ECOLOGICAL SPECIATION IN *NEOCHLAMISUS BEBBIANAE* LEAF BEETLES: THE ROLE OF POSTMATING ISOLATION AND THE GENETIC BASIS OF HOST USE TRAITS

By

Scott Patrick Egan

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

August, 2010

Nashville, Tennessee

Committee 2 (2009-2010)

Approved:

Professor David McCauley

Professor Patrick Abbot

Professor Jeffrey Feder

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Professor William Etges
Professor Jeffrey Feder

To my wife, Jennifer, for her love, support, and humor,

and

To my two sons, Jack and Sawyer, for their amazing ability to adapt

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CHAPTER I

INTRODUCTION

The origin and maintenance of biodiversity is of great interest to evolutionary biologists. More specifically, the role of ecologically driven natural selection has been a special focus of this work and recently has been shown to play a general role in generating new species (Funk et al. 2006). This phenomenon, referred to as ecological speciation, is defined as the process by which reproductive isolation arises between populations as a result of divergent selection between habitats (Schluter 2001). Prezygotic and postzygotic reproductive isolation builds between these populations as they climb separate adaptive peaks associated with each habitat (Gavrilets 2004). Ecological speciation has long been a central theme of the modern synthesis (Mayr 1942, 1947), linking microevolutionary processes to macroevolutionary patterns, that has over the last two decades, come under direct empirical test (Berlocher and Feder 2002, Coyne and Orr 2004, Rundle and Nosil 2005). In the study of ecological speciation, three core components are examined: ecologically based divergent selection, reproductive isolation, and the genetic link between them, either through pleiotropy or linkage disequilibrium (Kirkpatrick and Ravigne 2002, Schluter 2000, Rundle and Nosil 2005).

Examples of speciation events in which adaptation to different environments played a fundamental role in the evolution of reproductive isolation have come from both the lab and field experiments. Controlled laboratory experiments have shown that divergent adaptation to different environments can generate reproductive isolation between experimental populations both in allopatry (Rice and Salt 1990) and in sympatry (reviewed in Rice and Hostert 1993). There are also strong examples in nature within specific study systems that document the association of reproductive isolation, specifically prezygotic reproductive isolation, with adaptive ecological divergence (e.g., Feder et al. 1994, Funk 1998, Rundle et al. 2000, Nosil 2002, Vines and Schluter 2006). Some studies have documented that the same traits that are under divergent selection between environments directly cause reproductive isolation to occur, such as environment-dependent wing patterns used in defensive mimicry and mate selection in *Helioconius* (Jiggins et al. 2001). More generally, a consistent association between ecological divergence and reproductive isolation has been shown across disparate taxa spanning flowering plants, birds, amphibians, fishes, and insects (Funk et al. 2006). Reviews of this growing body of evidence documenting ecological speciation have concluded that more empirical evidence on two specific components to this process are still needed: (a.) the association of postmating reproductive isolation with ecological divergence and (b.) the genetic basis of traits involved in ecological speciation (Prowell 1998, Berlocher and Feder 2002, Coyne and Orr 2004, Rundle and Nosil 2005).

Herein, I use *Neochlamisus bebbianae* leaf beetle populations associated with Bebb’s willow (*Salix bebbiana*) and red maple (*Acer rubrum*) that are currently undergoing ecologically-driven divergence and speciation (e.g., Funk

1998, Funk et al. 2002) to further address the role that ecology plays in generating reproductive isolation and speciation. These two ”host forms” overlap in their geographic ranges (Karren 1972), exhibit preferences for their own host plants (Funk 1998; Egan and Funk 2006), exhibit fitness trade-offs between alternative hosts (Funk 1998), and show host-associated assortative mating in no-choice mating trials (Funk 1998; Funk and Egan, *in prep*). This dissertation involves two complementary projects aimed at (a.) understanding the genetic architecture of traits associated with ecological adaptation to different hosts that also contribute to known reproductive isolation (host preference and host associated performance), and (b.) examining the unknown nature of postmating isolation that may have evolved between these ecologically differentiated populations.

Genetic architecture

Evaluating the genetic architecture of host-use traits among host-associated populations of herbivores is a critical step to understanding the process of speciation (Lynch and Walsh 1998). Host-use traits for phytophagous insects include behavioral traits, such as feeding, oviposition, and mate-site preference, and performance traits, such as host-associated growth rates (developmental time), survival, and fecundity. The genetic architecture of these traits refers to the number of genes controlling a trait (few vs. many), gene action (additivity, dominance, epistasis), and the distribution of these genes across the genome (linkage). The architecture of these traits is important in the understanding of speciation in *N. bebbianae* and, more generally, to the assumptions within models of speciation, especially those allowing for gene flow (parapatric and sympatric speciation; Berlocher and Feder 2002; Coyne and Orr 2004).

Specific genetic architectures are more advantageous to speciation events were gene flow occurs to some degree (Berlocher and Feder 2002). Direct adaptation-isolation pleiotropy, where a gene that diverges under ecological adaptation directly effects reproductive isolation, seems the most conducive to speciation with gene flow. A close alternative is tight linkage between assortative mating and ecological adaptation genes. Along these lines, the actual number of genes involved in divergent adaptation and reproductive isolation needs to be evaluated in more study systems since it plays a critical role in models of speciation (Johnson and Gullberg 1998, Dieckman and Doebelli 1999, Kondrashov and Kondrashov 1999). Theory supports these scenarios, setting the stage for empirical investigations of sympatric speciation. Felsenstein (1981) proposed two models of sympatric speciation that relied on a small number of loci influencing mate selection. The one-locus, one-allele model permitted individuals to recognize and mate with those of a similar phenotype or genotype, while the one-locus, two-allele model permitted individuals to preferentially mate with others that carried the same allele at a given mate preference locus (Felsenstein 1981). Under the conditions of either of these models it is possible for selection to generate species in sympatry given that alleles at this mate preference locus become associated with alleles for preference or adaptation (i.e., linkage). Other models have considered multiple loci controlling both adaptation and mate preference and conclusions in these models differ based on the number and behavior of the loci involved. For example, Kondrashov and Kondrashov (1999) found that the number of genes underlying traits influences speciation with the probability of sympatric speciation increasing with increasing number of genes that affect fitness, but decreasing with the number of genes that affect mate choice. It should be noted that many of the theoretical papers on the opportunity for sympatric speciation assume additive behavior among loci, yet this assumption has rarely been tested (Dieckman and Doebelli 1999, Kondrashov and Kondrashov 1999). How the traits influencing ecological divergence and reproductive isolation are inherited among hybrids as ecological divergence progresses is also key. For example, if a trait influencing ecological adaptation exhibits dominance, then hybrids will not be intermediate between parents and will resemble more closely one parental population versus the other. This will either increase or inhibit divergence for this trait (versus additive inheritance), and thus, the speed and opportunity that reproductive isolation can arise and be maintained in the face of gene flow (Hendry et al. 2007). Along these lines, epistasis is also an underappreciated and understudied influence on trait inheritance. Epistasis refers to departures from independence of the effects of one locus by other loci (Lynch and Walsh 1998). Thus, the genetic background within which a specific locus resides can influence the way in which it is inherited among hybrids, again, influencing the opportunity and tempo of ecological divergence and speciation (Hendry et al. 2007).

From empirical data, host-use traits have been directly implicated in causing reproductive isolation between populations of herbivores feeding on different host plants. For example, host preference has been identified as an important trait in habitat isolation (e.g., Feder et al. 1994; Via 1999) and differential survival of migrants to alternative hosts has been identified as an important trait affecting reproductive isolation through ‘immigrant inviability’ (reviewed in Nosil et al. 2005). These traits have been specifically implicated as contributing to reproductive isolation among willow and maple host forms of *N. bebbianae* (habitat isolation – Funk 1998, Funk and Egan, in prep; immigrant inviability – Funk 1998, Nosil et al. 2005). However, the genetic basis of these traits important to ecological speciation has only been investigated in a few study systems (Rundle and Nosil 2005). Studies of the genetic basis of these traits comes from both classic quantitative genetic approaches (sibship or line cross analyses) and QTL analysis. Hatfield (1997) estimated the influence of additive, dominance, and epistatsis and number of loci among ecologically divergent populations of sticklebacks (*Gasterosteus aculeatus*) using line cross analyses. Among the four divergent morphological traits assessed, each behaved differently, demonstrating that additive genetic variance was enough to explain one trait, whereas additive + dominance + epistasis were involved with another trait. Furthermore, a modest number of genes were estimated to underlie these traits (range: 1 – 50 loci; Hatfield 1997). An investigation into the genetic basis of host odor discrimination in divergent host races of *Rhagoletis* flies demonstrated two important points. First, as few as three autosomal loci could explain the segregation patterns responsible for differences between host races in host odor discrimination and second, cytonuclear gene interactions influenced host odor discrimination towards the grandmaternal host (Dambroski et al. 2005). Etges (1993) used a sib analysis to investigate the genetic basis of developmental time, host associated survival, and body size for *Drosophila mojavensis* populations associated with two different groups of cactus. Additive and nonadditive effects, maternal effects, and genotype by environment interactions were all found to influence these host-associated traits. Etges et al. (2006) also found additive autosomal influences along with dominance effects from the Y-chromosome on courtship song variation between these same divergent taxa. In another study system, using a QTL analysis, investigations of linkage between host preference and performance in pea aphid host races concluded that very few loci are involved in host adaptation and reproductive isolation (as few as five loci) and that these critical loci were tightly linked (Hawthorne and Via 2001; Via and Hawthorne 2002), however this may be a case of where the same gene affects both traits (Coyne and Orr 2004). Another QTL analysis of benthic and limnetic sticklebacks showed that the morphological traits associated with divergent adaptation and associated reproductive isolation had just a few QTLs underlying each trait (Peichel et al. 2001).

To investigate the genetic architecture of the host-use traits documented to influence reproductive isolation between host associated populations of *N. bebbianae* (e.g., Funk 1998; Funk et al. 2002; Funk and Egan 2009), I used a mating design that follows fullsib families through F_1, F_2, and backcross generations and then compares the composite means for quantitative traits of interest using line cross analysis (Lynch and Walsh 1998, Fritz et al. 2003, Fritz 2006) to investigate the role of additive, dominance, and epistatic effects. Second, I used the Castle-Wright estimator on the same data to estimate the number of loci involved in divergent host adaptation, (Castle 1921, Wright 1968, Lynch and Walsh 1998). Third, I applied a correlation technique among traits to investigate possible associations between traits via pleiotropy or physical linkage (Butlin & Hewitt, 1988).

The role of postmating reproductive isolation

Although there have been important gains in the study of ecologically driven speciation, especially in documenting the effect of divergent adaptation on premating barriers to gene flow, data on the role of postmating isolating barriers is still rare. I am interested in documenting the role of all possible postmating barriers to gene flow (Coyne and Orr 2004). This includes postcopulatory aspects of “cryptic divergence” (Nosil and Crespi 2006), intrinsic postzygotic barriers (hybrid sterility and inviability due to genetic incompatibilities), and extrinsic postzygotic barriers (hybrid sterility and inviability due to ecological incompatibilities). I focused on the role that ecology plays in generating these barriers between host associated populations of *N. bebbianae*.

Postmating/prezygotic (cryptic) isolation occurs after copulation, but before fertilization and can be caused by poor sperm transfer or storage (Price et al. 2001) or differential sperm preference (Howard et al. 1998).

Postmating/prezygotic isolation is thought to involve only sexual selection, but has been documented to evolve as a by-product of natural selection between environments (Nosil and Crespi 2006). Intrinsic postzygotic isolation results from incompatibilities that evolve between genomes that cause reduced hybrid fitness (hybrid sterility or inviability). This form of reproductive isolation has been studied extensively and is thought to accumulate gradually with time (Coyne and Orr 1989, Coyne and Orr 1997) and typically not influenced by selection. However, recent work has implicated selection in promoting this form of reproductive isolation as well (Presgraves et al. 2003). Extrinsic postzygotic isolation is explicitly consistent with a role for ecological adaptation in speciation (Rundle and Nosil 2005) and involves a mismatch between hybrids and their parental environments. Few explicit examples exist that test the relative role of ecology in driving genetic (intrinsic) and ecological (extrinsic) incompatibilities in hybrids. MacNair and Christie (1983) found that *Mimulus* populations adapted to soils with high copper content expressed strong hybrid inviability when crossed with populations from normal soils. Lu and Bernatchez (1998) found increased embryonic mortality rates among hybrids between ecotypes of lake whitefish. Vamosi and Schluter (2002) found that hybrid sticklebacks had increased predation from trout, compared to their parental lineages. Other studies have been consistent with intrinsic postzygotic incompatabilities. For example, Linn et al. (2004) found that a portion of hybrid *Rhagoletis* flies could not orient to fruit odors under conditions in which parental individuals responded. The lack of responsiveness could have been due to genetic incompatibilities causing reduced chemoreception to host odors. Extrinsic postzygotic isolation causes the reduced hybrid fitness due to a mismatch between hybrid phenotype and environment. This is potentially a generally important issue for herbivorous insects that are adapted to different host plants, as an intermediate habitat (Schluter 2000) is rarely available. However, there is a difficulty in testing for extrinsic postzygotic isolation by teasing it apart from the effects of intrinsic post-zygotic isolation, due to subtle genetic incompatibilities that could be exacerbated in natural environments (Hatfield and Schluter 1999). Rundle and Whitlock (2001) proposed that comparing backcross hybrids between divergent environments can estimate ecologically-dependent isolation while controlling for these intrinsic differences. This comparison was undertaken using ecologically-divergent morphs of the three-spine stickleback, demonstrating that extrinsic isolation does contribute to post-zygotic isolation between these two morphs over and above any intrinsic incompatibilities (Rundle 2002).

To investigate the role that ecology plays in postmating isolation and to understand the role postmating isolation plays in *N. bebbianae,* I used crosses within and among populations adapted to each host plant (maple and willow) to assess the influence that divergent host associated adaptations play in postmating isolation between F1 and parental lineages. Following evidence consistent with postmating isolation in the F1 generation, I followed through to the F2 generation with backcross and F2 hybrids to test for an explicit role of ecology in postzygotic isolation, while controlling for genetic incompatibilities (Rundle and Whitlock 2001).

Collectively, using one consistent experimental design, I address two important and understudied aspects of ecological speciation and interpret the results of these studies within the bigger picture of speciation biology.

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CHAPTER II

ECOLOGICALLY DEPENDENT POSTMATING ISOLATION BETWEEN SYMPATRIC HOST FORMS OF NEOCHLAMISUS BEBBIANAE LEAF BEETLES [1]

Abstract

Ecological speciation is the promotion of reproductive isolation via the divergent adaptation of populations to alternative environments. A prediction peculiar to ecological speciation is that hybrids between such populations should be poorly adapted to parental environments, yielding reduced fitness and postmating isolation. However, F1 analyses alone cannot demonstrate that ecological ("extrinsic") factors contribute to such isolation. Rather, this requires documenting a 'switch' in the relative fitnesses of reciprocal backcrosses between environments. Specifically, each backcross should exhibit higher fitness in the environment of its pure parent, with which it shares the most genes – including environment-specific ones. By contrast, because genetic proportions are expected to be similar for all backcrosses (~¾ from one parental type, ~¼ from the other), the more general genetic incompatibilities responsible for "intrinsic" isolation predict no such environment-specific fitness-switches. Thus, although intrinsic isolation may contribute to the fitness reduction and variation underlying such patterns, it offers an insufficient explanation for them. Here, we present a

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quantitative genetic ‘backcross’ analysis of sympatric *Neochlamisus bebbianae* leaf beetle populations adapted to maple versus willow host plants. Results statistically supported ecological speciation predictions, notably the switch in relative fitness for backcross types, the expected rank order of cross type fitnesses, and appreciable extrinsic isolation. We additionally documented genetic variation in host-associated fitness, ruled out non-genetic maternal effects, and discuss the maintenance of ecological differentiation in sympatry. In sum, our study provides a rare and strongly supported demonstration of genetically based, ecologically dependent postmating isolation during ecological speciation.

Key words: divergent adaptation, ecological speciation, host races, hybrid fitness, reproductive isolation

Introduction

Understanding the mechanisms of speciation is a fundamental problem in evolutionary biology (1). “Ecological speciation” refers to the evolution of reproductive isolation as an incidental consequence of the divergent adaptation of populations to alternative environments (2,3,4). Such divergent natural selection has long been thought to play a part in the speciation process (5), but case studies have only recently begun to accumulate (3,6,7,). Nonetheless, a broad comparative analysis suggests that ecological divergence plays a taxonomically general role in speciation (8), while the isolation of ecological contributions via such comparative approaches is now being fruitfully applied to individual study systems (4, 9, 10,11,12). More generally, well developed model systems for evaluating ecological speciation have been developed in taxa as disparate as stickleback fishes (9,13), *Rhagoletis* fruit flies (14), and *Mimulus* monkey flowers (15).

Many advances have been made in the evaluation of ecologically associated premating reproductive barriers (6). Premating barriers are often clearly associated with ecological divergence and contribute to reproductive isolation, for example, via habitat isolation (4,14), temporal isolation (16), and premating immigrant inviability (17). Sexual isolation has also been shown to be influenced by ecological factors (4,18). Yet the potential ecological contributions to postmating barriers have been much less studied (7). Moreover, as multiple reproductive barriers have been investigated in relatively few study systems (17,19,20), the relative roles of postmating versus premating barriers in speciation are not well understood.

