites.

Trans-generational maternal effect. A spike in a natural enemy population (pathogen, parasitoid) leads to many individuals in the herbivore population attacked and presented with an immune challenge. Epigenetic mechanisms may occur such that the progeny of immune challenged and surviving parents are immune-primed against another attack. This may occur through mechanisms such as maternal effects, where the physiological result of the mother's developmental environment is passed on to offspring. Other genetic mechanisms are also possible, such as chromatin marking, where the structure of the DNA molecule is altered by an environmental stimulus (Jablonka and Lamb 2010). Trans-generational priming of the immune response has been shown in insects, although the exact mechanism is unclear (Little et al. 2003, Moret 2006, Freitak et al. 2009, Roth et al. 2010). The best evidence to date is that of Freitak et al. (2009), in which *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) progeny whose mothers were exposed to dietary bacteria exhibited trans-generational priming of enzyme activity, protein expression, and transcript abundance of immune functioning genes. While these results support maternal effects of the immune response, the offspring's response did not exactly mirror the mother's response. Studies have shown that the maternal effects are fleeting, and without the stimulus will not be maintained in the population (Jablonka and Lamb 2010). Thus, if natural enemy populations are not as high during the F1 generation, the

maternal effect is not as prominent in the population, and the outbreak subsides in the F2 generation.

Natural selection. Periods of insect outbreak following periods of high parasitism may be due to evolutionary processes such as natural selection. Regardless of maternal effects, individuals that have strong immune responses will be more likely to survive an attack, and if heritable, these genes will be passed to the next generation. Using both a model and experimental approach, Elderd et al. (2008) demonstrate that resistance to natural enemies plays a prime role in population fluxes of the *L. dispar* and that natural selection for resistance to these enemies drives population cycles. In this scenario, it is more difficult to explain the end of an outbreak if the progeny are strongly resistant; however, a wealth of additional factors, both physiological and ecological, can result in population crashes.

Conclusions

Outbreak species share traits and behaviors that implicate the immune response as having the potential for playing a significant role in insect outbreaks. Their gregarious behavior and egg-clustering, which increases the density of conspecifics in a given area, may increase their likelihood of density-dependent prophylaxis and melanism (which in turn strengthens their resistance to natural enemies). Outbreak species are often polyphagous, allowing for the potential of differential host plant selection, sometimes resulting in the selection of diets which maximize immune defense when needed. As research on the insect immune response continues to accumulate, the rather coarse picture that I have drawn of the role of the immune response will be refined such that exact

mechanisms of immunity on outbreaks can be defined and described. There are many avenues to explore on this topic, and the six hypotheses that I presented can be used as guidelines for future research (Table 3.1). These hypotheses are not mutually exclusive and most likely work in concert to influence outbreaks. Most of the examples pooled in this chapter come from research on forest insect pests, yet the hypotheses can likely be generalized to agricultural pests.