Two aspects of postmating isolation have been distinguished in the literature (1,7,21). First, “intrinsic” postmating isolation (hereafter referred to as “intrinsic isolation” for simplicity) reflects low hybrid fitness owing to general genetic incompatibilities between the genomes of divergent populations. For example, such intrinsic isolation may reflect negative epistatic interactions between the alternative alleles that have become fixed across loci between populations. Many studies have demonstrated intrinsic isolation (1). Second, “extrinsic” or ecologically dependent postmating isolation (hereafter, “extrinsic isolation") specifically refers to reduced hybrid fitness due to the maladaptive intermediacy of their ecologically relevant genotypes/phenotypes in parental environments (22). Thus, extrinsic isolation arises as populations divergently adapting to alternative environments climb different adaptive peaks, producing hybrids that fall into fitness valleys (23). Extrinsic isolation provides strong support for ecological speciation.

Despite belief in its importance and support from theoretical models (24), few studies have evaluated ecological contributions to postmating isolation, perhaps because of the labor-intensive nature of such research. Indeed, as pointed out by Coyne and Orr (1), "It has become fashionable to suggest that extrinsic, and especially ecological, postzygotic isolation is more common or more important than intrinsic in nature. This might well be true. But at present, such assertions rest more on intuition than data (p. 255)." Important examples invoking such ecological factors include an investigation of F1 hybrids between the benthic and limnetic forms of three-spine stickleback (22). These F1s were anatomically intermediate between parental morphologies and grew more poorly in parental environments than did each parent. Similarly, studies of three herbivorous insect species, each with populations specialized on one of two host plants, found F1s to perform more poorly than parentals on one (25) or both parental hosts (26-27).

These patterns could be explained by contributions from extrinsic isolation if: (a) F1 hybrids inherited the alleles underlying the (divergent) local adaptation of each parent population, and (b) the combination of these alleles in hybrids yielded phenotypes unsuitable to either parental environment. However, inferring extrinsic isolation from such results has been criticized (1, p. 250) on the grounds that they cannot rule out intrinsic factors as a sufficient explanation for observed postmating isolation. That is, F1 fitness reduction could simply reflect general genetic incompatibilities between alleles at loci that do not contribute to ecological adaptation. Even when F1 hybrid fitness is lower in parental environments (where they are exposed to natural ecological factors) than in the laboratory (where they are not) (e.g., 22) extrinsic isolation may not be the cause. This is because such results could instead reflect a general tendency for organisms to perform better in ‘benign’ lab environments as compared to ‘harsher’ natural ones (28,29).

A solution to the problem of rigorously documenting extrinsic isolation was provided by Rundle & Whitlock (30), who extended a quantitative genetic model of population crosses (31) to include two environments. The extended model demonstrates that whereas the analysis of F1 hybrids alone cannot distinguish the contributions of intrinsic versus extrinsic gene effects, extending the analysis to the next generation can. Specifically, evaluating both reciprocal backcross hybrids in each parental environment allows the additive-by-environment interaction ($\alpha_1\varepsilon$) to be isolated and evaluated.

The ‘backcross approach’ developed by Rundle & Whitlock on the basis of this model provides an experimental crossing design (Fig. 2.1) that allows potential extrinsic contributions to postmating isolation to be addressed empirically. This approach relies on three points. First, for each reciprocal backcross (i.e., crosses between one or the other ‘pure’ parental type and an F1 hybrid), ¾ of its genes ultimately derive from one pure parental type, and ¼ from the other. Second, if and only if extrinsic factors are contributing, environment-specific fitnesses are predicted to differ between the two reciprocal backcross types, depending on which pure parental type produced the backcross. This is because backcross type A will possess ¾ of the genes involved in ecological adaptation to the environment of its pure type A parent, and only ¼ of the genes involved in adaptation to the alternative environment of pure parental type B. The analogous pattern holds with respect to backcross type B. These observations underlie the inference of Rundle & Whitlock (30) that extrinsic isolation is demonstrated when the relative fitnesses of the two backcross types vary (switch) between parental environments. That is, each backcross type should exhibit relatively higher fitness in the environment of the parent to which it is most genetically similar. This result is statistically indicated by an interaction between Backcross Type and Parental Environment. Third, because each backcross type necessarily exhibits the same degree of hybridity, the two backcross types are, by contrast, not inherently predicted to exhibit considerable differences in environment-specific fitness if caused by intrinsic genetic incompatibilities alone.

In the present study, we adopt and extend the backcross approach in an analysis of sympatric populations of *Neochlamisus bebbianae* leaf beetles at a site in Vermont, USA, where their respective host plants intermingle in the same microhabitat. These study populations represented the ‘maple host form’ and the ‘willow host form’ of *N. bebbianae* (4), which specialize on red maple (*Acer rubrum*, Aceraceae) and Bebb’s willow (*Salix bebbiana*, Salicaceae) host plants, respectively. These host forms are partially ecologically differentiated in host preference and performance traits and exhibit partial premating reproductive isolation, apparently as a consequence of divergent host adaptation (4,6,32,33). They have thus provided an informative system for investigating ecological speciation.

Our experiments involved two generations of matings, yielding parental and hybrid offspring representing various cross types (see Fig. 2.1). For each beetle family in our experiments, an equal number of offspring were reared on each of the two host/test plants representing the parental environments in our study. This provided relative growth rate (RGR) (34) data. Faster insect development (greater RGR) may, for example, increase the likelihood of: survival to maturation in the face of predators, high reproductive success reflecting longer access to mates, or reaching the life history stage required for diapause when the growing season is short (35). Thus, we used RGR as our measure of relative viability/fitness (35), following Rundle (36). Our primary goal was (a) to use the backcross approach to rigorously document whether extrinsic isolation exists between study populations. Complementary objectives included: (b) evaluating genetic variation in host performance via the examination of family-level variation, (c) investigating possible non-genetic maternal effects (37), (d) comparing the contributions of various reproductive barriers to reproductive isolation, and (e) considering the relevance of these and prior findings with respect to the sympatric status of these populations.

Results

Generation 2: Performance of F1 hybrid and pure parental crosses

These four cross types demonstrated a significant Cross Type x Host Environment interaction (Table 2.1), and most pairwise cross type comparisons proved significant for each host (Fig. 2.2). Fitness was greatest for pure parental types on their native host plant, followed by both hybrid crosses, with pure parentals on the foreign host doing most poorly. Both reciprocal hybrids performed best on their maternal host (see below). The reduced fitness of hybrids compared to pure parental types on their native host, and the switching of relative performance of cross types across hosts (i.e., the interaction term mentioned above) were consistent with the possibility of extrinsic postmating isolation, if not proving it.

Generation 3: Performance of backcross hybrid and pure parental crosses

As in the generation two results, cross type fitness varied significantly on both maple ($F_{3,46} = 20.5$, $P = 0.0004$) and willow ($F_{3,51} = 58.8$, $P < 0.0001$), with backcrosses consistently exhibiting lower fitnesses than pure parental types on their native hosts, and thus a degree of postmating isolation. Most centrally to this study, these patterns were exactly as predicted by the ecological speciation hypothesis and documented extrinsic isolation between these maple- and willow host form populations. Two results support this conclusion. First, the relative fitnesses of these reciprocal backcrosses switched order across test plants (Fig. 2.3), as also indicated by a highly significant Backcross Type x Host Environment interaction term (Table 2.1). Specifically, the relative fitnesses of each backcross type on a given test plant corresponded to its genetic similarity to the pure parental type natively associated with that plant. A genetic basis for these results was further supported by a significant Family x Host Environment term (Table 2.1), indicating genetic variation in host-specific performance. For the sake of completeness, we also evaluated possible variation among the four unique crosses that comprise each backcross type (Fig. 2.1) via a hierarchical ANOVA. However, this factor did not affect the Backcross Type x Host Environment interaction that is the focus of our study, so the simpler analysis is presented. Second, the rank order of cross type fitnesses is precisely as expected in each host environment (Fig. 2.3), and six of eight adjacent cross type fitnesses differed significantly (Fig. 2.3). The likelihood of this ranking pattern being observed by chance is $P = (1/4!)^2 = 0.0017$.

Generation 3: Evaluation of possible maternal effects

To evaluate the possibility that maternal rearing environment influenced offspring fitness, we added Maternal Host Plant as a fixed effect to the ANOVA model. No such influences were observed (Maternal Host Plant: $F_{1,\,347} = 1.8393$, P = 0.1759) and the Backcross Type x Host Plant interaction term remained significant. In a complementary approach, we performed two separate analyses, one each for individuals whose mothers had been reared on maple versus willow, respectively. These analyses thus removed any maternal contributions to the results of our earlier ANOVAs. Nonetheless, both analyses again revealed highly significant Backcross x Host Plant interaction terms, indicating that our evidence for extrinsic isolation was not due to such maternal effects (Table 2.2). A lack of non-genetic maternal effects was further supported by the similar offspring fitnesses across maternal rearing environments within each test plant environment (Fig. 2.4).

In this context, it is intriguing that both reciprocal F1 hybrid cross types nonetheless performed better on their maternal host than their paternal host. In the apparent absence of an environmental explanation for this pattern, a genetic one might be sex linkage. This is consistent with the tendency for male leaf beetles to be the heterogametic sex and for genes responsible for host plant adaptation in herbivorous insects to be located on the X-chromosome (38). However, the lack of sex-based differences in hybrids observed here suggests that any such X-linked genes likely exhibit dominance.

Generation 3: Quantifying and comparing reproductive isolation for backcross hybrids

We quantified the "individual contribution" of extrinsic isolation – that is, the magnitude of reproductive isolation it would produce if acting alone, on a scale of 0.0 to 1.0 – and compared it to values previously calculated for other reproductive barriers between these host forms (17, 19), yielding the following:

habitat isolation = 0.39, premating immigrant inviability = 0.58, sexual isolation = 0.61, extrinsic postmating isolation = 0.36. We further found the fitness decline in both backcrosses to be appreciable when reared on the ‘wrong’ test plant. It was also asymmetric. Specifically, the ‘maple-like’ backcross type grew >45% faster on maple than on willow, whereas the ‘willow-like’ backcross type grew >70% faster on willow than on maple.

Discussion

Rundle & Whitlock (30) emphasize that “any isolation detected by a comparison of F1 or F2 hybrids to the native parental form in each habitat can involve contributions of both intrinsic genetic and environment-dependent gene effects (p. 200)” and thus conclude that “a reduction of F1 or F2 fitness relative to parental forms in a transplant experiment is not sufficient evidence for ecological speciation (p. 201)”. This argument is critical for two reasons. First, few studies of postmating isolation in natural populations have acquired data on the backcross generations necessary to make this distinction. Thus, little rigorous documentation of extrinsic postmating isolation exists. Second, while intrinsic isolation can readily evolve by various mechanisms (e.g., by genetic drift or even as a consequence of divergent selection), extrinsic postmating isolation is most easily explained as a byproduct of ecologically adaptive divergence (7). Thus, rigorously documenting extrinsic isolation offers especially strong corroboration of the ecological speciation hypothesis. It has been further argued that extrinsic isolation may commonly evolve as populations initially adapt to different environments, thus playing a critical role in the early stages of speciation (1,2). Indeed, various models of speciation with gene flow rely on assumptions of reduced fitness in phenotypically intermediate hybrids (39).

Our study of the maple and willow host forms of *Neochlamisus bebbianae* leaf beetles was inspired by the papers that first developed (30) and empirically tested (36) the backcross approach for evaluating extrinsic isolation. Our rationale and results are thus most readily compared to this original investigation (36) plus a recent paper (40) representing the only other application of this approach. These prior studies both treat ecologically divergent pairs of fish species – benthic and limnetic forms of three-spine stickleback, and killifish adapted to divergent salinity regimes, respectively – and provide varying degrees and forms of evidence for extrinsic isolation. The present study extends the backcross approach by incorporating family-level effects and maternal rearing environment in our ANOVA models. These allowed us to evaluate genetic variation and potential non-genetic maternal influences on our results, respectively. Unlike the prior studies, all 14 possible cross types (Fig. 2.1) were evaluated.

Most importantly, our investigation provides strong statistical documentation of genetically based, ecologically dependent postmating isolation (i.e., extrinsic isolation) between the ecologically divergent maple- and willow-associated host forms of *N. bebbianae* leaf beetles. That is, we demonstrate that the relative fitnesses of the two reciprocal backcrosses switch across host environments (Fig. 2.3). Specifically, the ‘maple-like’ backcross type (having a pure maple-associated parent) grew >45% faster on maple than on willow, whereas the ‘willow-like’ backcross type grew >70% faster on willow than on maple. Such patterns cannot be sufficiently explained by intrinsic factors (30). Further, the complete match of rank fitnesses among cross types in each environment to predictions of ecological speciation had not previously been demonstrated. (30,36,40). These results further corroborate accumulating evidence that these host forms are undergoing ecological speciation.

Multiple reproductive barriers, especially postmating barriers, to reproductive isolation have been evaluated for only a modest number of taxa, one being the present study system. (17). Comparisons among four such barriers revealed extrinsic isolation to be 62% as strong as the strongest among them, indicating its appreciable contribution to ongoing speciation. The various forms of strong support for extrinsic isolation’s importance provided by this study might partly reflect the nature of the alternative beetle environments evaluated here. For specialized insect herbivores, such as these beetles, the host plant is often the site of all life activities. Further, alternative host plants represent environments that are rather biologically discrete, as opposed to continuously varying. This is especially true when, as here, these alternative hosts belong to phylogenetically disparate plant families. In such situations, not only are hybrid-friendly biologically intermediate habitats nonexistent, but host-related selection pressures are expected to be especially specific, strong, and divergent between host-associated populations. In turn, this may predispose such populations to divergent adaptation and the reproductive isolation predicted to accompany it under models of ecological speciation. This contrasts with hybridizing taxa that inhabit more continuous and less starkly differentiated environments. For example, sticklebacks inhabit benthic and open-water lake habitats that grade into each other (22), while big sagebrush experience habitat gradients along mountain slopes (41).

Nonetheless, despite the arguments for host-associated differentiation reviewed so far, other current findings combine with prior results to suggest that – and perhaps help explain why – these host forms have not progressed to biological species status. For example, some backcross individuals and families exhibited higher growth rates on the pure parental host than did the pure parentals themselves (Fig. 2.3). This illustrates within-host-form genetic variation of a kind that might facilitate gene flow between host forms. Indeed, such gene flow is indicated not only by incomplete reproductive isolation, but also by a recent AFLP-based study of these and additional maple- and willow host form populations (42). That study revealed genetic homogenization and close phylogenetic relationships between the present study populations at putatively neutral loci. Indeed, these sympatric maple and willow host form populations proved more similar to each other than to allopatric populations of the same host form at these loci, consistent with local gene flow. The study also found that a subset of loci apparently evolving under divergent host-related selection was highly differentiated between these sympatric host forms. Such results indicate that host-specific divergent selection is strong enough to maintain differentiation at genomic regions associated with host adaptation in the face of recurrent gene flow. Thus, the phenotypic results of the present study corroborate recent molecular ones in explaining how sympatric maple and willow host forms can remain ecologically differentiated despite incomplete reproductive isolation. They further satisfy various criteria hinting at their possible status as host races (43).

To summarize: recent reviews have noted the lack of compelling tests of ecologically-dependent postmating isolation (1,7) even though it is a specific prediction of ecological speciation (30). Here, we rigorously document a clear example of such extrinsic isolation among sympatric, ecologically divergent leaf beetle populations representing two *N. bebbianae* host forms. We do so while controlling for potential contributions from intrinsic genetic incompatibilities, maternal effects, and family-level variation. Future work on this system will, for example, evaluate temporal changes in the proportion of hybrids across the life cycle of these populations using molecular markers. Such data will allow the quantification of hybrid cross type frequencies, the strength of selection acting on them, and patterns of gene flow between host forms (44,45). These and additional investigations will provide further insights into the little-studied contributions of extrinsic isolation to ecological speciation.

Materials and Methods

Natural history of N. bebbianae *host forms*

Neochlamisus bebbianae (Brown) is an eastern North American leaf beetle (Coleoptera: Chrysomelidae) that is univoltine and uses specific host plant species from six genera in five different families (46,47). The suite of populations associated with each host plant is referred to as a particular "host form" (4), each of which exhibits host-specific adaptations (4,33). The maple and willow host forms studied here are sympatric in moist and disturbed habitats across northeastern North America. All life activities, from oviposition through larval development and adult emergence, feeding, and mating, occur on the host plant, although adults fly between individual plants to find oviposition sites and mates. The normally obligate adult winter diapause of these beetles can be broken by manipulating greenhouse conditions, allowing the continual production of new generations for experiment.

Experimental crosses and larval performance/fitness assays

All Generation 1 test animals were collected on their host plants during the summer of 2007 from a site in Caledonia County, Vermont, USA (44.402° N, 71.917° W). These were brought to Vanderbilt University and individually raised to maturity on cuttings of their native host plants (for details, see (4)). From these adults, F_1 hybrid and pure parental offspring cross types were generated to form Generation 2. In turn, adults from this generation were used to create the backcross and pure parental cross types of Generation 3. See Figure 2.1 for further details on the crossing design.

All test families were derived from individual male/female matings and no beetle was mated more than once. Beetles were paired in 5-cm Petri dishes lined with moistened filter papers and continually observed for two hours. If copulation was visually confirmed during this period, the pair was left together overnight to facilitate further insemination. In the absence of copulation, females were later paired with a male of the same type as its original partner. Mated Generation 1 and Generation 2 females were then individually housed in 30 cm x 15 cm mesh bags that were tied over a meristem on a sapling tree of its native host plant, on which they oviposited, thus providing the next generation of test offspring. Bags were constructed of DelNet (DelStar Technologies, Inc., Middletown, DE). All saplings represented genotypes native to northeastern North America and were maintained in the Vanderbilt University greenhouse. Greenhouse conditions were maintained at 21-24°C during the day, 18-21°C at night, a 14:10 light:dark cycle, and > 70 % relative humidity, mimicking summer conditions at the collection locality. These conditions yielded the continual production of newly flushed leaves that prompted oviposition and provided test foliage during the September 2007 – May 2008 period of this experiment.

Eggs were harvested from bags on a weekly basis and maintained in family-specific, filter-paper-lined Petri dishes that were stacked in sealed plastic boxes lined with moist paper towels. These boxes were kept in an incubator at 24° C and a14:10 light-dark cycle, and eggs were checked daily for larval emergence. Individual larval offspring were weighed (= wt1) using a Mettler Toledo MX5 microbalance (Mettler Toledo, Inc., Columbus, OH) on the day of emergence (= t1) and those from each family were alternately assigned to either maple or willow as the plant on which they would be reared, following their order of emergence. Each test larva was then individually maintained in a 5-cm Petri dish lined with moist filter paper and a cutting of its test plant. Dishes were cleaned and foliage replaced every two days. Larval weight (= wt2) was again measured on day 14 (= t2) of rearing to calculate relative growth rate (RGR). RGR = $[((\ln wt_2) - (\ln wt_1)) / (t_2 – t_1)]$ and represents the proportional increase in mass per unit time, accounting for initial size and the nonlinear nature of growth over time (34).

Statistical analysis

Fitness comparisons of offspring from the reciprocal F_1 hybrid and pure parental cross types of Generation 2 employed an ANOVA model that included Cross Type (MM, MW, WM, WW), Host Environment (maple or willow), and Cross Type x Host Environment as fixed effects and Family nested within Cross Type and Family x Environment nested within Cross Type as random effects. The focal Generation 3 analyses employed two different approaches. First, separate ANOVAs using data for individuals from each rearing host were conducted to evaluate the effect of Cross Type per se on fitness. Here, Cross Type was a fixed effect and Family nested within Cross Type was a random effect. Second, to more specifically test whether backcross fitness was ecologically dependent, an ANOVA model was used that included Backcross Type (BC_{maple} or BC_{willow}), Host Environment, and Backcross Type x Host Environment as fixed effects, and Family nested within Backcross Type and Family x Environment nested within Backcross Type as random effects. Additionally, two analyses were performed to assess potential contributions of non-genetic host-associated maternal effects on our results. These used ANOVAs in a manner analogous to the main analyses just detailed and are otherwise adequately described in the main text. All ANOVA models including random effects were fit using the Restricted Maximum Likelihood method (48) and F-ratios were constructed to test effects of a partially nested design (49). All post-hoc treatments of means comparisons were conducted using a Tukey HSD test, which accounts for multiple comparisons. All statistical analyses were performed using the program JMP 5.0.1a (50).

Quantifying reproductive isolation

We calculated the individual contribution of extrinsic isolation to total reproductive isolation (following (17)) as: EI = backcross fitness – (backcross fitness/pure parental fitness), where backcross and pure parental fitnesses were each estimated using mean RGR across all possible combinations of cross type and test plant environment. Analogous values from other reproductive barriers estimated for this system (17) using previously published formulas (19) were also compiled. Finally, we calculated the relative fitness reduction of the reciprocal backcross types on their non-native test plants by comparing the relative growth rates of each backcross on each of the two test plants.

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Table 2.1. ANOVA on RGR demonstrating extrinsic postmating isolation.

Effects	df	MS	*F*	*P*
Generation 2: F1 hybrid and pure crosses				
Cross Type	3	0.0019442	1.29	0.3022
Host Environment	1	0.0151450	30.51	**< 0.0001**
Cross Type x Host Env.	3	0.0855936	172.4	**< 0.0001**
Family (Cross Type)	22	0.0015056	1.28	0.1869
Family (Cross Type) x Env.	16	0.0004964	0.42	0.9764
Residual	249	0.001179		
Generation 3: Backcross hybrids				
Backcross type	1	0.0009819	2.19	0.1469
Host Environment	1	0.0006451	0.4284	0.5171
Backcross type x Host Env.	1	0.0399871	26.55	**< 0.0001**
Family (Cross Type)	38	0.0004478	0.5778	0.9794
Family (Cross Type) x Env.	35	0.0015060	1.943	**0.0015**
Residual	348	0.000775		

Table 2.2. ANOVA on RGR for backcrosses analyzed separately by maternal host plant.

Effects	df	MS	*F*	*P*
		Offspring with maple maternal host plant		
Cross Type	1	0.0001655	0.05	0.8259
Host Environment	1	0.0001513	30.88	**< 0.0001**
Cross Type x Host Env.	1	0.0682933	13937.4	**< 0.0001**
Family (Cross Type)	16	0.0032175	4.875	**< 0.0001**
Family (Cross Type) x Env.	16	0.0000049	0.007	0.99
Residual	173	0.000660		
		Offspring with willow maternal host plant		
Backcross type	1	0.0015837	1.03	0.3217
Host Environment	1	0.0013904	1.57	0.2262
Backcross type x Host Env.	1	0.0523520	59.26	**< 0.0001**
Family (Cross Type)	21	0.0015371	1.57	0.0614
Family (Cross Type) x Env.	18	0.0008835	0.901	0.5781
Residual	173	0.000981		

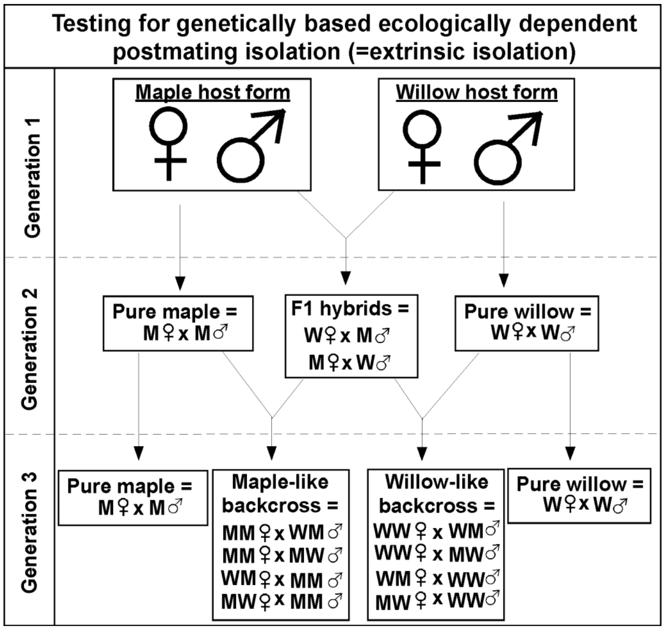

Figure 2.1. Experimental crossing design to create F1 and backcross hybrids, along with ‘pure parental’ (i.e., within-host-form) crosses, for tests of extrinsic isolation. Generation 1 animals were collected from the field as immatures and reared to maturity in the laboratory, whereas generations 2 and 3 were propagated entirely in the lab/greenhouse.

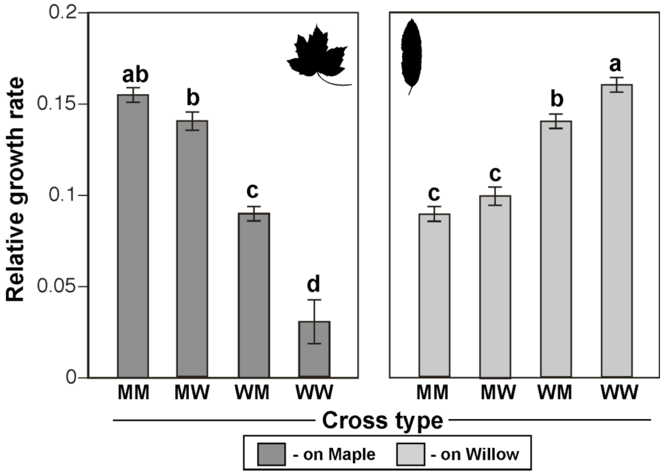

Figure 2.2. Mean ± s.e.m. of the relative growth rate to day 14 for F1 hybrid and pure parental cross types on red maple (dark grey) and Bebb's willow (light grey) foliage. Different letters over bars indicate significant differences between cross types, based on a Tukey HSD test at $P < 0.05$. Abbreviations applied to offspring of each cross type: MM = maple female x maple male; MW = maple female x willow male; WM = willow female x maple male; WW = willow female x willow male.

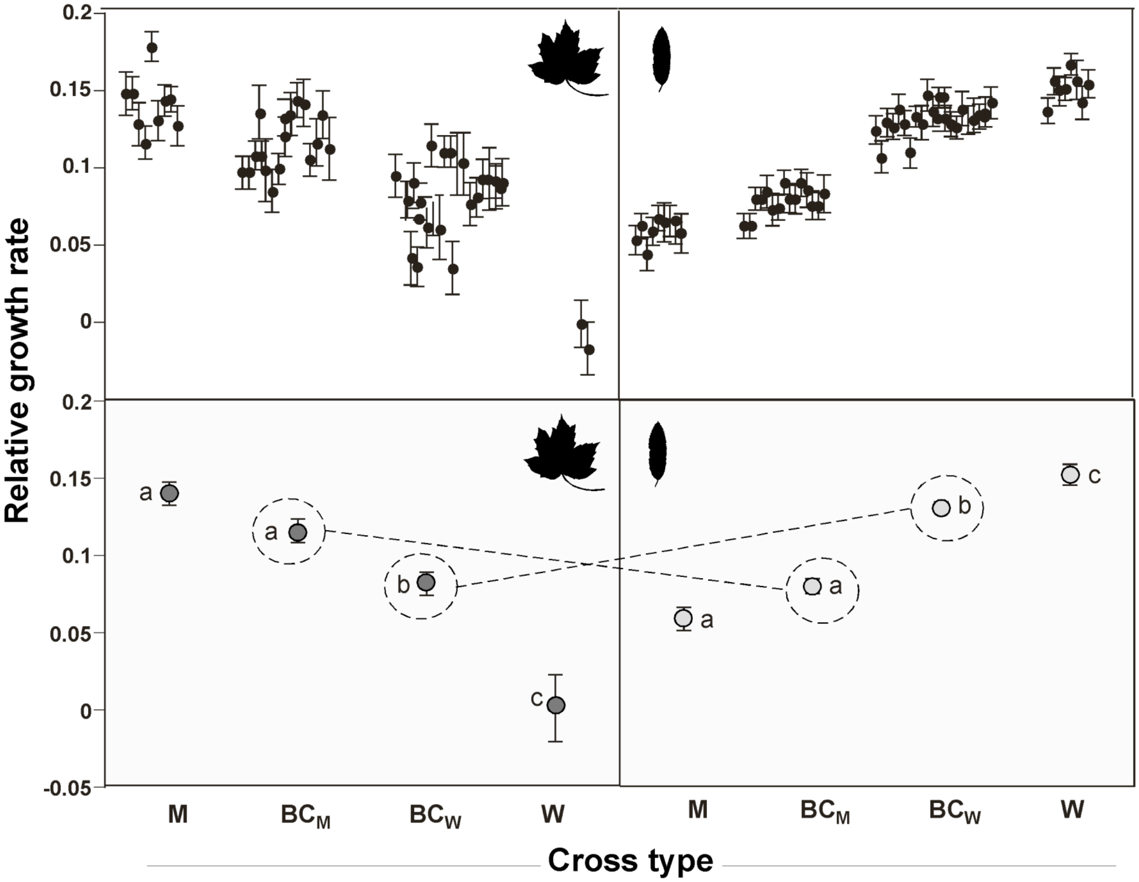

Figure 2.3. Mean ± s.e.m. of the relative growth rate to day 14 for backcross hybrid and pure parental cross types on red maple (left panels) and Bebb's willow (right panels) foliage. Individual family means are presented (top panels) as are the means of these family means (bottom panels). In the bottom panels, each backcross type is circled and connected between panels by a dashed line to illustrate the ecologically dependent fitness of backcross hybrids (i.e., 'switching'). This pattern documents extrinsic postmating isolation, a critical prediction of ecological speciation. The small number of families representing the pure parental willow cross type on maple reflects the general incapacity of this host form to survive on maple foliage. Different letters show significant differences from a Tukey HSD test at *P* < 0.05

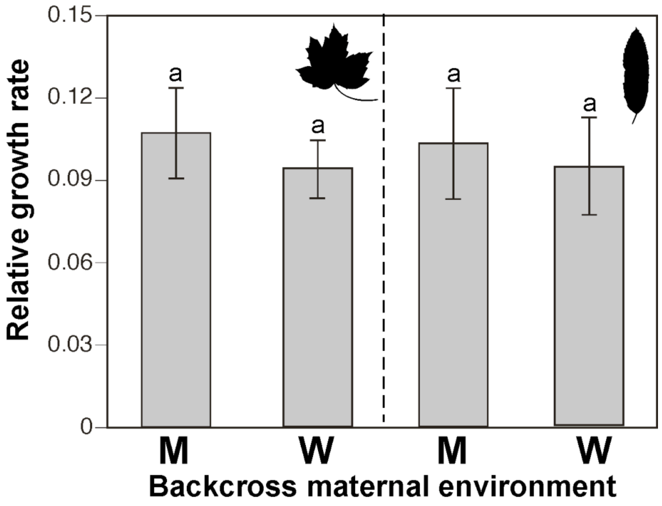

Figure 2.4. Mean ± s.e.m. of the relative growth rate to day 14 for offspring deriving from dams that had been reared on either maple (left bars) or willow (right bars) test plants. This plot demonstrates a lack of effects on offspring performance as a function of maternal environment, as no differences were observed for any comparison (*P* > 0.05, see Results and Table 2.2 for details).

CHAPTER III

NOVEL ASPECTS OF POSTMATING ISOLATION AMONG HOST FORMS OF *NEOCHLAMISUS BEBBIANAE* LEAF BEETLES

Introduction

The study of ecological speciation is most clearly tested through the study of reproductive isolating barriers (Coyne & Orr 2004). Reproductive barriers can arise prior to mating, generally termed premating barriers, that inhibit the initiation of gene exchange, or barriers can arise after mating, generally term postmating barriers, which inhibit gene flow by removing those ‘hybrid’ individuals from the gene pool prior to any mating events, thus not allowing them to move genes between taxa. Prezygotic and postzygotic reproductive isolation builds between these populations as they climb separate adaptive peaks associated with each habitat (Gavrilets 2004), but the relative role and order of these different types of barriers for a given system has been rarely explored in great depth (see Nosil et al. 2005).

Examples of speciation events in which adaptation to different environments played a fundamental role in the evolution of reproductive isolation have come from both the lab and field experiments. Controlled laboratory experiments have shown that divergent adaptation to different environments can generate reproductive isolation between experimental populations both in allopatry and sympatry via premating and postmating barriers (Rice and Salt

1990; Rice and Hostert 1993). There are also strong examples in nature within specific study systems that document the association of reproductive isolation, specifically prezygotic reproductive isolation, with adaptive ecological divergence (e.g., Feder et al. 1994, Funk 1998, Rundle et al. 2000, Nosil 2002, Vines and Schluter 2006). Some studies have documented that the same traits that are under divergent selection between environments directly cause reproductive isolation to occur, such as environment-dependent wing patterns used in defensive mimicry and mate selection in *Helioconius* (Jiggins et al. 2001). More generally, a consistent association between ecological divergence and reproductive isolation has been shown across disparate taxa spanning flowering plants, birds, amphibians, fishes, and insects (Funk et al. 2006).

During previous dissertation research using experimental crosses between divergently adapted populations of the leaf beetle, *Neochlamisus bebbianae*, I noticed that larvae from hybrid crosses were dying at two different life stages more often than pure crosses. First, as newly hatched larvae (day 1 – 4), the hybrid offspring were falling out of their maternally inherited egg case (see Natural History section for details), desiccating, and dying, whereas pure crosses were not. It appeared that there might be a mismatch in the size of the maternally inherited egg case and the size of the hybrid larvae. To address this possibility, I tested (A.) whether there was a difference in the size of the egg cases constructed by the females from the willow and maple host forms, (B.) whether females from each host form construct different size egg cases when they mate within versus between host forms, (C.) whether hatchling larval size differed among offspring from pure maple and willow crosses and hybrid crosses, and (D.) whether there is an association between the degree of mismatch between egg case and larval size and the observed phenomenon of falling out of the case.

Second, through larval growth (day 4 – pupation) hybrid offspring were growing at a slower pace than pure crosses and during feeding preference trials hybrids were consuming less plant material than pure crosses (Egan, Janson, Brown, and Funk, unpublished manuscript, and Chapter 2). To investigate behavioral differences among crosses that might be associated with decreased feeding amounts and growth rates, individual larvae from each of the four cross types used throughout this dissertation work (MM, MW, WM, WW) were observed in 2-hour assays of foraging behavior. Crosstypes were then compared for time efficiency (time to reach a given host plant), time economy (time spent on a specific host plant), and decision making behaviors (time spent crawling, number of times leaving a give host plant).

Each of these types of reproductive barriers are rarely reported in the speciation literature, and thus, offer novel insights into the many aspects of speciation influenced by ecological adaptation.

Materials and Methods

Study system

Neochlamisus bebbiane (Brown) is an eastern North American species of chrysomelid leaf beetle that uses particular tree species from six genera as host plants (Karren 1972; Funk 1998; Brown and Funk 2005). *N. bebbianae* populations associated with red maple (*Acer rubrum L.*; Aceraceae) and Bebb's willow (*Salix bebbiana* Sarg.; Salicaceae) are the focus of the present study and overlap geographically in northeastern North America (Karren 1972). These two types of host-associated populations, referred to as 'host forms', most readily prefer, grow, survive, and oviposit on the foliage from the plant they use in nature (the 'native host'), but accept the native hosts of other host forms to a lesser degree (Funk 1998; Egan and Funk 2006; D.J. Funk, unpublished data). The Willow and Maple host forms exist at modest densities, all life activities occur on the native host, adults will fly among individual plants, and females lay eggs singly, wrapping each in fecal material (Brown and Funk 2005, D.J. Funk, personal observation). The *Acer* host form contains a cryptic asexual lineage, so all females from Maple used in experimental crosses to generate the offspring were confirmed *a posteriori* to be from the sexual lineage using PCR-RFLP (Egan, McCauley, & Funk, unpublished data).