Hypotheses	Immunity Measure	Hypothesis support	Comments	Species	References
Density-dependent	M	+	Larval transmission of viruses declined as density increased.	*Lymantria dispar**	D 'Amico et al. 1996
prophylaxis	M	+	Larger egg masses had higher resistance to NPV.	*Malacosoma californicum p.**	Rothman & Myers 1996
(response	EN, PO	+	Immunity measures increased with increasing gregariousness.	*Spodoptera exempta**	Wilson et al. 2001
as density	AB, EN, HM, PO	+	Increased pathogen resistance under crowded conditions.	*Shistocera gregaria**	Wilson et al. 2002
increased)	EN, P	-	No difference in EN of moths from different sites.	*Epirrita autumnata**	Klemola et al. 2006
	M	+	Disease resistance increased as larval density decreased.	*Lymantria dispar**	Reilly & Hajek 2008
	M	-	Resistance to disease was not related to size of egg mass.	*Malacosoma californicum p.**	Cory & Myers 2009
Melanin	M	+	Melanic morphs 2 time more resistant to NPV.	*Mythimna separata**	Kunimi & Yamada 1990
(response as	M	-	Melanic morphs were more susceptible to NPV.	*Mamestra brassicae*	Goulson & Cory 1995
as melanin	PO	+	Melanic morphs 4 times more resistant to NPV.	*Spodoptera exempta*	Reeson et al. 1998
increased)	EN, HM	+	Positive association between size of wingspot & immunity.	*Calopteryx splemdens*	Rantala et al. 2000
	EN, PO	+	Melanic larvae more resistant to fungal disease.	*Spodoptera exempta**	Wilson et al. 2001
	M, P	-	Parasitism was positively associated with melanism.	*Operophtera brumata**	Hagen et al. 2006
	AB, PO	+	Causative relationship between melanism & immune response.	*Spodoptera littoralis**	Cotter et al. 2008
	EN	+	Melanic morphs have stronger EN then pale morphs.	*Lymantria monacha*	Mikkola & Rantala 2010
	EN	+	Melanic morphs have stronger EN then pale morphs.	*Lymantria monacha*	Mikkola & Rantala 2010
Thermal effects	EN	+	Encapsulation of parasitoid highest during warmer months.	*Protopulvinaria pyriformis*	Blumberg 1991
(response as	M	0	Virulence of fungus is strongly affected by temperature.	*Zonocerus variegatus*	Thomas & Jenkins 1997
temperature	M	-	Melanization of silica beads decreased with increasing temperature.	*Anopheles gambiae*	Suwanchaichinda & Paskewitz 1998
increased)	HM	+	Phagocytosis by hemocytes enhanced with increased temperature.	*Locusta migratoria*	Ouedraogo et al. 2002
	M	+	High temperature reduced susceptibility to parasitism.	*Acyrthosiphon pisum*	Blanford et al. 2003
	AB, AF	+	Heat shocked larvae had enhanced immune response.	*Galleria mellonella*	Wojda & Jakubowicz 2007
Host plant	EN	+	Host plant on which larvae grew fastest also had highest EN.	*Pieris rapae*	Benrey & Denno 1997
(response as	AB, EN, HM. PO	+	High protein content enhances NPV resistance & immunity.	*Spodoptera littoralis**	Lee et al. 2006
diet provides	EN	-	Hydolysable tannins have negative effects on EN.	*Epirrita autumnata**	Haviola et al. 2007
higher nutrients	EN, PO	-	Encapsulation was higher on low quality foliage.	*Epirrita autumnata**	Klemola et al. 2007b
and/or	EN, P	-	Larvae fed high & low quality diets did not differ in parasitism.	*Epirrita autumnata**	Klemola et al. 2008
less toxins)	PO, AB	+	Larvae on high protein diet had higher AB activity.	*Spodoptera littoralis**	Lee et al. 2008
	AB, HM, M, PO	+	Increasing protein:carb diet increased resistance to infection.	*Spodoptera exempta**	Povey et al. 2009
	EN	+	Differential effects of amides on generalist vs. specialists.	*Eois nympha, S. frugiperda*	Richards et al. 2010
	EN	-	Encapsulation not associated with resistance to pathogen.	*Orgyia antiqua**	Sandre et al. 2011
Key natural enemies	AB, EN, PO	+	Parasitoid benefits from melanization of respiratory tube.	*Teleogryllus oceanicus*	Bailey & Zuk 2008
(response of natural	EN, PO	+	Parasitoid "hides" from host immune response.	*Trichoplusia ni*	Caron et al. 2008
enemy to the host	PO	-	Polydnavirus inhibits phenoloxidase cascade	*Manduca sexta*	Beck & Strand 2007
immune system)	HM	-	Polydnavirus disrupts hemocyte function	*Pseudoplusia includens*	Strand et al. 2006
Maternal effects	HM	+	Higher HM in populations from different potato fields.	*Leptinotarsa decemlineata*	Ots et al. 2005
(response as	AB, PO, M	+	Parental immune challenge enhanced offspring immunity.	*Tenebrio molitor*	Moret 2006
as impact of	-	+	Natural selection for host resistance effects insect outbreaks.	*Lymantria dispar**	Elderd et al. 2008
parental resistance	M	+	Transgenerational effect of crowding on pathogen resistance.	*Schistocera gregaria**	Miller et al. 2009
or exposure on	PO, AB	+	Progeny of mothers exposed to bacteria exhibited priming.	*Trichoplusia ni**	Freitak et al. 2009
offspring)	AB, PO,	+	Mothers & fathers can transfer resistance to offspring.	*Tribolium castaneum*	Roth et al. 2010

Table 3.1. Summary table of potential hypotheses on insect outbreaks and immune responses. Abbreviations: EN, encapsualtion/ melanization;, HM, hemocytes; PO, phenoloxidase, AB, lysozyme activity/ antibacterial activity; AF, anifungal activity; M, mortality (or survival); P, Parasitism, NPV, nuclear polyhedrosis virus; and *, outbreak or pest species.

CHAPTER 4:

Ecological Consequences of Viral-Allelochemical Interactions in *Manduca sexta* Linnaeus (Lepidoptera: Sphingidae)

Abstract

Larval insect hosts escape from natural enemies using a variety of defenses, including those that are behavioral, morphological, chemical, and physiological. Another common defense is resistance, i.e. encapsulation of the parasitoid egg. Polydnavirus (PDV) allows the parasitoid to escape encapsulation by the host herbivore. Numerous empirical studies have quantified the insect host immune response without taking into account the counter-defense of the parasitoid, specifically the effect of PDV on the larval immune defense. Since the focal organisms of many of these studies are attacked by braconid and/or ichneumonid parasitoids, many of which carry PDV, estimates of encapsulation generated without including the anti-hemocyte factors (such as PDV) may not reflect the complete picture of the immune response.

The objective of this study was to use a model system, *M. sexta* and *C. congregata*, to directly test the effect of the allelochemical nicotine and the presence or absence of PDV on the larval immune responses, using silica beads as parasitoid egg proxies. The three questions addressed were, (1) how does the presence of PDV impact the immune response of M. sexta, (2) is its immune response affected by levels of nicotine in its diet, and (3) is there a differential effect of PDV due to nicotine level on larval immune response?