These beetles have an interesting life history where the females cover their egg cases with fecal material to form a protective egg case (Brown and Funk 2005, Chaboo et al. 2008). When the larva hatches from the egg it cuts its way out of the maternal egg case through the top, partially emerges, but leaves its abdomen folded up inside of the case. As the larva grows, it adds to the case in length and width with its own fecal material to allow it room to grow. At the end of larval growth, the larva seals itself within the case, pupates, and then emerges as an adult to complete the life cycle.

Testing hybrid mismatch with maternal egg case

To test for differences in size between egg cases constructed by females from the willow and maple host form and to test for differences within each host form for females mated with males from their own or the alternative host form, I measured different aspects of the size of the unhatched egg cases of *N. bebbianae* females. Two perpendicular measurements of the diameter of the egg case opening were measured using an Axioskop 2 Plus universal microscope with a Nikon DS-U1 camera and NIS-Elements BR software. Egg case length and midwidth (= width taken at half length) were measured with an Olympus SZ40 dissecting microscope using a fitted ocular ruler and converted to millimeters by hand.

To address size differences among larvae from each crosstype generated, I measured different aspects of the size of newly hatched larvae that either fell out of their egg cases or remained in their egg case through day 4. I measured individuals from pure maple crosses (maple male x maple female), pure willow crosses (willow male x willow female), and hybrids crosses (maple male x willow female; willow male x maple female). Using the Olympus SZ40 dissecting microscope, I measured the length and width of the larval head capsule and the length of the front tibia.

To then quantify the mismatch between larval size and egg case size, I subtracted the average head capsule size (average of the length and width) from the average of the egg case diameter (average of the two perpendicular measurements). All comparisons were done using a t-test or a one-way ANOVA followed by Tukey's test for posthoc means comparisons within the program JMP 5.0.1 (SAS).

Foraging behaviors: Test animals and controlled matings

Individuals used in crosses originated from field collections across eastern North America in the summer of 2006 and 2007. Because no collection from one geographic population contained enough individuals to perform the crosses, different localities were pooled together for each host form. All foliage used for beetle rearing and female oviposition was grown in the Vanderbilt greenhouse from plants originating from multiple localities (red maple: Warren Co., TN; Amherst, MA; Bebb's willow: Amherst, MA, Walsingham, ON).

Pure and hybrid genotypes were created from controlled crosses in the lab. Four types of crosses were created: MM = Maple mother x Maple father; MW = Maple mother x Willow father; WM = Willow mother x Maple father; WW = Willow mother x Willow father (these abbreviations will be used throughout). All pairings occurred between one male and one female in 5 cm Petri dishes lined with a moistened piece of filter paper and pairings lasted 2 – 4 hours under continuous observation. If copulation occurred (visually confirmed by the male aedeagus extended and inserted into the female), the pair was left overnight. In unsuccessful pairings (no copulation occurred), each individual was removed and added to a new pairing from the same type of cross. All successful pairings were unique. After copulation, females were added individually into a 0.5 mL eppendorf tubes with small holes in the top for airflow, placed in cardboard tube boxes (VWR), and placed in an incubator at 4° C for 2-6 weeks in 2004 and 6 weeks in 2005 to mimic winter diapause (Janson and Funk, unpublished data). After artificial diapause, females were individually housed within 30-cm x 15-cm bags constructed of DelNet (DelStar Technologies, Inc.) on to the female's native host in the Vanderbilt greenhouse and eggs were harvested weekly. When eggs were harvested, they were stored by family in 5 cm Petri dishes lined with dry filter paper in an incubator at 24° C and 14-10 light-dark schedule. Eggs were checked daily.

In 2006, there were 317 individuals measured across 23 families (6 WW, 8 WM, 3MW, 6 MM); in 2007, there were 165 individuals measured across 28 families (3 WW, 5 WM, 10 MW, 10 MM). All individuals were reared and bagged individually, thus information could be linked between parent and offspring.

Foraging behaviors: Petri dish assays

Foraging behavioral assays were performed in an environmental chamber (26 - 28°C; >75% humidity) within 5-cm Petri dishes lined with moistened filter paper in which one leaf disc from each of three test plants was added. The three test plants were the two parental host species, *Acer rubrum* and *Salix bebbiana*, and a third sympatric host plant, alder (*Alnus serrulata*; Betulaceae), on which *Salix* and *Acer* host forms of *N. bebbianae* accept to a lesser degree than their native host in feeding trials (Egan and Funk 2006). Notably, there is a predominantly asexual host form of *N. bebbianae* that resides on many species within the genus *Alnus* (Karren 1972, Funk, unpublished data). These trials were initiated by the addition of a single test animal to each Petri dish. The ‘location’ (on *Alnus*, *Acer*, or *Salix* discs, or the test arena) and ‘behavior’ (stationary, walking, feeding) of each individual was recorded at five-minute intervals over two hours for a total of 24 observations. An individual was considered to be “on” a disc when at least two legs were in contact with it, “crawling” if it was in motion with legs ambulating, and “feeding” if mouthparts were in contact with the leaf disc. Data from individuals that were observed on discs for less than 2 of the 24 observations were discarded prior to analysis. From the two hour observation, I compared mating classes (hybrid vs. pure) under three broad categories: (a.) response time or efficiency (time to reach a type of leaf disc; any, paternal host, maternal host) , (b.) time economy (total time spent on a host; any, paternal, maternal), and (c.) movement patterns (time spent crawling, number of times leaving a plant disc, number of times switching species of leaf disc).

For all comparisons of foraging behavior, crosses were pooled by class as either pure (*Salix* x *Salix*, *Acer* x *Acer*) or hybrid (*Salix* x *Acer*, *Acer* x *Salix*). Due to violations in the assumptions of parametric statistics common in behavioral studies, data was converted to ranks and analyzed using a Wilcoxon sign rank test (Zar 1996). Data between years was not different when year was included in an initial model, thus data was combined for the analysis presented here.

Results

Hybrid mismatch to maternal egg case

Egg cases constructed by maple host form females are larger in size than egg cases constructed by willow host form females (Figure 3.1.A-C) based on measures of diameter ($t_{df=168} = 3.343$; $P = 0.0008$), length ($t_{df=169} = 6.67$; $P < 0.0001$), and midwidth ($t_{df=169} = 14.07$; $P < 0.001$). Furthermore, egg cases constructed by females of either host form do not differ when they are mated with a male from their own host form or the alternative host form (all $P > 0.05$; Figure 3.2.A-F). Comparisons of hatchling larval size among crosstypes showed significant differences using all three measures of body size (Table 3.1). Pure hatchling larvae from the maple host form are larger than pure hatchling larvae from the willow host form (Figure 3.3.A-C) based on measures of head capsule length and width and front tibia length. Hybrid hatchling larvae from matings between willow and maple host forms were found to intermediate in size although it was not always significantly intermediate (Figure 3.3.A-C).

Comparing the mismatch between egg case diameter and larval capsule demonstrated that WW and MM crosses were similar, yet MW crosses showed a larger mismatch and WM crosses showed a smaller mismatch ($F_{3,100} = 29.15$, $P < 0.0001$, Figure 3.4.A). However, it is possible that hybrids are just less vigorous or healthy than pure crosses, which makes them more likely to fall out regardless of the degree of mismatch. So, I also compared individuals that did and did not fall out of their case within each of the two hybrid crosstypes (MW and WM). Those individual hybrid offspring that did fall out of their case had a greater degree of mismatch between body size and maternal egg size than individual hybrids that did not fall out of their cases (Table 3.2, Figure 3.4.B).

Foraging behaviors

The number of individuals that were observed on a leaf disc less than two of the twenty-four observations did not differ between hybrid and pure classes ($\chi^2_{df=1} = 1.22$, $P = 0.2702$) and were dropped from the analyses to remove less vigorous individuals. In response times, hybrid and pure classes did not differ in the time it took them to reach a leaf disc of any species ($\chi^2_{df=1} = 2.52$, $P = 0.1126$; Figure 3.5.A), nor did they differ in the time it took to reach the leaf disc of a specific species (time to alder: $\chi^2_{df=1} = 2.17$, $P = 0.1408$; time to maple: $\chi^2_{df=1} = 0.14$, $P = 0.7036$; time to willow: $\chi^2_{df=1} = 0.15$, $P = 0.7023$). Interestingly, hybrids took marginally longer to reach the maternal host ($\chi^2_{df=1} = 2.71$, $P = 0.0998$; Figure 3.5.B) and significantly longer to reach the paternal host ($\chi^2_{df=1} = 9.79$, $P = 0.0018$; Figure 3.5.C), but did not differ in the time it took to reach either parental host when pooled ($\chi^2_{df=1} = 0.01$, $P = 0.9243$).

In time economy, hybrids spent just as much time as pure classes on plant discs versus in the test arena ($\chi^2_{df=1} = 0.03$, $P = 0.8519$; Figure 3.5.D), however spent 50% less time on the maternal host ($\chi^2_{df=1} = 5.49$, $P = 0.0191$; Figure 3.5.E) and 14% less time on the paternal host ($\chi^2_{df=1} = 17.23$, $P < 0.0001$; Figure

3.5.F). Interestingly, hybrids spent more time on the non-host alder, than pure classes ($\chi^2_{df=1}$ = 6.34, *P* = 0.0118), but did not differ in time spent on maple ($\chi^2_{df=1}$ = 0.01, *P* = 0.9497) or willow ($\chi^2_{df=1}$ = 0.33, *P* = 0.5626) separately, or when pooled ($\chi^2_{df=1}$ = 1.06, *P* = 0.3024).

In movement patterns, hybrids were observed crawling around 33% more than pure classes ($\chi^2_{df=1}$ = 6.88, *P* = 0.0087; Figure 3.5.G) and they left plant discs 25% more often ($\chi^2_{df=1}$ = 5.66, *P* = 0.0174; Figure 3.5.H). Hybrids did not switch between the discs of different species more often than pure classes ($\chi^2_{df=1}$ = 1.32, *P* = 0.2513), although the trend of the data was consistent with that pattern (Figure 3.5.I).

Discussion

Postmating barriers promote ecological speciation in N. bebbianae

Divergent ecological adaptation has lead to the evolution of multiple barriers among *N. bebbianae* host forms, including premating barriers such as habitat isolation, immigrant inviability, and sexual isolation (Funk, 1998; Funk *et al.*, 2002; Egan & Funk, 2006; Funk & Nosil, 2007) and postmating barriers such as extrinsic hybrid inviabiblity (Chapter 2; Egan and Funk 2009). The present study addresses the role that two novel aspects of reproductive isolation play in generating barriers to gene flow among willow and maple host forms of *N. bebbianae*.

Hybrid mismatch to maternal egg case

When divergently-adapted willow- and maple-associated populations of *N. bebbianae* hybridize, I observed that the hybrid offspring fell out of their cases more often than offspring from pure crosses. These uncased hybrids go on to desiccate and die. This study has demonstrated that this phenomenon is likely due to a mismatch between the larval size and the egg case size. This reduced hybrid fitness is an example of postmating reproductive isolation that will reduce gene flow between these populations and promote ecological speciation.

Maternal effects, in general, can play an important role among a diverse array of organisms (Mousseau and Fox 1998, Mousseau et al. 2009) and specifically, to insects (Fox and Mousseau 1998). However, observations of maternal effects are commonly put in the context of ‘adaptations’, whereas the role it plays between host forms of *N. bebbianae* is contributing towards reproductive isolation and speciation. To my knowledge, this is the first explicit evidence that maternal effects can contribute towards reproductive isolation and speciation, although the idea has been suggested in theory (Wade 1998). Although evidence is lacking, it is possible that maternal effects could commonly contribute to reproductive isolation. If a maternal effect is defined as an effect on the phenotype of offspring that originates from the phenotype or environment of its mother (Falconer 1989), then there are many aspects of the life history of plant feeding insects that could be influenced. For example, developmental timing of the pupa of the flesh fly *Sarcophaga bullata* is strongly influenced by the experience of the mother (Denlinger 1998). When the *S. bullata* mother is raised on a long day length, the offspring can respond to short day lengths by entering diapause, however, if the *S. bullata* mother is raised on a short day length, the offspring can not respond to short day lengths and does not enter diapause (Denlinger 1998). The adaptive significance of this relationship is related to the ability of the fly to take advantage of early spring, where day length is shorter and going into diapause would not allow the offspring to take advantage of this favorable time of year and not go into an untimely diapause when resources are available. Whether larger or smaller egg cases is adaptive, per se, for *N. bebbianae* on maple and willow respectively, is unknown. However, the mismatch between hybrid offspring and the maternal effect (i.e., the egg case) causes clear barriers to gene flow between these ecologically-divergent populations.

Foraging behaviors

There were also differences in the foraging behaviors of hybrids compared with pure crosses that might contribute to decreased gene flow between host forms. Evidence for behavioral differences in hybrids is rare, but have been documented to contribute to lower hybrid fitness, and thus, reproductive isolation among a handful of closely related taxa (Stratton and Uetz 1986, Helbig 1991, Linn et al. 2004, Coyne and Orr 2004). *N. bebbianae* hybrids reached maternal and paternal host plants slower than pure crosses. They also spent less time on maternal and paternal host plants than pure crosses,. Hybrids also spent more time crawling around and left host plants more often than pure crosses. These patterns suggest that hybrids appear to be slightly less responsive to host cues than pure crosses. Under natural conditions, if hybrids are slower to respond and less decisive, they might be more visible to predators and natural enemies, and thus have even higher reductions in fitness than just host-dependent growth and survival demonstrated in Chapter 2.

The foraging dysfunctions exhibited by hybrid *N. bebbianae* are likely a rare example of 'behavioral extrinsic hybrid inviability' (Coyne and Orr 2004). This reflects a decrease in hybrid fitness due to some type of 'intermediate' behavior. Helbig (1991) demonstrated that F_1 hybrids between two populations of the black-cap bird *Sylvia atricapilla* displayed a migratory behavior that was intermediate of either parental form, which would decrease hybrid fitness. One blackcap population would migrate in a southeasterly direction, while another would migrate in a southwesterly direction during the winter. When these two populations were hybridized, F_1 hybrid offspring exhibited an intermediate direction that would deliver the bird to unsuitable wintering grounds (Helbig 1991). Another variant of this type of reproductive barrier is 'behavioral extrinsic hybrid sterility' (Coyne and Orr 2004). Here, the intermediate behavior relates to mate acquisition, rather than resource acquisition. An unique example of this occurs between two species of wolf spider, *Schizocosa ocreata and S. rovneri*. These morphologically similar species have males that exhibit different courtship displays and songs (Stratton and Uetz 1986). When F_1 hybrid males court a female, they drift between intermediate displays and back and forth between each species specific display. All females from each species and the F_1 hybrids usually reject them. It would be interesting to follow up with behavioral assays of mating behaviors in *N. bebbianae* to determine if the intermediate behaviors in foraging are also shown during mating.

In addition to generating reproductive barriers, hybridization can be a creative source of variation that can move novel behaviors among species, or isolate the hybrids themselves into a novel environment. The observation that hybrid *N. bebbianae* spent more time on a host different from either parent is consistent with a pattern of transgressive hybridization (Rieseberg et al 1999), where hybrids have a host range that expands beyond that of either parent. This allows them to expand into new environments, and become reproductively isolated form either parent (Welch and Rieseberg 2002). Interestingly, there is an asexual lineage of *N. bebbianae* that is a host form of alder in northeastern North America (D.J. Funk, unpublished data). It might be that the alder host form of *N. bebbianae* originated from hybridizations between the willow and maple host forms. Although more detailed experimentation would be required to determine this possibility in *N. bebbianae*, a similar story has been demonstrated in the *Rhagoletis pomonella* species complex (Schwarz et al. 2005). Here, a new form of *Rhagoletis* appeared on the host plant *Lonicera*, and it was determined that the origin of this new putative host race was a hybridization between *R. mendax* (host: blueberry) and *R. zephyria* (host: snowberry).

Conclusions

As populations adapt to different environments and reproductive isolation builds, hybrids between populations may suffer reductions in fitness due to intrinsic genetic incompatibilities that can evolve as a consequence of a number of mechanisms, including drift (Dobzhansky, 1937), or due to extrinsic ecological incompatibilities that most likely evolve as a consequence of divergent natural selection (e.g. Hatfield & Schluter, 1999; Rundle 2002). In this study, it is unclear whether these two barriers are extrinsic or intrinsic, and in fact, the maternal effect (egg case) barrier does not appear to be described in the literature. Further study and additional generations, as applied in Chapters 2 and 4 may help with understanding these barriers further.

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Table 3.1. ANOVA tables for testing the effect of cross types (MM, hybrid, WW) on larval body size using three different measures: (A.) front tibia length, (B.) head capsule width, and (C.) head capsule height. Results of Tukey's post-hoc means comparisons tests are presented in Figure 3.3.

Size Measurement	**Factor**	**DF**	**SS**	**F**	**P**
(A.) Front tibia length	Crosstype	2	0.047	35.09	< 0.0001
	Error	156	0.104		
(B.) Head capsule width	Crosstype	2	0.077	107.21	< 0.0001
	Error	156	0.056		
(C.) Head capsule height	Crosstype	2	0.091	97.77	< 0.0001
	Error	156	0.075		

Table 3.2. ANOVA table for testing the effect of hybrid cross type (WM or MW), egg type (OOC or nonOOC), and their interaction on the mismatch in size between the egg case opening and the larval head capsule size. Results of Tukey's post-hoc means comparisons tests are presented in Figure 3.4.B.

Factor	**DF**	**SS**	**F**	**P**
Cross type	1	0.0119	5.97	0.0162
Egg type	1	0.0031	1.54	0.2175
Cross type X Egg type	1	0.0618	31.06	< 0.0001
Error	106	0.2110		

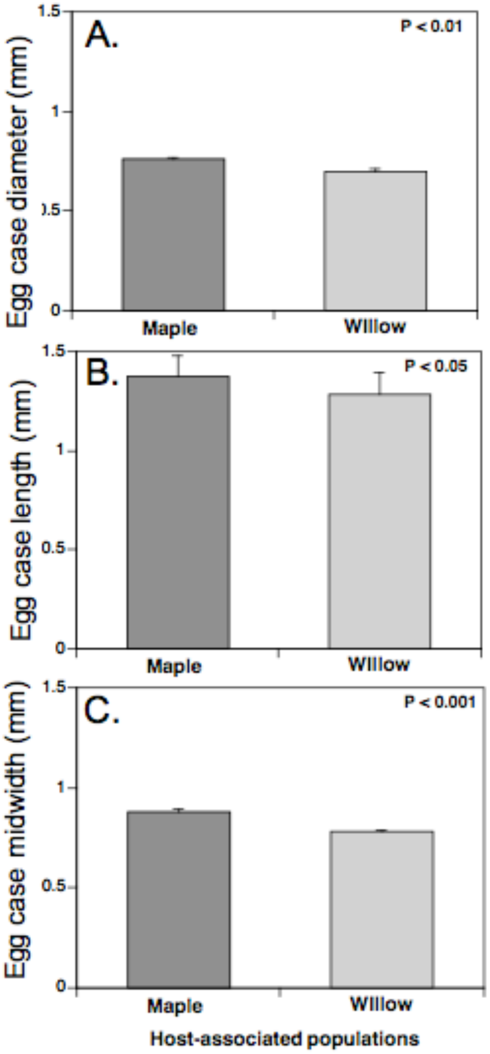

Figure 3.1. (A.) Egg case diameter, (B.) egg case length, and (C.) egg case midwidth constructed by females from the willow and maple host forms of *N. bebbianae*.

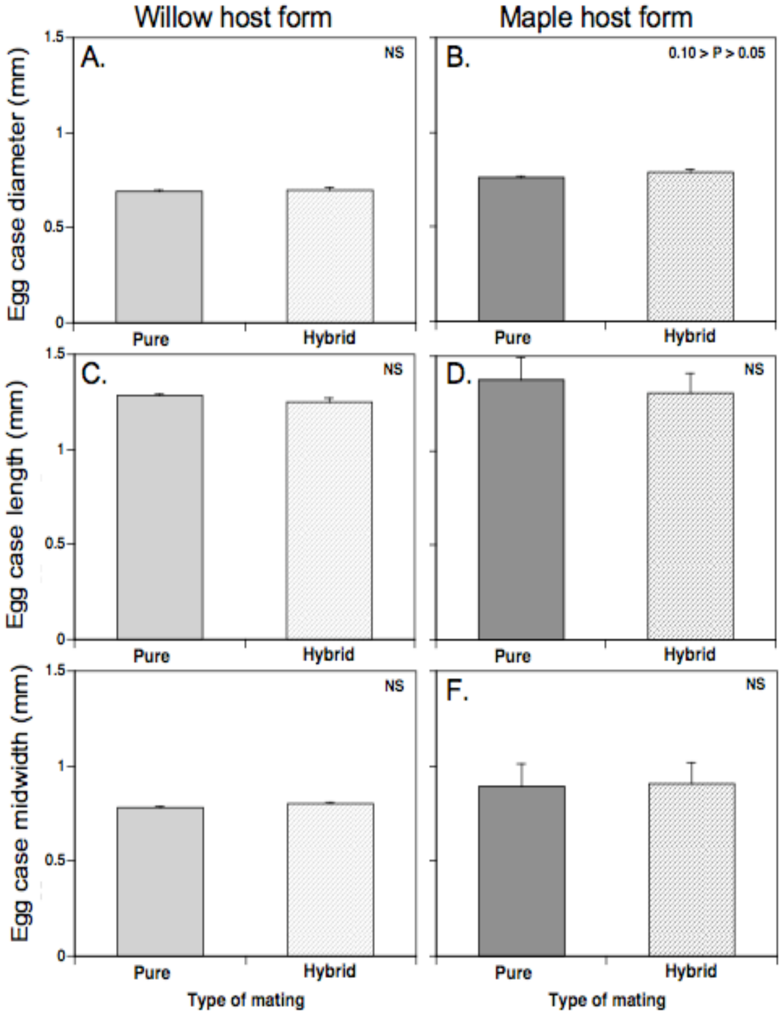

Figure 3.2. (A. & B.) Egg case diameter, (C. & D.) egg case length, and (E. & F.) egg case midwidth constructed by females from the willow (left) and maple (right) host forms of *N. bebbianae* that were mated with males from their own host plant (pure matings) or the alternative host plant (hybrid matings).

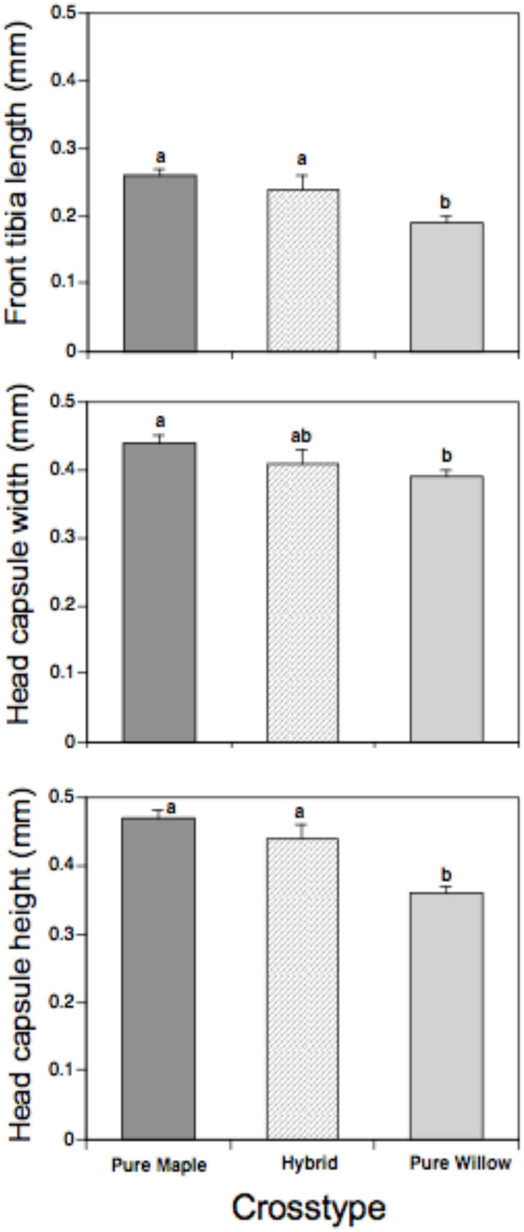

Figure 3.3. (A.) Front tibia length, (B.) head capsule height, and (C.) head capsule width for newly hatched offspring from pure crosses and hybrid crosses. Different letters above bars indicates a significant difference from an ANOVA and Tukey's post-hoc test ($P < 0.05$).

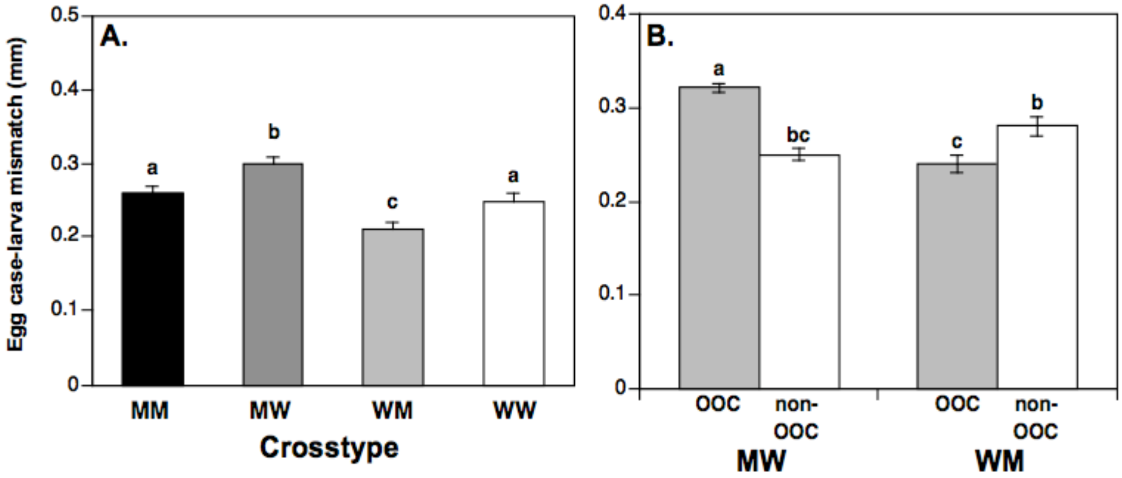

Figure 3.4. Mismatch in diameter between the maternal egg case diameter and the larval head capsule diameter among: (A.) the four crosstypes generated in this study and (B.) the larvae that fell out of their cases (Out-Of-Case; OOC) versus the larvae that remained in their cases (non-OOC) for the two types of hybrid crosses. Different letters above bars indicates a significant difference from an ANOVA and Tukey's post-hoc test ($P < 0.05$). (MM = maple male x maple female; MW = maple male x willow female; WM = willow male x maple female; WW = willow male x willow female)

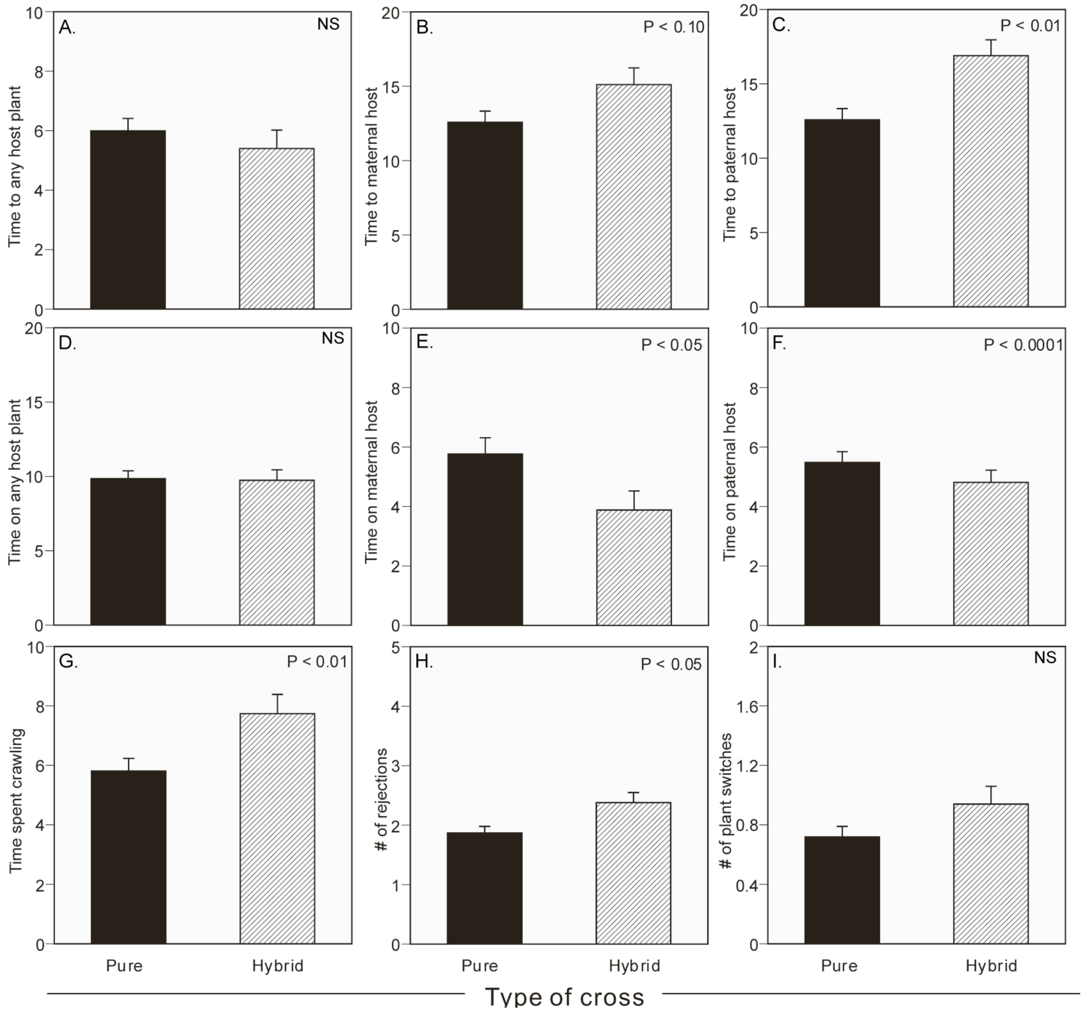

Figure 3.5. Comparison between pure crosses (maple x maple; willow x willow) and hybrid crosses (maple x willow) in behavioral foraging assays. Row 1 depicts the average time to reach a given host plant (panels A – C). Row 2 depicts the average time spent in a given locality during the assay (panels D – F). Row 3 depicts three aspects of the decision making process (panels G – I). See Methods and Results for more details.

CHAPTER IV

ANALYSIS OF THE GENETIC ARCHITECTURE OF HOST-USE TRAITS INVOLVED IN ECOLOGICAL SPECIATION BETWEEN SYMPATRIC WILLOW AND MAPLE HOST FORMS OF *N. BEBBIANAE* LEAF BEETLES

Introduction

Populations adapted to different environments can exhibit large phenotypic differences. The genetic basis of phenotypic divergence between populations is of general interest for several reasons. First, the mean phenotype of offspring from interpopulation crosses (F1 hybrids) influences patterns of hybridization and introgression among populations. If traits involved in local adaptation to each environment are not inherited additively, then asymmetric gene flow can occur, with hybrids conforming more to the locally adapted phenotype from one environment, but not the other. If these traits are inherited additively, then hybrids will exhibit intermediate phenotypes that may not match with either parental environment (Rundle 2002, Fuller 2008, Chapter 2). Second, there may be general forms of outbreeding depression (low hybrid fitness) or outbreeding enhancement (high hybrid fitness) in F1 or later hybrid generations that also influence patterns of gene flow between populations. When these ecologically divergent populations have the opportunity to interbreed in nature, selection may favor dispersal strategies or reproductive isolation to enhance or discourage outcrossing. Thus, understanding the genetic basis of interpopulation differentiation is a key to understanding the genetic basis of speciation (Lynch and Walsh 1998).

This pursuit can become even more important when the trait diverging between populations is directly influencing reproductive isolation, either because it is a ‘magic’ trait that itself influences both the locally adapted phenotype and reproductive isolation, or through some genetic association via pleiotropy or tight linkage. When the evolution of reproductive isolation is ultimately driven by divergent natural selection on traits between environments this process is described as ‘ecological speciation’ (Schluter 2000, 2001). This model of speciation and its general importance has received much recent attention (see reviews, Funk et al. 2002, Rundle and Nosil 2005, Schluter 2009). Although progress has been made in understanding the association between ecological divergence and reproductive isolation in many individual study systems (i.e., Funk 1998, Rundle et al. 2000, Nosil 2007) and more generally across taxa (Funk et al. 2006), a detailed understanding of the genetic basis of divergence during ecological speciation is still needed (Coyne and Orr 2004, Rundle and Nosil 2005). This is likely due to two important reasons: (1) that the understanding of ecological speciation in the wild comes from non-model organisms where genetic and laboratory resources are limited and (2) generating the types of interpopulation crosses necessary to address genetic aspects is difficult and work intensive.

Premating barriers tend to play a more important role than postmating barriers during ecological speciation (Nosil et al. 2005, Rundle and Nosil 2005), yet much more empirical evidence is available on the genetics of postmating barriers than for premating barriers (Coyne and Orr 2004, Rundle and Nosil 2005). This is underscored by the difference in the patterns of evolution of prezygotic versus postzygotic isolation (Coyne and Orr 1989, 1997, 1998, 2004) suggesting that each has general differences in their genetic basis. Moreover, understanding the genetic basis of divergence and reproductive isolation will also allow a better understanding of the geographic context (Kondrashov and Kondrashov 1999, Dieckmann and Doebeli 1999) and tempo (Hendry et al. 2007) of ecological speciation. Specifically, assumptions in models of speciation with gene flow commonly assume that traits diverging between populations are inherited additively, which is not known to be a generality across taxa.

In addition to interactions between alleles at a given locus (additive to dominance effects), interactions between loci via epistasis can also play an important role during phenotypic divergence and speciation. The most common reference to epistasis during speciation occurs via intrinsic genetic postzygotic isolation, commonly referred to as Dobzhansky-Muller (D-M) incompatibilities in honor of its original investigators (Coyne and Orr 2004). These D-M incompatibilities arise when alleles at two different loci arise independently in the two different populations and are compatible with their natal populations. Yet, when brought back together due to hybridization, these alleles cause some form of fitness decrease (Dobzhansky 1937). This form of epistasis can arise via any model of speciation (drift or selection), but also most commonly describes postzygotic reproductive isolation. It is still unclear from the literature how general a role epistasis plays in prezygotic isolation. However, in few specific study systems, epistasis has been shown to play a role in the divergence of ecologically important traits that are involved in prezygotic reproductive isolation, such as immigrant inviability caused by salinity tolerance in killfish (Fuller 2008) or predator defense in sticklebacks (Hatfield 1997). At a minimum, this might mean that multiple loci are necessary for these ecological traits to diverge. Alternatively, it may be that the changes in one locus in one divergent population work well only in their natal genetic background and when brought back together with the alternative population, this locus changes its effect. This would be a form of D-M incompatibility, but for prezygotic traits under selection. More data on this subject is needed.