I found a significant interaction between nicotine diet (treatment) and the effect of the presence of virus. Larvae had a lower percent encapsulation when injected with PDV,

except when they fed on diet with 0% nicotine. This experiment revealed that *M. sexta* larval immune response depended on the quality of its host plant. Furthermore, the importance of PDV in the interpretation of immune response when using a proxy for parasitism such as silica beads was clearly demonstrated.

Introduction

Parasite-host interactions are the classic example of the Red Queen hypothesis, setting the stage for the evolutionary arms race of adaptation and counter-adaptation (VanValen 1973, Dawkins and Krebs 1979). Organisms are forced to defend themselves, usually from not just one threat, but from multiple natural enemies, thus requiring that resources available for defense be partitioned among multiple defense tactics and strategies. Adaptation of a host against one parasite may make it vulnerable to another (Kraaijeveld, and Godfray 1999). Limited resources will eventually lead to trade-offs between defense and other fitness traits. Further, trade-offs among defenses might influence immune efficiency (Rigby and Jokela 2000).

Larval insect hosts escape from natural enemies using a variety of defenses, including those that are behavioral (Gross 1993, Gentry and Dyer 2002, Smilanich et al. 2011), morphological (Gentry and Dyer 2002, Barbosa and Caldas 2007), chemical (Dyer 1995, Gentry and Dyer 2002, Nishida 2002), and physiological (Carton et al. 2008, Smilanich et al. 2009a). For example, the generalist caterpillar *Estigmene acrea* Drury (Lepidoptera: Arctiidae) feeds on a less nutritious plant that contains pyrrolizidine alkaloids when parasitoids are present, even though it has superior growth on alternative foodplants (Singer et al. 2004). *E. acrea* sequesters the alkaloid toxins as an

antiparasitoid chemical defense (Singer et al. 2004). Another common defense is resistance, i.e. encapsulation of the parasitoid egg. These defenses may interact or be subject to trade-offs. An important consideration when estimating these trade-offs is to attempt to mimic the ecological strength of each attack, in this case by including polydnavirus (PDV).

A larval host attacked by parasitoids normally mounts an immune response to any object (fungi, bacteria, parasitoid eggs, etc.) introduced into its hemocoel. The immune response is composed of a humoral and cellular response that work in concert to defend against parasitoids, parasites, and pathogens (reviewed by Carton et al. 2008, Strand 2008, Beckage 2008). Endoparasitic wasps in the two families Braconidae and Ichneumondae have evolved counter-defenses to these immune responses (Beckage 1998, Webb 1998). During oviposition the female wasp injects calyx fluid containing polydnavirus (PDV), venom, and ovarian proteins along with her eggs into the host (Beckage et al. 1994, Alleyne and Beckage 1997). These components act individually or interact with each other to trigger immunosuppression of the host (Stand and Luckhart1994, Luckhart and Webb 1996). Many wasps in the family Braconidae, including *Cotesia congregata* Say (Hymenoptera: Braconidae), also require the presence of PDV for successful parasitism of their lepidopteran host (Stoltz 1993, Lavine and Beckage 1995, Stand and Pech 1995, Beckage 1998).

The cellular response of larvae to infection is directed by specialized cells called hemocytes. Briefly, these hemocytes, called granular cells, contact a foreign target and lyse releasing materials that promote attachment of plasmatocytes. Multiple layers of plasmatocytes form a capsule around the object, which is then melanized by the humoral

response. The combination of asphyxiation from encapsulation and the cytotoxic by-products produced during melanization is thought to contribute to killing foreign bodies such as parasites or parasitoid eggs (Strand 2008). PDV, as well as potentially other factors in the calyx fluid, allow the parasitoid to escape this fate. PDV can cause hemocyte dysfunction in some species, including *Manduca sexta* when parasitized by *Cotesia congregata* (Lavine and Beckage 1995). These hemocytes are damaged and not mobilized for defense or unable to lyse or attach and form a capsule around the egg. This effect on the hemocytes is temporary in some species, and larvae have been shown to recover their immune function, but usually by that point the egg has successfully evaded encapsulation and is able to develop in the hemocoel (Agari et al. 1996, Yin et al. 2003). On the other hand, one study suggests that the hemocytes of *M. sexta* parasitized by *C. congregata* are not able to recover from the parasitoid attack and remain dysfunctional throughout the rest of the host larval development (Amaya et al. 2005).