In contrast to the interpopulation analyses, intrapopulation analyses comparing parents and offspring for the heritability of a given trait of interest are also quite useful in understanding the process of speciation and the opportunity that a population can change in the future (Lynch and Walsh 1998). Heritabilities measure the proportion of phenotypic variation in a population that is generated by genetic variation among individuals and thus estimate the relative contributions of genetic and non-genetic (environmental) factors to total phenotypic variance (Falconer 1989). This is particularly interesting when combined with estimates of the strength of selection (S) to then get an estimate of a population's response to selection ($R = h^2 S$) (Falconer 1989, Lynch and Walsh 1998).

Historically, there has also been a general interest in the number of genes affecting a trait or phenotype under selection. The answer to this question has important implications for models of speciation, especially models of speciation with gene flow, where a few genes of major effect are a common assumption (Felsenstein 1981, Kondroshov and Kondroshov 1999, Dieckman and Doebeli 1999, Servedio and Noor, 2003). In addition to the geographic context, it can greatly influence the speed of ecological speciation (Hendry et al. 2007). From previous analyses (Egan, Janson, Brown, and Funk, unpublished manuscript), it appears that host-associated populations of the leaf beetle *Neochlamisus bebbianae* analyzed in the present study are consistent with the expectations of speciation with gene flow. Within this previous study of just a first generation of crosses in the lab (crosses within and between host-associated populations), analyses of host preference data, a surrogate for habitat isolation, demonstrated genetic variation for this trait and evidence for genes of major effect. Since habitat isolation is the first barrier chronologically affecting gene flow and also one of the strongest in *N. bebbianae* (Funk 1998, Funk et al. 2002, Funk and Egan, unpublished data), further analysis of the underlying genetic architecture of this trait and others involved in prezygotic isolation will aid in the understanding of the genetic basis for ecological speciation in *N. bebbianae*.

More generally, populations of herbivorous insects feeding on different host plants offer excellent systems to better understand the genetic basis of ecological divergence and reproductive isolation (Funk et al. 2002). Further, Coyne and Orr (2004) discuss the important distinction in what can be learned from post-speciation divergence versus that which occurred during the actual process of speciation. Herbivorous insect populations feeding on different plants can be partially differentiated, incompletely isolated, and produce interfertile crosses and thus offer a unique opportunity to understand genetic divergence during the actual process ecological speciation as these populations fall along the speciation continuum between panmixia and good species.

Neochlamisus bebbianae (Brown) [Coleoptera: Chrysomelidae] is an eastern North American species of leaf beetle that uses specific tree species from six taxonomically disparate genera as host plants and offers just such an opportunity. All life activities – from oviposition to adult feeding and mating – are conducted on the host plant (Karren 1972, Funk 1998). Populations associated with different host plants are partially differentiated in host preference and performance traits, and exhibit partial reproductive isolation as a consequence of divergent host adaptation (Funk 1998, 1999, Funk et al. 2002, Egan and Funk 2006, Funk and Nosil 2008). The present study evaluates *N. bebbianae* populations associated with red maple (*Acer rubrum*; Aceraceae) and Bebb's willow (*Salix bebbianae*; Salicaceae) from a sympatric locality in Caledonia County, Vermont.

This system allows me to address four interesting questions in regards to the genetic basis of ecological speciation using traditional quantitative genetic approaches: (1) To test how traits involved in ecological divergence and reproductive isolation are inherited (additively, dominance, epistasis), I used a line cross analysis (Lynch and Walsh 1998), (2) To estimate the number of loci that control these same traits, I used a Castle-Wright estimator (Lynch and Walsh 1998), (3) To test whether the host-use traits of host preference and host-associated performance covary via pleiotropy or tight linkage within the genome, I used a correlation approach (Saldamando et al. 2005), and lastly, (4) to estimate heritabilities (i.e., additive genetic variance) with in each host form, parent-offspring regressions were performed (Falconer 1989).

Materials and Methods

Field collections and lab husbandry

During the summer of 2007, eggs and young larvae of *N. bebbianae* were collected on Bebb's willow and red maple plants at a sympatric locality in Caledonia County, Vermont (44.4018° N, 71.9173° W). Animals from this collection site were brought back to the laboratory at Vanderbilt University and individually raised on cuttings of their native host plant within 5-cm Petri dishes lined with moistened filter paper and sealed within plastic shoeboxes lined with moistened paper towels. As individuals pupated, pupal cases were placed on dry filter paper in 5-cm Petri dishes and sealed within plastic shoeboxes. As adults emerged, they were maintained exactly as the larvae. These lab-raised adults originating from field collections were used to create reciprocal F_1 hybrids and pure offspring as a control. The offspring from these lab crosses were raised as mentioned above until adulthood, where these next generation adults were used to create eight reciprocal backcross types, F_2s, and additional pure crosses as a control.

Experimental crosses and oviposition

All crosses occurred between a single male and female in a 5-cm Petri dish lined with a moistened filter paper. If copulation was visually confirmed (by observation of aedeagal insertion into the female) within a 2-hr period of continuous observation, the pair was left together overnight to facilitate insemination. In unsuccessful pairings (i.e., no copulation occurred), the unmated female was removed and later paired with a male of the same type as its original partner. After copulation, females were individually housed within 30 cm x 15 cm mesh bags constructed of DelNet (DelStar Technologies, Inc., Middletown, DE) that were tied over the meristem of a sapling native plant. All saplings were native genotypes to northeastern North America and were grown within the Vanderbilt University greenhouse facility. Greenhouse conditions were set to: day temperature range = 21 – 24 °C, night temperature range = 18 – 21 °C, > 70 % relative humidity, and a 14:10 light:dark schedule to mirror summer conditions in Caledonia County, Vermont, USA. These conditions provided constant new leaf growth throughout the timing of this experiment for feeding and oviposition (September 2007 – May 2008).

Mated females from *generation 1* were placed on their native host plant for oviposition of the eggs that would make up *generation 2*. Due to the experimental design, *generation 2* offspring were split evenly and raised on each of the two host plants (see below). Thus, mated females from *generation 2* were placed on the plant from which they were raised upon for the oviposition of the eggs that would make up *generation 3*. Eggs from each generation were harvested weekly. All eggs were separated and maintained by family in 5-cm Petri dishes lined with dry filter paper that were stacked in plastic boxes lined with moist paper towels and sealed with a layer of plastic wrap and a plastic top. These boxes were kept in an incubator (24° C;14-10 light-dark) and eggs were checked daily for hatching larvae. Upon larval emergence, an individual’s weight was measured using a Mettler Toledo MX5 microbalance (Mettler Toledo, Inc., Columbus, OH) sensitive to 0.001 mg. After initial weight was measured, all hatchling larvae were assayed for feeding preferences and then went directly into performance assays (detailed below).

Host preference assays

Upon emergence from eggs, naïve individuals participated in sequential 24-hr no-choice feeding tests to measure host preference. Feeding tests occurred in 5-cm Petri dishes lined with moistened filter paper and provided with two leaf discs removed from foliage using a standard hole-punch tool. Each individual participated in two consecutive feeding trials, with each trial using discs from a different host plant (maple or willow). Order of test plant presentation across the two trials was systematically varied across replicates to avoid possible bias due to prior testing. Following each trial, total feeding damage on leaf discs was scored under a dissecting microscope fitted with an ocular with a 20 x 20 grid. The magnification was set so that one disc was the width of the grid and thus a disc represented 314 grid units. Data from larvae consuming less than five units of foliage was discarded to eliminate data from non-vigorous individuals.

Reciprocal rearing and performance assays

For each generation, larvae were maintained individually in 5-cm Petri dishes and were provided with a cutting of one of the two host plants. Individuals from *generation 1* that were harvested in the field as immatures were reared to adulthood on their native host plant. For *generation 2* & *3*, the test plant an individual from a given family received was systematically alternated as they hatched so that larvae from each family were evenly split between willow and maple rearing treatments, which were also equally distributed in time. Foliage and moistened filter papers were replaced every two days. Larval weight was measured on the day of emergence and at two weeks and was then converted into a *relative growth rate* (RGR), which was calculated as: $RGR = [((\ln wt_2) - (\ln wt_1)) / (t_2 – t_1)]$, where wt_1 and wt_2 were the larval weights at the beginning and end of the performance assay, and t_1 and t_2 were the times of the initial and final weight measurements. RGR represents the proportional increase in mass per unit time and adjusts for initial size and the nonlinear relationship between growth over time (Hunt 1982).

Parent – Offspring regression for intrapopulation analysis

The heritability of larval weight at hatch, host plant feeding preference, and host-associated growth rate was estimated independently within the willow and maple host forms of *N. bebbianae* with multiple approaches to a parent-offspring regression (Falconer 1989). A traditional parent-offspring regression of the average offspring value by family on the average value of the two parents (the midparent) was performed. However, this method can be influenced by maternal effects rather than just by genetic contributions (Falconer 1989, Keller et al. 2001). Subsequently, I attempted to control for host-associated maternal effects by conducting father-offspring regression (Falconer 1989, Lynch and Walsh, 1998). Because paternal effects are unlikely in general and would offer a very implausible explanation for variation in host preferences (Futuyma et al. 1993), this analysis can not only provide evidence for a genetic contribution per se, but also for the strength (e.g., heritability) of that contribution (Falconer 1989, Lynch and Walsh 1998). To estimate heritability from the midparent, the slope of the regression was used ($h^2 = b$); to estimate heritability from one parent, twice the slope of the regression was used ($h^2 = 2b$, Falconer 1989).

Line cross analysis of genetic effects for interpopulation comparisons

A line cross analysis was used to estimate the genetic basis of divergence in initial larval weight, host plant preference measured by feeding preference assays, and host plant performance measured by relative growth rate, which strongly influences survival. The analysis used the principle of the joint scaling test (Mathers and Jinks 1982, Lynch and Walsh 1998), which assigns coefficients of determination to each cross that describes the expected contribution from each genetic effect (Table 1). Genetic effects analyzed here are additive (α) and dominance (δ) effects, and the effects of the three possible digenic epistatic effects, additive x additive ($\alpha\alpha$), additive x dominance ($\alpha\delta$), and dominance x dominance ($\delta\delta$) (Lynch and Walsh 1998, Rundle and Whitlock 2001, Demuth and Wade 2007a, 2007b, Fitzpatrick 2008, Fuller 2008). For example, for the test of the additive effect, one parental population (maple) is assigned a coefficient of 1, the other population (willow) is assigned a -1, F1s and F2s are expected to be exactly intermediate and thus assigned a valued of 0, backcrosses to the maple population were assigned 0.5, and backcrosses to the willow population were assigned -0.5. In addition to the fixed genetic effects, I also tested for the effects of families (all offspring from a given female) within crosses which was treated as a random factor.

The full model, including all five fixed genetic effects and the random effect of family, was fit using a REML mixed-model ANOVA. Starting with a simple model, I first tested for a significant additive effect. Using the 'stepwise' regression feature within the 'Fit Model' window in JMP, I added the dominance and digenic epistatic effects and tested whether they significantly improved the fit of the model using a partial F-test (Zar 1996). Nonsignificant terms were dropped and the order of significant effects was varied to assure that it did not change the final model. The final model was chosen for each trait that included the minimum set of significant terms.

Estimation of the minimum number of genes

For the estimation of the effective number of genes or gene regions underlying the phenotypic variation between lines the estimator 'n_e' created by Castle (1921) was used. This assumes that: (1) all the genetic variation between the lines is additive, (2) the segregating factors studied are unlinked, and (3) all alleles have equal effects on the trait considered. The equations given by Lande (1981b, equations 4d and 8d) were used. In addition to the traditional approach of Castle (1921), I used a modification suggested by Zheng (1992) that takes into account linkage and variation in allelic effects.

This method has previously been used to estimate the number of genes underlying trait differences between closely related species of *Mimulus* (Fenster and Ritland 1994), Hawaiian *Drosophila* (Templeton 1977), and many other traits between closely related taxa (reviewed in Lynch and Walsh 1998). This method was originally proposed by Castle (1921). The formula is:

$$\mathbf{n_e = \underline{[(z_{Pmaple} - z_{Pwillow})^2 - var(z_{Pmaple}) - var(z_{Pwillow})]} \,/\, 8var(s}$$

where z_i is the trait mean for line i, var (z_i) is the variance of the trait mean for line i, and var(s) is the segregation variance. Wright's (1968) original equation for segregation variance, which I have used here, is:

$$\mathbf{var\ (s) = var(F_2) - \{[2var(F_1) + var(P_{maple}) + var(P_{maple})]/4\}}$$

where var(i) is the phenotypic variance among individuals in line i. A correction for family effects on line variances will be performed by pooling within-family variance estimates (Sokal and Rohlf 1995, Hatfield 1997).

Wright's estimate of gene number contains many assumptions, which will undoubtedly be violated in an analysis of a non-model organism in wild populations. These assumptions include a constant effects among loci and parental line means differing by ≥ 2 standard deviations of each lines mean (Zeng 1992). However, failure to meet assumptions of this method generally bias the estimate downward and thus this estimate approximates the minimum number of “effective factors” (Zeng 1992). This estimate should be taken as a first assessment of the number of loci underlying host-use traits in *N. bebbianae* to determine if these traits are controlled by many genes or very few and can be used to compare with other studies in which this method has been employed to estimate the number of genes underlying divergent traits between closely related taxa (Templeton 1977, Fenster and Ritland 1994), especially those suspected of ecological speciation (Hatfield 1997). In addition to the Castle-Wright estimations, there are tests that can be applied to the structure of this type of data to estimate the presence of genes of large effect.

Analysis of pleiotropy or tight linkage

Correlation analyses were carried out to test for pleiotropic effects or tight linkage between genes influencing weight at larval hatch, host plant preference, and host-associated performance (i.e., growth rate). This method is similar to one adopted by Butlin & Hewitt (1988) to test for similar effects on genes influencing multiple song traits and a related morphological character (peg number) (Butlin and Hewitt 1988). Parametric correlations were calculated for all pairs of characters within each individual (or family for relative growth rate). Individuals were divided in two groups: segregating (backcross and F2 hybrids) and nonsegregating (parental and F1 generations). The correlations obtained for each pair of characters were tested on the z-transformed values (Sokal and Rohlf 1995). Any correlation in the nonsegregating generation is most likely due to common environmental effects between the characters (or possibly genetic variation within populations) whereas correlations in the segregating generations include these environmental components plus genetic correlations produced by either pleiotropy or linkage between genes underlying the traits analyzed. Therefore, I tested for significant differences between the estimates of correlations obtained from the segregating and nonsegregating generations (Butlin and Hewitt 1988). A Bonferroni correction was applied to control for table wide Type I error (Sokal and Rohlf 1995).

Results

Parent – offspring regressions

Parent-offspring regressions revealed no significant heritability estimates for the three traits analyzed in this study. While clearly these traits are heritable in nature, it was the general lack of variation of a specific trait within each host form that generated these null results. For larval weight at hatch, analyses within the willow host form (midparent-offspring regression: h^2 = - 0.001 ± 0.002, $F_{1,12}$ = 0.17, $P > 0.50$; father-offpsring regression: h^2 = - 0.005 ± 0.006, $F_{1,12}$ = 0.37, $P > 0.10$) and maple host form (midparent-offspring regression: h^2 = 0.07 ± 0.11, $F_{1,16}$ = 0.29, $P > 0.50$; father-offpsring regression: h^2 = 0.11 ± 0.19, $F_{1,16}$ = 0.49, $P > 0.50$) showed no significant associations. For host plant preference, analyses within in the willow host form (midparent-offspring regression: h^2 = 0.004 ± 0.002, $F_{1,12}$ = 2.95, $P > 0.10$; father-offpsring regression: h^2 = - 0.14 ± 0.30, $F_{1,12}$ = 0.22, $P > 0.50$) and maple host form (midparent-offspring regression: h^2 = - 0.60 ± 0.95, $F_{1,16}$ = 0.40, $P > 0.50$; father-offpsring regression: h^2 = 0.83 ± 1.33, $F_{1,16}$ =

0.39, $P > 0.60$) showed no significant associations. For host associated performance (growth rate), analyses within the willow host form (midparent-offspring regression: $h^2 = -0.09 \pm 0.12$, $F_{1,12} = 0.88$, $P > 0.10$; father-offpsring regression: $h^2 = -0.08 \pm 0.17$, $F_{1,12} = 0.37$, $P > 0.50$) and maple host form (midparent-offspring regression: $h^2 = 0.07 \pm 0.10$, $F_{1,16} = 0.40$, $P > 0.50$; father-offpsring regression: $h^2 = 0.31 \pm 0.42$, $F_{1,16} = 0.84$, $P > 0.10$) showed no significant associations.

Line cross analysis of genetic effects

For hatching larval weight, a model including additive and $\alpha\delta$ epistasis was sufficient to explain the variation between the willow and maple host forms of *N. bebbianae* and their hybrids using the joint scaling approach (Table 4.2, Figure 4.1.A). The proportional effect of each term on hatchling larval weight was equivalent (Table 2). For feeding preference on maple, a complex model including additive, dominance, and two types of epistasis ($\alpha\delta$ and $\delta\delta$) were required to fully explain the variation (Table 4.2, Figure 4.1.B). The proportional effect of each term on maple feeding preference varied widely with the dominance term having the largest effect and the additive and $\alpha\delta$ epistasis terms having the smallest, but equivelant effect, while the $\delta\delta$ epistasis term had an intermediate effect (Table 4.2). For feeding preference on willow, a model including additive, dominance, and $\alpha\delta$ epistasis terms best explained the variation (Table 4.2, Figure 4.1.C). The proportional effect of each term on willow feeding preference also varied widely, but in a similar way to maple feeding preference with dominance term having the largest effect, the additive term having the smallest significant effect, and $\alpha\alpha$ epistasis term having an intermediate effect (Table 2). For growth rate, in general, models required fewer terms and the additive effect explained the majority of the variation (Table 2). For relative growth rate on maple, an additive and $\alpha\delta$ epistasis terms best explained the variation (Table 4.2, Figure 4.1.D). The proportional effect of the $\alpha\delta$ epistasis term was slightly larger than the additive term on the relative growth rate on maple (Table 4.2). For relative growth rate on willow, an additive model was sufficient to explain the variation between the willow and maple host forms of *N. bebbianae* and their hybrids (Table 4.2, Figure 4.1.E). Thus, no other genetic component significantly increased the explanatory power of the model.