Numerous empirical studies have quantified the insect host immune response without taking into account the counter-defense of the parasitoid, specifically the effect of PDV on the larval immune defense. There are two prevalent techniques that have been used to quantify melanization/encapsulation in the literature. The first involves the insertion of a ~2mm nylon microfilament ~0.2mm in diameter that is sanded and knotted. The larva or pupa is allowed to react and then it is frozen after a preset period of time. The implant is dissected from the insect and the degree of encapsulation and/or melanization is quantified measuring the gray scale value using photographs and imaging software (Doums et al. 2002, Rantala and Kortet 2003, Zuk et al. 2004, Kapari et al. 2006, Rantala and Roff 2006, 2007, Klemola et al. 2007, Stoehr 2007). The second

technique involves the injection of 40-120 :m silica beads that are dyed with Congo red dye to enhance visibility during dissections. These beads are suspended in a buffer solution using a modified glass pipette or sterilized syringe. The insect is also frozen after a preset period of time and the beads are dissected from the individual. The melanization (i.e. the degree of darkening) of beads is determined by measuring the red value of the beads in photographs and using imaging software (Lavine and Beckage 1996, Lovallo et al 2002, Rantala & Roff 2007, Smilanich et al. 2009a, 2009b, Diamond & Kingsolver 2010). These techniques are used to estimate the immune response of the insects against parasitoids, parasites, and other pathogens (Rantala and Roff 2007, Smilanich et al. 2009b). Since the focal organisms of many of these studies are attacked by braconid and/or ichneumonid parasitoids (Zuk et al. 2004, Kapari et al. 2006, Klemola et al. 2007, Rantala and Roff 2007, Stoehr 2007, Smilanich et al. 2009a, 2009b, Diamond and Kingsolver 2010), estimates of encapsulation generated without including the anti-hemocyte factors (such as PDV) may not reflect the complete picture of the immune response.

The objective of this study was to use a model system, *M. sexta* and *C. congregata*, to directly test the effect of the allelochemical nicotine and the presence or absence of PDV on the larval immune responses, using silica beads as parasitoid egg proxies. The effects of PDV on immunosuppression have been extensively studied in *Manduca sexta* parasitized by *C. congregata* (Lavine and Beckage 1996, Alleyne and Beckage 1997, Amaya et al. 2005). Further, nicotine is known to affect the survival of *C. congregata* larvae its effect varies with the concentration of nicotine in the in the host caterpillar diet (Thurston and Fox 1972, Thorpe and Barbosa 1986, Bentz and Barbosa

1990, Barbosa et al.1986, 1990, 1991). Levels of nicotine in synthetic diet were varied to address three questions. The three questions addressed in this study were, (1) how does the presence of PDV impact the immune response of *M. sexta*, (2) is its immune response affected by levels of nicotine in its diet, and (3) is there a differential effect of PDV due to nicotine level on larval immune response?

Methods

Study organisms

The tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), feeds primarily on plants in the family Solanaceae (Hodges 1971), particularly tobacco, as its name would suggest. Solanaceous plants contain a diverse array of alkaloids including nicotine, in tobacco. *Manduca sexta* has a wide distribution throughout temperate and tropical regions of the Nearctic (Rothschild and Jordan 1903).

Cotesia congregata Say (Hymenoptera: Braconidae) is a gregarious endoparasitoid of *M. sexta*, and other sphingids. *Cotesia congregata* has been shown to have higher mortality when its host, *M. sexta*, is feeding on diets with nicotine or plants with higher nicotine (Thurston and Fox 1972, Thorpe and Barbosa 1986, Barbosa et al. 1986, 1990, 1991). This may be due, in part, to nicotine absorbed in the host larvae hemolymph on which *C. congregata* larvae feed (Self et.al 1964). Furthermore, the effects of nicotine on *C. congregata* appear to be dependent upon the amount of nicotine the host larvae ingest (Barbosa et al. 1991)

Insect rearing

Manduca sexta eggs were obtained from the University of Washington colony. This colony moved from Harvard to University of Washington in 1973 and originated from eggs sent from the USDA-ARS colony in Fargo NC in 1969. These eggs are collected from tobacco plants that are placed in cages with adult moths, so it is assumed that they represent the offspring of many different matings between males and females within the colony. Larvae were reared on an artificial diet that is a modification of the Yamamoto (1969) hornworm diet. Three types of artificial diet were made; no nicotine, 0.1% nicotine, and 0.3% nicotine (see Table 4.1 for modifications of diet ingredient and amount nicotine). These levels of nicotine are similar to levels of nicotine of high and low nicotine tobacco cultivars (Sisson and Saunders 1983). Diet was stored at 4° C.

A petri dish of *M. sexta* eggs was placed in a covered glass dish and provided 0% nicotine synthetic diet until they hatched and crawled onto the food to feed. After 2-3 days of feeding on 0% nicotine synthetic diet, the larvae were haphazardly assigned to one of the three diets. Larvae were reared in large glass dishes with diet placed on wire stand so the larvae could feed and not contaminate the food with frass (see Figure 1, in Yamamoto 1969). Dishes were covered by plastic wrap poked with holes to allow air flow. Diet was changed and the glass dishes were cleaned with bleach solution and rinsed thoroughly every two days. Dishes of larvae were reared in an environmental chamber at 23°C, 16h L: 8h D.

When larvae molted to 4^{th} instars, they were weighed and placed in petri dishes with a small cube of the appropriate diet. When larvae were 0.3-0.5g, they were randomly assigned to the virus or no-virus treatment.

Cotesia congregata parasitoids were obtained as pupae from Dr. Karen Kester at Virginia Commonwealth University. Her colony was established from tobacco crop populations in Blackstone, VA. Each summer new wasps are collected and added to the colony. Wasp pupae are placed in ~500 ml Solo® deli cups with a cotton ball moistened with honey water until wasps emerged. Female wasps were dissected for PDV ~2-4 days after emerging.

PDV Extraction and Injections

Female *C. congregata* were dissected and calyx fluid containing PDVs was extracted as described by Beckage et. al. (1994) and Lavine and Beckage (1996). In brief, wasps were knocked out by placing them in 70% ethanol for 30 seconds and then transferred to phosphate buffered saline (PBS) solution to remove residual alcohol. Each wasp was then placed in a 10:l droplet on a glass petri dish under a dissecting microscope. Ovaries were removed from the wasp by pulling on the ovipositor. The ovaries and venom sack were clearly visible (see Figure 4.1) and the ovaries were carefully separated from the venom sack . The ovaries were macerated to empty the calyx fluid into the PBS droplet. Then the droplet was pipetted into a microfuge tube and kept on ice. Two wasp-equivalents (macerated ovaries from two females) were dissected and injected into every *M. sexta* larvae for the virus treatment. Once the desired number of ovaries was dissected and placed in the tube, the tube was centrifuged for a few seconds to form a pellet of the solid particles and the supernatant liquid was removed and placed into another tube. PBS was added to the tube to obtain the desired volume of two wasp-equivalents for every 5:l of PBS.

Immune Response Measurements

A technique of injecting silica beads as a proxy for the oviposition of parasitoid eggs as described by Hu et al. (2003) and modified by Smilanich (2009a) was used. I modified the technique by incorporating PDV in assessments if immune responses. Silica beads (DEAE SephadexA25, 40-120 :m, Sigma Aldrich®, St Louis, MO, USA), dyed with 0.1% dilution of Congo red dye, were suspended in PBS. Approximately 2.5mg of beads were suspended in 1000ul of PBS yielding a mean of ~20-25 beads in each 5:l injection. The equivalent mass of dyed beads was added to the PDV solution. The bead solution was vortexed before each injection to insure total suspension of beads within liquid. Five :l were injected into 4th instars using a sterilized Hamilton 1702RN syringe with an 26 gauge beveled point needle (Hamilton Company®, Reno, NV, USA). Injections were always performed by the same person (J.G.S.). During each injection period approximately the same number of virus and no virus individuals were injected to avoid bias. The no-virus individuals were injected before the virus individuals to prevent contamination. The syringe was sterilized with 70% ethanol between each injection and then rinsed in PBS to avoid injecting larvae with any amount of alcohol. After each day of injections the syringe was disinfected with 70% ethanol and rinsed with 10% sodium hypochlorite (bleach solution) to avoid contamination with virus.

Only recently molted 4th instar individuals with a mass between 0.03 g to 0.05 g were used to measure immune response (see Table 4.2 for sample sizes). This mass range

provided enough time for larval growth after injection but avoided the possibility of the larvae beginning to molt into 5th instar.

Larvae were placed on a surface cooled to ~4° C and then injected just below the proleg between the 2nd and 3rd abdominal segment. The incision in the larva's integument was sealed after injection using Liquid Bandage (CVS Pharmacy®, Woonsocket, RI, USA) to prevent bleeding and infection. Larvae were allowed to feed on their respective diet for 48 hours. Forty-eight hours post injection their condition was noted and if they had eaten and frass was present in their cups, they were frozen in a -20° C freezer until they were dissected. Beads were recovered during dissection of larvae in PBS solution and photographed using a Zeiss AxioCamMRc5 digital camera at 80x magnification on a Zeiss dissecting microscope using imaging software (Discovery v.8, AxioCam software®; Carl Zeiss, Oberkochen, Baden-Wurttemburg, Germany). Using photographs of beads changes in the darkening of beads were evaluated using Adobe Photoshop software® (v.6.0), i.e., I quantified changes in red intensity after melanization. The strength of melanization is measured based on the degree to which the red color is obscured by blackened hemocytes. The Photoshop software measures the red value (r-value) for each bead. A completely non-melanized red bead will have the maximum r-value of 255 (pure red), however, a completely melanized bead with have an r value of 0 (black). For ease of interpreting data, I used the following equation to change the r-values to percent melanization: *1- (r-value/maximum r-value)* (Smilanich et al. 2009a). For each larva encapsulation (percent melanization) was quantified, as well as mass at the time of injection. The latter measurement of the mass was includes to test whether encapsulation was correlated to mass at time of injection.

Statistical Analysis

The data were analyzed in a randomized 2x3 factorial analysis of variance (ANOVA) using a mixed linear model to compare mean r-value across diets in the presence or absence of virus (i.e. PDV). (PROC MIXED, SAS 1997). All models were checked for normality and homogeneity of variances and data did not need to be transformed. Percent melanization was calculated for each individual from the mean of the r-values of the beads recovered from each individual.