Estimation of the minimum number of genes

The Castle-Wright and Zeng estimators suggest that each trait analyzed here is controlled by a few genes of large effect, which is consistent with previous results (Egan, Janson, Brown, and Funk, unpublished manuscript). However, two patterns did emerge from these results. First, it appears that there are more loci involved with feeding preferences (a behavioral trait) than with relative growth rate (a physiological trait) (Table 3). Second, it also appears that preference for and performance on maple involve more loci than these same host-use traits on willow (Table 3).

Analysis of pleiotropy or tight linkage

The relationship among individual traits and between segregating and nonsegregating generations within a specific trait comparison varied widely. Most notable were the five trait comparisons that generated significantly different correlations between the segregating and nonsegregating generations. This is evidence for genetic correlations due to pleiotropy or tight linkage. Weight at day 1 was strongly associated with host preference for maple and for willow (Table 4). Host preference for each host plant was strongly and positively associated with relative growth rate on that host plant (Table 4). Lastly, host preference for one host plant was strongly, but negatively associated with preference for the other host plant (Table 4). Relative growth rate on each host plant (by family) was also negatively associated, however did not remain significant after Bonferroni correction.

Discussion

Three interesting patterns emerged from the analysis of interpopulation genetic effects on hatchling weight, host plant preference, and host-associated performance. First, I found that each trait differed in the way it is inherited, but that all had strong additive components and most had epistatic components. Second, models of inheritance were more complex for maple-associated traits than willow-associated traits. Third, models of inheritance were more complex for feeding preference than they were for host-associated performance.

Epistatic effects were significant for several traits, but varied in their overall effect size from 0.10 to 0.56. Finding some epistasis is plausible given the possible complexity of behaviors such as feeding preferences and physiological adaptations to host plant consumption and digestion. Krahe et al. (2002) observed that in the cricket *C. biguttulus* that the behavioral trait of acoustic signaling involved the interaction of two or more auditory interneurons that seem to be decisive in coordinated functions of excitement and inhibition in the generation of sound. During divergence, sequential substitutions must not disrupt the complex control of such complex behaviors but they may not function well in an alternative genetic background, leading to epistatic effects in hybrids (similar to Dobzhansky-Muller interactions that underlie postzygotic isolation; Orr 1997). Hatfield (1997) also investigated the genetic architecture of traits associated with ecological adaptation between benthic and limnetic sticklebacks. He found epistatic effects for the traits involved in feeding (gill raker number and length) and predator defense (lateral plate number and pelvic spine length).

My correlation analysis suggests that there were some genetic associations. Most notable were the strong positive associations between preference for and performance on each host plant and the negative association between preference for each host plant (Table 4). Correlations between preference for and performance on a given host are assumed to be common among locally adapted insect populations, yet evidence is still building (Funk et al. 2002). The strong negative association between preference for willow and maple is consistent with genetic trade-offs (Fry 1996). Genetic trade-offs in preference, a surrogate for habitat isolation, is strong evidence that adaptation for preference to one environment entail reduce preference to the alternative enviroment through some type of antagonistic pleiotropy (Futuyma and Moreno 1988, Jaenike 1990, Fry 1996). This should increase reproductive isolation over an expectation under on genetic trade-offs where the response of one trait is independent of the response of another.

Although epistatic and dominance effects were detected (Table 2), Lynch and Walsh (1998) suggest caution in applying the Castle-Wright estimator in these circumstances. However, they argue that it can, nevertheless, provide a valuable initial guide to the genetic architecture of a trait. The estimation of the number of genes or gene regions that control divergence among the willow and maple host form of *N. bebbianae* suggests a low number of ‘factors’. Similar results of a few genes of large effect were found in previous transgenerational studies in *N. bebbianae* using the Fain’s test (Egan, Janson, Brown, and Funk, unpublished manuscript). QTL analyses of certain behavioral traits in *Drosophila* have also suggested a small number of loci with large effects (*D. melanogaster* - Gleason et al. 2002, *D. virilis* - Huttunen et al. 2004). In contrast, Hatfield (1997) found a wide range (1 – 50) in the number of loci affecting traits involved in feeding (gill raker number and length) and predator defense (lateral plate number and pelvic spine length) associated with ecological speciation in sticklebacks. It might be that behavioral and physiological traits have a more simple genetic basis than morphological traits. Interestingly, those traits that appeared more complex from tests for epistasis, dominance, and additivity were consistent with the results from the Caste-Wright estimator, which showed slightly more loci influencing those traits.

Inferences from the effective number of factors measured in this study should be treated with caution as the estimators used tend to underestimate the true number of genes (Zeng 1992, Shaw and Parsons 2002). Simulations suggest that this bias is not a major problem where the true number of loci is small, as in this case (Otto and Jones 2000). The strong additive effects on most traits and the suggestion that genes of large effect are present provide a strong platform from which to undertake further work using QTL approaches, for which AFLP markers are also available (Egan et al. 2008). The challenge will be to attain better survival in the F2 and backcross generation, so that sample sizes can be increased to the numbers required for such investigations.

The number of genes underlying differentiation may be influenced by the types or strengths of selection that the traits compared have experienced. Butlin (1996) considered that the small number of substitutions inferred to underlie song divergence in *Nilaparvata lugens* was still consistent with drift given the broad acceptance range displayed by females for the trait in question, pulse repetition frequency. However, in the case of the two closely related host form of *N. bebbianae* analyzed here, previous studies have sought to isolate the role of host plant adaptation and control for genetic drift (Funk 1998, Funk et al. 2002). By comparing allopatric populations on the same host plant versus those on alternative host plants, these studies showed that the majority of ecological divergence and reproductive isolation was associated with host plant use and not drift and non-host-associated selection (i.e., abiotic factors). Given the background in this system, I believe the loci found in this analysis to be associated with host-associated selection.

One initial interest of this study was to investigate whether there was sex-linkage for any of the traits involved with host use. Given the design required for a line cross analysis, the sex of the F2 and backcross hybrids must be determined (Lynch and Walsh 1998). However, the generation of F2 and backcross hybrids did not survive in sufficient numbers to analyze adult data for each cross. This is regrettable, since other studies among insects have found evidence for sex linkage. For example, in the Hawaiian crickets *Laupala paranigra* and *L. kahalensis* sex-linkage was observed for puse rate in backcross generations (Shaw 1996) and in *Drosophila pseudoobscura* and *D. persimilis* eight genes that contral interpulse intervals in communication were found on the X chromosome (Williams et al. 2001).

In contrast to the interpopulation analyses using data from the experimental crosses, the intrapopulation (within host form) analysis comparing parents and offspring for the heritability of larval weight at hatching, host plant preference, and host associated performance did not find evidence of a genetic basis. This was due to the low amount of variation in each trait expressed within each host form, which is common in studies addressing hertibility (Lynch and Walsh 1998). In fact, it actually suggests that the other analyses (line cross and Castle-Wright estimator) were the optimal analyses since they took advantage of the between host form variation to address similar topics of genetic architecture.

It is possible that as populations originally diverged in host use, divergent natural selection in each environment used up all the within population variation in among the traits analyzed here. Alternatively, there was some aspect specific to context of original divergence that expressed very strong stabilizing selection on these traits, possibly due to reinforcing selection. Specific knowledge of the geographic context of the origin of divergence among host forms of *N. bebbianae* are unknown, but strong selection against variation within host forms during divergence might be more critical under scenarios of geographic contact where gene flow was occurring. Lastly, while this lack of variation within host forms is not surprising, other studies of these *N. bebbianae* host form have found slightly higher intrapopulation variation (i.e., Funk 1998, Egan and Funk 2006). This raises the possibility that while it is likely that the populations studied here truly do have lower amounts of variation, it is possible that the quality of the experiments, such as the quality of the host plants used in the experiments might have biased the results. For example, if the alternative host plant was stressed and of lower quality or the native host plant was of higher quality relative other individuals available, this would generate false positives that were not associated with the factors of interest.

Overall, there appears to be a simple genetic architecture with a small number of genes interacting. This is consistent with the possibility that these taxa could diverge while undergoing gene flow based on models (Kondroshov and Kondroshov 1999, Dieckmann and Doebeli 1999).

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Table 4.1. Expected contributions of additive and non-additive genetic effects to generation means (after Mather and Jinks 1982, Lair et al. 1997, Demuth and Wade 2007a). Effect symbols: μ=cross mean (intercept); α=additive component; δ=dominance component; $\alpha\alpha$=additive x additive epistasis; $\alpha\delta$=additive x dominance epistasis; $\delta\delta$=dominance x dominance epistasis. Generation notation: P_W = parental willow generation (pure willow x pure willow); BC_W = willow backcross hybrid (F$_1$ hybrid x pure willow); F_1 = first generation hybrid (willow x maple); F_2 = second generation hybrid (F_1 hybrid x F_1 hybrid); BC_M = maple backcross hybrid (F_1 hybrid x pure maple); P_M = parental maple generation (pure maple x pure maple).

Generation	Contribution to generation mean	μ	α	δ	$\alpha\alpha$	$\alpha\delta$	$\delta\delta$
P_W	$\mu+\alpha+\alpha\alpha$	1	1	0	1	0	0
BC_W	$\mu+½\alpha+½\delta+¼\alpha\alpha+¼\alpha\delta+¼\delta\delta$	1	0.5	0.5	0.25	0.25	0.25
F_1	$\mu+\delta+\delta\delta$	1	0	1	0	0	1
F_2	$\mu+½\delta+¼\delta\delta$	1	0	0.5	0	0	0.25
BC_M	$\mu-½\alpha+½\delta+¼\alpha\alpha-¼\alpha\delta+¼\delta\delta$	1	-0.5	0.5	0.25	-0.25	0.25
P_M	$\mu-\alpha+\alpha\alpha$	1	-1	0	1	0	0

Table 4.2. Results of linear mixed model analyses on genetic effects (i.e., line cross analysis). Only significant terms from the REML linear mixed effects model are shown. Proportional effect represents the proportion of the total effects that are due to each specific type of effect (i.e., absolute value of additive effect/sum of absolute values for all genetic effects (following Demuth and Wade 2007a).

	Effects					
Trait	μ	α	δ	$\alpha\alpha$	$\alpha\delta$	$\delta\delta$
Hatching larval weight	0.3073	-0.045			-0.044	
F-ratio	99.71	128.5			10.10	
P-value	<0.0001	<0.0001			0.0015	
Proportional effect		0.51			0.49	
Feeding preference for maple	-2.83	-7.2509	37.7196		-7.8116	-21.05
F-ratio	0.2476	84.7489	9.0778		8.5736	8.4661
P-value	0.6188	<0.0001	0.0026		0.0035	0.0037
Proportional effect		0.10	0.51		0.10	0.29
Feeding preference for willow	16.36	3.298	-18.599	-9.3378		
F-ratio	27.13	56.41	7.222	9.011		
P-value	<0.0001	<0.0001	0.0073	0.0027		
Proportional effect		0.10	0.60	0.30		
Growth rate on maple	0.0755	-0.076			0.095	
F-ratio	4.381	88.2			22.45	
P-value	0.0371	<0.0001			<0.0001	
Proportional effect		0.44			0.56	
Growth rate on willow	0.1157	0.0467				
F-ratio	22.55	218.2				
P-value	<0.0001	<0.0001				
Proportional effect		1.0				

Table 4.3. Effective number of factors (n_e) contributing to divergence in traits between sympatric willow and maple host forms of *N. bebbianae*. (C-W = Castle-Wright method; Z = Zheng method)

Trait	Estimation method	n_e ±SE
Hatchling weight	C-W	2.38 ±1.07
	Z	2.94 ±0.96
Feeding preference on maple	C-W	3.89 ±1.17
	Z	4.21 ±1.86
Feeding preference on willow	C-W	1.52 ±0.66
	Z	2.79 ±0.74
Relative growth rate on maple	C-W	1.69 ±1.71
	Z	2.11 ±2.79
Relative growth rate on willow	C-W	0.92 ±0.55
	Z	1.57 ±0.86

Table 4.4. Correlations among z-transformed trait values within the segregating (backcross and F_2 hybrids) and nonsegregating (parentals and F_1 hybrids) generations and a test for differences between correlations of the segregating and nonsegregating generations. Bold P-values with asteriks denote significance after Bonferroni correction. (Gen = Generations; Seg = Segregating, NonSeg = nonsegregating; WT_1 = larval weight on day 1; HP_W = host preference on willow; HP_M = host preference on maple; RGR_W = relative growth rate on willow; RGR_M = relative growth rate on maple)

Gen	Trait comparison	N	r	P	difference ($r_n – r_s$)	Z	P
NonSeg	WT_1 – HP_W	464	-0.15	0.0011	- 0.2524	- 4.29	**<0.0001***
Seg		753	0.10	0.0055			
NonSeg	WT_1 – HP_M	465	0.54	<0.0001	0.2730	5.59	**<0.0001***
Seg		745	0.27	<0.0001			
NonSeg	WT_1 – RGR_W	163	-0.55	<0.0001	0.0818	1.25	0.1065
Seg		266	-0.63	<0.0001			
NonSeg	WT_1 – RGR_M	132	-0.10	0.2702	- 0.0171	- 0.15	0.4387
Seg		215	-0.08	0.2453			
NonSeg	HP_W – HP_M	463	-0.16	0.0006	- 0.2175	- 3.69	**0.0001***
Seg		743	0.06	0.1145			
NonSeg	HP_W – RGR_W	162	0.30	0.0001	0.3129	3.19	**0.0007***
Seg		256	-0.01	0.8854			
NonSeg	HP_W – RGR_M	125	-0.32	0.0002	- 0.1720	- 1.61	0.0541
Seg		213	-0.15	0.0267			
NonSeg	HP_M – RGR_M	162	-0.44	<0.0001	0.2706	2.98	**0.0014***
Seg		255	-0.17	0.0054			
NonSeg	HP_M – RGR_M	124	0.26	0.0042	0.0896	0.82	0.2055
Seg		213	0.17	0.0153			
NonSeg	RGR_W – RGR_M	20	-0.59	0.0065	- 0.5626	- 2.18	0.0145
Seg		37	-0.02	0.8865			

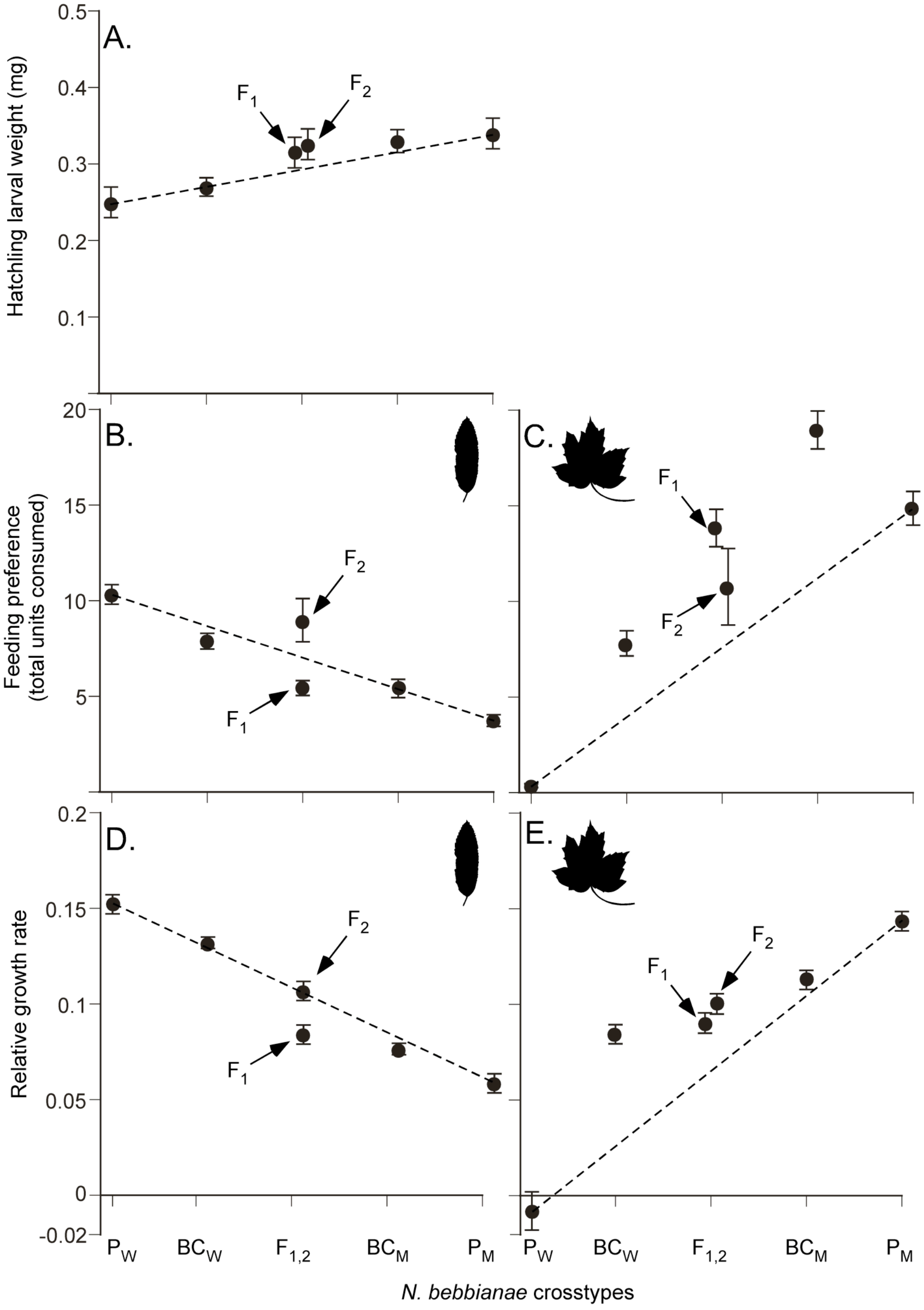

Figure 4.1. Least square means (± SE) of (A) hatchling larval weight, (B & C) feeding preferences on willow and maple, respectively, and (D & E) relative growth rates on willow and maple, respectively, by the different crosstypes of *N. bebbianae*. Dashed line represents expectations under a purely additive model of inheritance. F_1 and F_2 hybrids fall along the same point on the relatedness scale and are moved left or right if mean or standard errors overlap. (P_W – pure cross from willow host form; BC_W – backcross to willow host form; BC_M – backcross to maple host form; P_M – pure cross from maple host form)

CHAPTER V

CONCLUSIONS

Ecological speciation is defined as the process by which reproductive isolation arises between populations as a result of ecologically based divergent natural selection between environments (Schluter 2000, 2001). Selection is ecological when individual survival and reproduction are dependent on interactions with its environment. Selection is divergent when it works in contrasting directions on specific traits between populations or individuals. In this dissertation, I used populations of the leaf beetle *Neochlamisus bebbianae* associated with Bebb's willow (*Salix bebbianae*) and red maple (*Acer rubrum*) that are currently undergoing ecologically-driven divergence and speciation to address specific questions about the role that ecology plays in generating reproductive isolation. There were two main aspects of this work: (a.) understanding the genetic architecture of traits associated with ecological adaptation to different hosts that contribute to reproductive isolation and (b.) examining the role of postmating isolation that has evolved between these ecologically differentiated populations. In doing so, I was able to uncover and contrast the genetic basis for host use on each plant and the role of different postmating barriers that arise during the speciation process.