Results

There was a significant interaction between nicotine diet (treatment) and the effect of the presence of virus (Table 4.3). The mean percent melanization on the 0%, 0.1%, and 0.3% nicotine was 31.43%, 33.70% and 34.44% melanization respectively (Table 4.2). Larvae had a lower percent encapsulation when injected with PDV, except when they fed on diet with 0% nicotine. The mean percent melanization on the 0%, 0.1%, and 0.3% nicotine diets with virus were 31.23%, 24.51%, and 24.42% melanization respectively (Table 4.2). This experiment suggests that there is an interaction between the PDV of *C. congregata* and the nicotine in the diet that causes some immunosuppression of *M. sexta* challenged by parasitoids (Figure 4.2).

Discussion

This experiment revealed that *M. sexta* larval immune response depended on the quality of its host plant. Furthermore, the importance of PDV in the interpretation of

immune response when using a proxy for parasitism such as silica beads was clearly demonstrated. If this experiment had been performed without including PDV, one might conclude that nicotine had no effect on *M. sexta* immune response because the percent melanization in the absence of virus on all three diets were not significantly different (Table 4.2 and 4.3).

The influence of allelochemicals, specifically nicotine, on the growth of *M. sexta* and survival of its parasitoid, *C. congregata*, has been extensively studied (Benz and Barbosa 1990, Barbosa et al. 1991, Krischik et al. 1991, Harvey et al. 2007, Thompson and Redak 2007).

Although *M. sexta* larvae are able to develop successfully on diet with nicotine, they generally prefer diet without nicotine (Thompson and Redak 2007). This is the case even when feeding by parasitized larvae on nicotine-rich food could increase their chance of survival (Thompson and Redak 2007). Allelochemicals, such as nicotine, significantly affect species in the third trophic level, affecting the size, development and survival of a parasitoid (Thurston and Fox 1972, Campbell and Duffey 1979, Barbosa et al. 1986, Thorpe and Barbosa 1986). The results of this experiment suggest larval *M. sexta* may face a trade-off in anti-parasitoid strategies. Increased encapsulation ability may be associated with the loss of anti-parasitoid chemical defense in the caterpillar.

Likewise the results could reflect an ecological trade-off for gregarious parasitoids. Although nicotine can reduce parasitoid survival within the host, the reduction in fitness in parasitoids, such as *C. congregata*, may be minimal given that a female may lay 30-300 eggs into a larval host in a single oviposition (Fulton 1940, Beckage and Riddiford 1978, Godfray 1994, Beckage 1998). Even with a lower survival

rate, many parasitoid larvae still survive and pupate. Further, some mortality may increase the probability of survival of siblings by preventing overcrowding and intense competition for limited resources within the host. Fitness penalties for overshooting optimum clutch size have been suggested by models, and in the absence of counterbalancing measures (i.e. encapsulation of some parasitoid larvae), high precision in the number of eggs laid would be necessary (Godfray and Ives 1988, Godfray 1994). Therefore, some encapsulation by the larval host or decreased survival due to nicotine may actually increase the overall population survival of the parasitoid's offspring.

In addition, researchers have experimentally quantified larval immune responses in parasitized hosts by either using nylon threads or silica beads as surrogates for the encapsulation of parasitoid eggs.

PDVs are closely tied to the parasitoid species and are incorporated into the parasitoid's genome (Godfray 1994, Beckage 1998). Although closely related wasps have genetically similar PDVs (Stoltz et al.1981, Stoltz and Whitfield 1992), different species of lepidopteran hosts parasitized by related wasps may have differential immune responses to PDVs associated with the same species of parasitoid (Lovallo et a. 2002). Therefore, the presence of PDVs from wasp species that attack the host insect may alter or weaken the intensity of the insect host's immune response and may be dependent on the presence or absence of primary or secondary compounds acquired from the herbivore host plant.

Endoparasioids in the superfamily Ichneumonoidea, such as *C. congregata*, share a long evolutionary history with their larval hosts and the association between PDV and parasitoids arose 73 ± 11 mya (Wharton 1993, Belshaw and Quicke 2002, Pennacchio

and Strand 2006). The PDV/parasitoid interaction has greatly contributed to the evolutionary success and high species diversity of the wasp lineages Braconidae and Ichneumonidae (Whitfield 2002). Given the long evolutionary interaction between host, parasitoid, and its PDV and the integration of the PDV genome within its parasitoid host, one would expect that both PDV and parasitoid should be highly adapted to the herbivore hosts they attack. Since *M. sexta* primarily feed on plants in the family Solanaceae containing plants rich in alkaloids (Hodges 1971), the expectation would be that the parasitoid/PDV complex that attacks them would be adapted to alkaloids, such as nicotine. Nicotine is the most abundant alkaloid, in that the second most abundant alkaloid nornicotine is only about 4.5% of the quantity of nicotine. However, the tobacco hornworm copes with this allelochemical by egesting most of the nicotine it ingests, leaving only about 2% nicotine in the blood, where the immune response proceeds (Self et al. 1964). Although there are no studies to my knowledge that address the mechanistic effect of nicotine on PDV and encapsulation, my results suggests that in some cases nicotine may enhance the activity of PDV or reduce the activity of herbivore defense. Further studies that directly test the interactions between PDV immunosuppression and toxic compounds, such as nicotine, are necessary to better understand the interplay between parasitoid, PDV, and its host herbivore's diet and possible implications for parasitoid/host herbivore range shifts.

Table 4.1. *Manduca sexta* artificial diet with ingredients for three levels of nicotine used. This diet is a modified Yamamoto (1969) tobacco hornworm diet.

General ingredients	
1650 ml. dH_20**	
Dry ingredients	
60 g. Casein	
20 g. Wesson salts	
40 g. Alphacel	
55 g. sugar	
1 g. Cholesterol	
3 g. Sorbic Acid	
9 g. L-Ascorbic Acid	
7.5 g. USDA vitamin premix	
100 g. Wheat Germ	
Refrigerated ingredients	
4.4 g. Choline	
0.58 g. Streptomycin	
Levels of nicotine diets	Nicotine* added
0.0%	0.0 ml.**
0.1%	1.5 ml.**
0.3%	4.5 ml.**

* MP Biomedicals, LLC. (-) - Nicotine Free Base
** dH_20 was removed for amount of nicotine added.

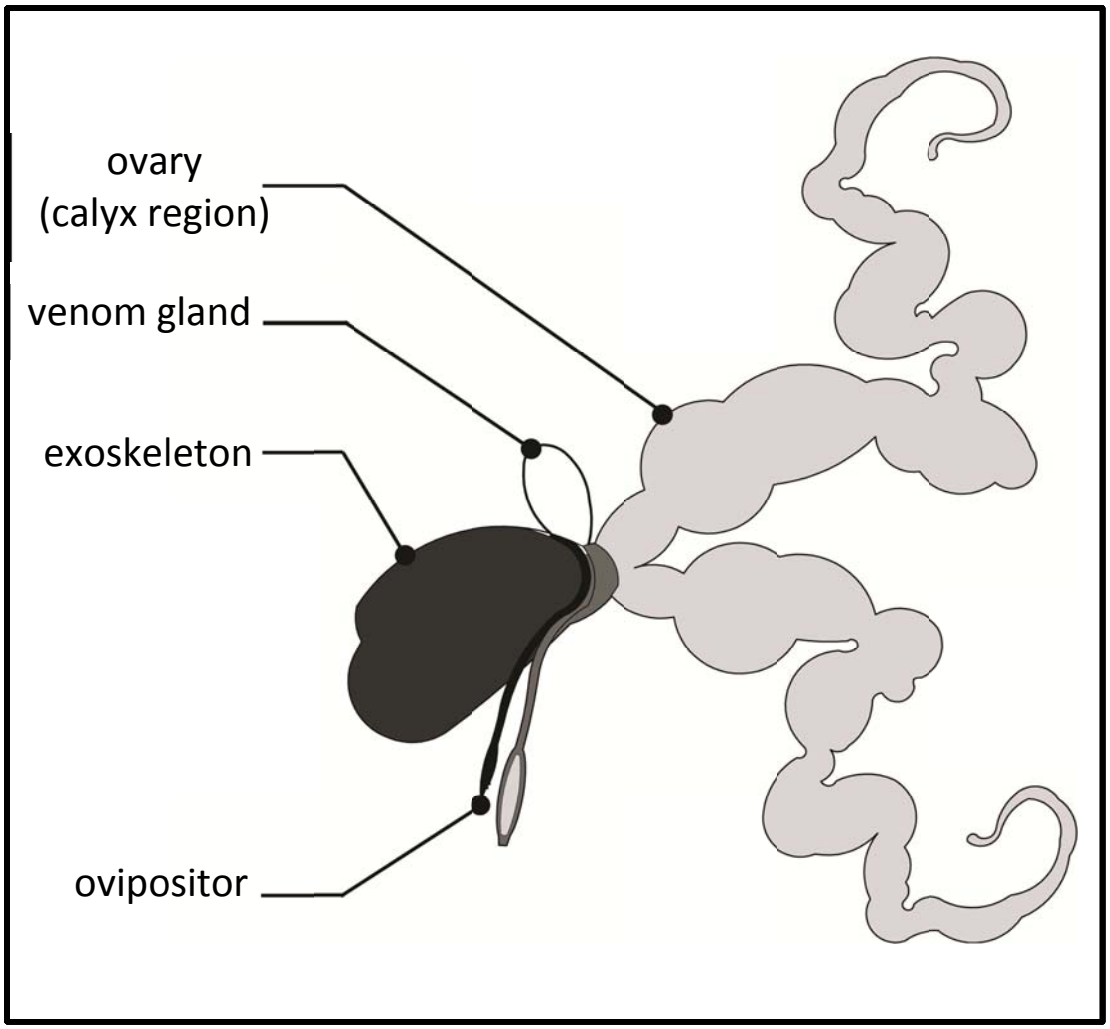

Figure 4.1. Diagram of reproductive tract of *Cotesia congregata* removed during wasp dissection. Illustrated are a pair of ovaries with the calyx region located above the common oviduct connected to the ovipositor (Illustration by J. Riordan).

Table 4.2. Sample size and mean percent melanization of beads in *Manduca sexta* on all levels of nicotine in the presence and absence of polydnavirus.

	0.0% Nicotine		**0.1%Nicotine**		**0.3% Nicotine**	
	Sample size	Mean (percent)	Sample size	Mean (percent)	Sample size	Mean (percent)
No Virus	**25**	**31.43**	**20**	**33.70**	**21**	**34.44**
Virus	**20**	**31.23**	**33**	**24.51**	**32**	**24.42**

Table 4.3. Analysis of variance (ANOVA) of percent melanization. Treatment indicates the three levels of nicotine in the artificial diet (0.0%, 0.1%, and 0.3%). Virus refers to the presence or absence of polydnavirus.

Source of variation	Num d.f.	Den d.f.	F	P
Treatment (Nicotine concentration)	2	145	0.67	0.5124
Virus	1	145	15.23	0.0001*
Treatment*Virus	2	145	3.46	0.0342*

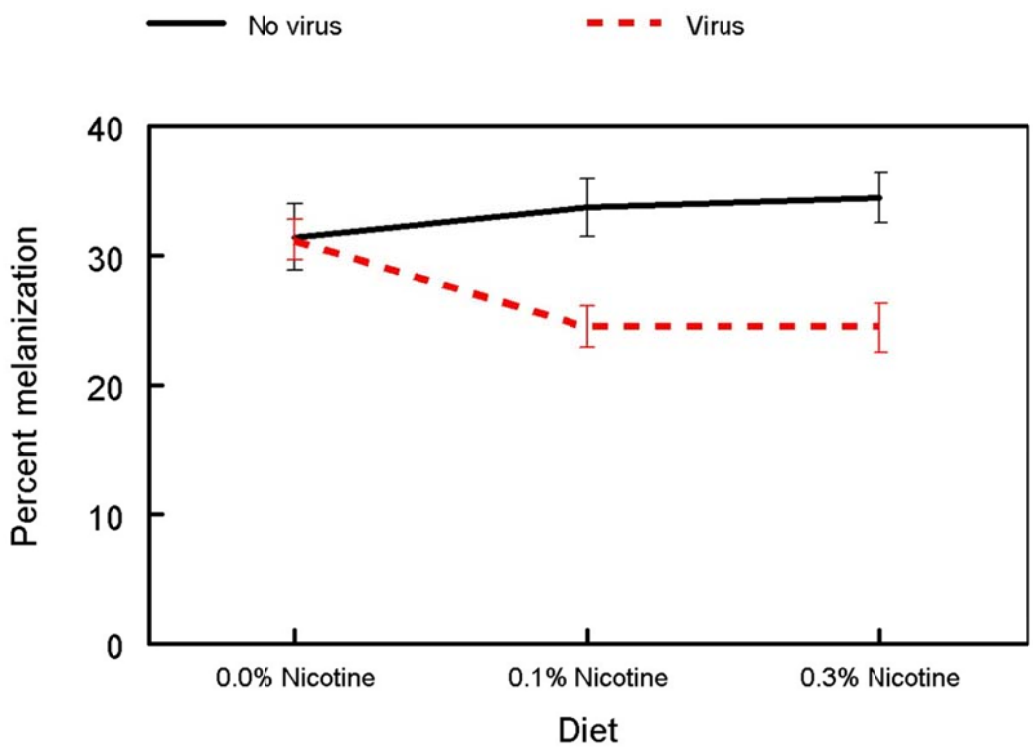

Figure 4.2. Percent melanization by *Manduca sexta* on artificial diet with 3 levels of nicotine. The percent melanization is significantly lower for larvae feeding on 0.1% and 0.3% nicotine diets that have been injected with polydnavirus from *Cotesia congregata*.

APPENDIX A:
Co-Author Acknowledgements

Chapter 1 : J.G. Shlichta, R. F. Medina, and P. Barbosa. True generalists: Polyphagous lepidopteran herbivores show no host-plant associated differentiation. *Entomologia Experimentalis et Applicata*, *in prep.*

Chapter 2: J.G. Shlichta and P. Barbosa. Host plant-associated immune response in polyphagous forest Lepidoptera. *Ecology*, *in prep.*

Chapter 3: J.G. Shlichta and A. M. Smilanich. Immune Responses and Their Potential Role in Insect Outbreaks. *In P. Barbosa, D. K. Letourneau* and *A. A. Agrawal (Eds.) Insect Outbreaks Revisited.* Wiley-Blackwell, Oxford OX4 2DQ, UK, *in press*.

Chapter 4: J.G. Shlichta, K. Kester, and P. Barbosa. Ecological consequences of viral-allelochemical interactions in *Manduca sexta* Linnaeus (Lepidoptera: Sphingidae). *Ecol. Entomol., in prep.*

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