This work fits within a greater framework of the study of ecological speciation that I pursued during my dissertation work. The study of ecological speciation is best understood through the pursuit of three complementary themes: ecological divergence, reproductive isolation, and genetic differentiation (Figure 5.1, Funk 2009). Each of these components can have influences on the two others. For example, the genetic basis of a trait under divergent ecological selection can influence the chance that it can diverge under some level of gene flow (see Chapter 4 for details) and generate reproductive isolation under geographic contact. I have addressed aspects of each of these components during my work at Vanderbilt University to better understand the entire process of ecological speciation.

Ecological divergence

The study of ecological divergence can address the pressures that might drive divergence between populations or the response of those populations to divergent selection. In addition to the work I have described in Chapter 2 and 3 on measures of ecological divergence (feeding and habitat preferences; host-associated growth rates), I addressed a putative selection pressure that might drive *N. bebbianae* to specialize onto willow and maple. This addressed the information-processing hypothesis (IPH), which posits that specialist herbivores should make host-associated decisions more effectively than generalists and thus enjoy associated fitness advantages that may help explain the evolutionary prevalence of host-specific insects (Egan & Funk 2006). This is because generalists must evaluate a greater diversity of host plants/cues than specialists and thus face a cognitive challenge that is predicted to constrain the efficiency and accuracy of their choices. Potential selective advantages to ecological generalism are easily imagined. For example, generalists will often have access to a greater resource base, a more nutritionally balanced diet, and a greater capacity to confront variable environments than ecological specialists (e.g. Bernays & Minkenberg 1997, Bernays et al. 1994, Funk & Bernays 2001, respectively). Nonetheless, in the most species-rich animal taxa—the herbivorous insects—most species are quite ecologically specialized, using host plants from a single plant family, genus, or even species, while ignoring most locally available plant taxa (Futuyma & Moreno 1988, Jaenike 1990, Bernays & Chapman 1994). The phylogenetic finding that transitions from herbivore generalism to specialization occur at higher rates than the alternative (Nosil 2002) offers a historical accounting for this pattern and underscores long-standing interest in its evolutionary causes. Many hypotheses have been offered to explain the somewhat counterintuitive prevalence of herbivore specialization as an evolved ecological strategy (Jaenike 1990). One hypothesis that has only recently come under empirical test despite the age and esteem of its conceptual origins (Levins & MacArthur 1969) is the ‘neural constraints’ or ‘information-processing’ hypothesis (IPH, Bernays & Wcislo 1994, Bernays 2001). This hypothesis is based on two assumptions that generate a specific prediction. The first assumption is that constraints exist on the amount of information that can be processed and the number and accuracy of decisions that can be made in a given period of time. The second assumption is that generalist herbivores encounter more host-associated information/decisions than specialists.

To test this hypothesis, I experimentally quantified the specificity, efficiency, and accuracy of host selection, as both larvae and adults, for many individuals representing each of three ‘host forms’ of *N. bebbianae* leaf beetles. These experiments provided several significant findings: (1) host forms differed in larval specificity, (2) the more specialized host forms more efficiently and accurately their native host as both larvae and adults, and (3) using individual level data, positive correlations between larval specificity and both efficiency and accuracy across test individuals provided the most direct evidence to date for a biological association between these variables. My results thus provided strong and consistent support for the IPH at the level of both populations and individuals. Because individual *N. bebbianae* make many host-associated decisions in nature, my results suggest that cognitive constraints may play a major role in the evolutionary dynamics of ongoing ecological specialization and diversification in this species.

In addition to the pressure to specialize on a specific host plant species due to the cognitive constraints of generalism, there is also the possibility of trade-offs in fitness on each host plant as a population becomes more specialized (i.e., Agrawal 2000). Evidence in support of these types of trade-offs did not come from trade-offs in performance (i.e., growth rate, RGR) as has been traditionally shown, but from trade-offs in feeding preferences (Chapter 4). I would not categorize this as a fitness trade-off per se, but it could be that an adaptation for a behavioral preference for host plant 1 comes with a negative feedback to preferences to host plant 2 (Forbes et al. 2005), which itself may decrease gene flow between populations inhabiting each host plant and allow for greater divergence between them.

Reproductive isolation

Divergent ecological adaptation has lead to the evolution of multiple premating barriers among *N. bebbianae* host forms: habitat isolation, immigrant inviability, and sexual isolation (Funk 1998, Funk et al. 2002, Egan & Funk 2006, Funk & Nosil 2008). Premating reproductive isolation has been more thoroughly studied than postmating isolation even though postmating isolation could play an important role (see review, Rundle & Nosil 2005). For the modest number of studies that have addressed some aspect of postmating isolation among ecological divergent taxa, data suggest that it can play an important role in promoting speciation during ecological divergence (Hatfield & Schluter 1999, Via et al. 2000, Pappers et al. 2002, Rundle 2002, Vamosi & Schluter 2002, Forister 2005, Nosil & Crespi 2006, Craig et al. 2007). In Chapter 2 and 3 and in previous work (Egan, Janson, Brown, and Funk *unpublished manuscript*), I focused on the role that postmating isolation plays in reproductive isolation among maple and willow host forms of *N. bebbianae*. This work showed that decreased fecundity from hybrid crosses and reduced hybrid offspring fitness both appear to play an important role in reproductive isolation among these ecologically divergent taxa. Cryptic barriers, such as reduced female fecundity, can have an important impact on overall reproductive isolation, yet have only recently been shown to be associated with ecological divergence (Nosil & Crespi 2006). Furthermore, females from hybrid matings were less likely to oviposit than those from pure matings (Egan, Janson, Brown, and Funk, unpublished manuscript). One possible explanation for the existence and strength of this barrier could be mechanical isolation due to an interaction between size difference between maple and willow host forms, where the maple host form is slightly larger (Adams & Funk 1997, Funk, unpublished data), and the sexual dimorphism exhibited by all *Neochlamisus*, where females are slightly larger (Karren 1972, Adams & Funk 1997). The cross type with the least success, maple females x willow males, was the larger sex from the larger host form with the smaller sex from the smaller host form. The specific underlying reason for this pattern, such as whether mechanical isolation plays an important role here, will require additional manipulative experiments to understand.

As populations adapt to different environments and reproductive isolation builds, hybrids between populations may suffer reductions in fitness due to intrinsic genetic incompatibilities that can evolve as a consequence of a number of mechanisms, including drift (Dobzhansky 1937), or due to extrinsic ecological incompatibilities that most likely evolve as a consequence of divergent natural selection (e.g., Hatfield & Schluter 1999, Rundle 2002). For those *N. bebbianae* females that did oviposit (regardless of the origin of the male partner), there were no differences in egg number, oviposition rate, or egg hatch rates (Egan, Janson, Brown, and Funk, unpublished manuscript). This suggests that intrinsic postmating incompatibilities between *N. bebbianae* host forms did not manifest at the earliest developmental stages, even though this type of barrier has been shown to act early in development in other ecologically divergent taxa (Lu & Bernatchez 1998) and illustrates it possible influence here.

Measures of hybrid fitness in each parental environment, via relative host-associated growth rate and survival, did document a pattern of reduced hybrid fitness between these divergently adapted populations. Previous studies have argued that if hybrids can perform equally well to parents in some environment, either in the lab (Hatfield & Schluter 1999) or one of the two parental environments (Forister 2005), but exhibit reduced fitness relative to a parent in another habitat, then extrinsic postmating isolation has been shown. Thus, hybrid and pure *Mitoura* butterflies that grew and survived equally well on one host plant but not on the other (Forister 2005) or hybrid *Eurosta* gall flies performed equally well as the pure host race on some more 'benign' genotypes of each host plant, but much less on others (Craig et al. 2007) were consistent with extrinsic isolation. A somewhat parallel example can be demonstrated in *N. bebbianae* as all F_1 hybrid and pure cross types survived equally well on willow, while not on maple (Egan, Janson, Brown, and Funk, unpublished manuscript), demonstrating an influence of the environment.

As highlighted and described in Chapter 2, these differences are the result of extrinsic incompatibilities due to an ecological mismatch between hybrid phenotype and environment and thus the direct results of ecological divergence of their parents (Hatfield & Schluter 1999, Rundle & Whitlock 2001). Using the backcross hybrids suggested by Rundle & Whitlock (2001), we addressed the criticism of studies that only observe decreased fitness in F1 hybrids that could be explained by genetic incompatibilities expressed in the harsh conditions in nature (Rundle & Whitlock 2001). In Chapter 2, I also extended the backcross approach by incorporating family-level effects and maternal rearing environment in our ANOVA models (Egan & Funk 2009). These allowed me the ability to evaluate genetic variation and potential non-genetic maternal influences on my results, respectively.

Most importantly, this investigation provides strong statistical documentation of genetically based, ecologically dependent postmating isolation (i.e., extrinsic isolation) between the ecologically divergent maple- and willow-associated host forms of *N. bebbianae* leaf beetles. That is, we demonstrated that the relative fitnesses of the two reciprocal backcrosses switch across host environments (Chapter 2, Figure 2.3). Specifically, the ‘maple-like’ backcross type (having a pure maple-associated parent) grew >45% faster on maple than on willow, whereas the ‘willow-like’ backcross type grew >70% faster on willow than on maple. Such patterns cannot be sufficiently explained by intrinsic factors (Rundle & Whitlock 2001). These results further corroborate accumulating evidence that the willow and maple host forms of *N. bebbianae* are undergoing ecological speciation.

Maternal effects and reproductive isolation

Interestingly, the reduction in hybrid fitness in growth rate and to some degree survival, is not as strong if the hybrid is in its maternal environment among the F_1 hybrid generations (Egan, Janson, Brown, and Funk, unpublished manuscript). In fact, on average, fitness as measured by growth rate is 3.5 times greater if a hybrid finds itself in the maternal environment versus the paternal environment. The expression of this pattern in nature would be that the offspring of females colonizing the alternative host, mating with the resident males, and staying to oviposit would suffer harsher consequences than males colonizing the alternative host, mating with resident females, who would then remain on their native host to oviposit. This should manifest as asymmetric male-biased gene flow between the host forms. This ecological pattern is very interesting, given a recent study of genetic variation in these same *N. bebbianae* host forms that is also consistent with male-biased dispersal and introgression (Funk, Egan, & Nosil, unpublished data). Interestingly, this is not a pattern seen in all herbivorous insect host races. Hybrid offspring showed no differences in survival on the maternal and paternal host in the water lily leaf beetle (Pappers et al. 2002) and in *Mitoura* butterflies (Forister 2005), although in the latter example the trends were in the direction of better growth and survival on the maternal host. This suggests that sex-linked (Prowell 1998, Janz 1998) or nongenetic maternal effects (Mousseau & Fox 1998) may play a larger role in the speciation process of *N. bebbianae* than in other herbivorous insects systems. Strangely, these maternal effects completely disappear in the next generation of hybrids (F_2 and backcross hybrids; Chapter 2)

However, the clearest aspect of maternal effects on offspring that contributes to reproductive isolation comes from the mismatch of the maternal egg case to the hybrid larvae (Chapter 3). Because it is a protective structure created by the mother and passed along to her offspring, it is an excellent example of this somewhat abstract phenomenon. Given that *N. bebbianae* is one species within the case bearing group, the Camptosomata, it is possible that these type of maternal effects play an important role in reproductive isolation between closely related taxa.

Continued opportunity for gene flow

When combining the current studies of postmating barriers with previous studies of premating barriers (Funk 1998, Funk et al. 2002), I do not believe that the combination of premating and postmating barriers has produced complete reproductive isolation. Previously collected AFLP genotype data and mtDNA sequence data both suggest either there is continuing gene flow between these *N. bebbianae* host forms or that the host shift occurred very recently (Funk 1999, Egan et al. 2008, Funk et al., unpublished data). My data suggests that if hybrid matings do occur, it is possible for the hybrid offspring to grow and survive in each habitat, especially in the maternal habitat. Moreover, a small proportion of *N. bebbianae* hybrids from each direction of the cross did attain growth rates similar to those attained by pure crosses on their native host. If reproductive isolation is not complete, then this opens the opportunity for reinforcement to act on population experiencing gene flow, given that hybrids are less fit than offspring from pure matings.

Genetic differentiation during ecological speciation

Ever since Darwin (1859), the study of natural selection has been a primary focus of evolutionary biology (Fisher 1930, Mayr 1942, Endler 1986, Kingsolver et al. 2001). Investigations of selection include attempts to demonstrate its occurrence, identify the various roles it plays, and evaluate its relative importance as a cause of biological differentiation.

To complement the quantitative genetic studies in Chapter 2 (the isolated effect of comparing backcrosses isolates the role of the additive x environment genetic effect) and Chapter 4 (using line crosses and Castle-Wright estimations), I also addressed questions of the genetic basis of ecological divergence and speciation through the use of a comparative genome scan. The purpose of using this method was to evaluate the contributions of host plant related divergent selection to the genome wide patterns of genetic differentiation during ecological speciation between maple- and willow-associated populations of *Neochlamisus bebbianae* leaf beetles (Egan et al. 2008). For each of 15 pairwise population comparisons, we identified “outlier loci” whose strong differentiation putatively reflects divergent selection. Of 447 AFLP loci, 15% were outliers across multiple population comparisons, and low linkage disequilibrium indicated that these outliers derived from multiple regions of the genome. Outliers were further classified as “host-specific” if repeatedly observed in “different-host” population comparisons but never in “same-host” comparisons. Outliers exhibiting the opposite pattern were analogously classified as “host-independent.” Host-specific outliers represented 5% of all loci and were more frequent than host-independent outliers, thus revealing a large role for host-adaptation in population genomic differentiation. Evidence that host-related selection can promote divergence despite gene flow was provided by population trees. These were structured by host-association when datasets included host-specific outliers, but not when based on neutral loci, which united sympatric populations. Lastly, three host-specific outliers were highly differentiated in all nine different-host comparisons. Because host-adaptation promotes reproductive isolation in these beetles, these loci provide promising candidate gene regions for future molecular studies of ecological speciation. [It should be noted that this work was done in parallel in another study system that generated somewhat different, but equally interesting results (Nosil, Egan, & Funk 2008).]

Summary

Overall, postmating barriers do play a role in reproductive isolation between these partially isolated, divergently adapted host forms of *N. bebbianae*, but the strength of these barriers varies and some depend on the ecological context. On average, hybrid offspring between the maple and willow host forms display slower growth rates on each host relative to the native parent. These differences contribute to a strong role of reduced hybrid fitness in the reproductive isolation between them (Chapter 2). Furthermore, I demonstrate a genetic basis to many of the traits involved in reproductive isolation, including host preference (Chapter 2 and 4). Host preference, a surrogate for habitat isolation, is a strong isolating mechanism in herbivorous insects (e.g., Feder et al. 1994) because the host plant is commonly the location of mating (Funk & Nosil 2008) and this barrier acts early in chronology of individuals barriers (Ramsey et al. 2003). Lastly, I have shown some novel aspects of reproductive isolation that have not been clearly addressed in the literature (Coyne & Orr 2004), yet appear to play important roles between the ecologically divergent populations investigated here and appear to be general factors among many ecologically divergent pair of populations. Overall, the ability to address the general questions of how species form and what role ecology play during this process have been aided by addressing each of the aspects of this process (ecological divergence, reproductive isolation, and genetic divergence; Figure 5.1; Funk 2009) and what I come away with is a better understanding of the complexities of the speciation.

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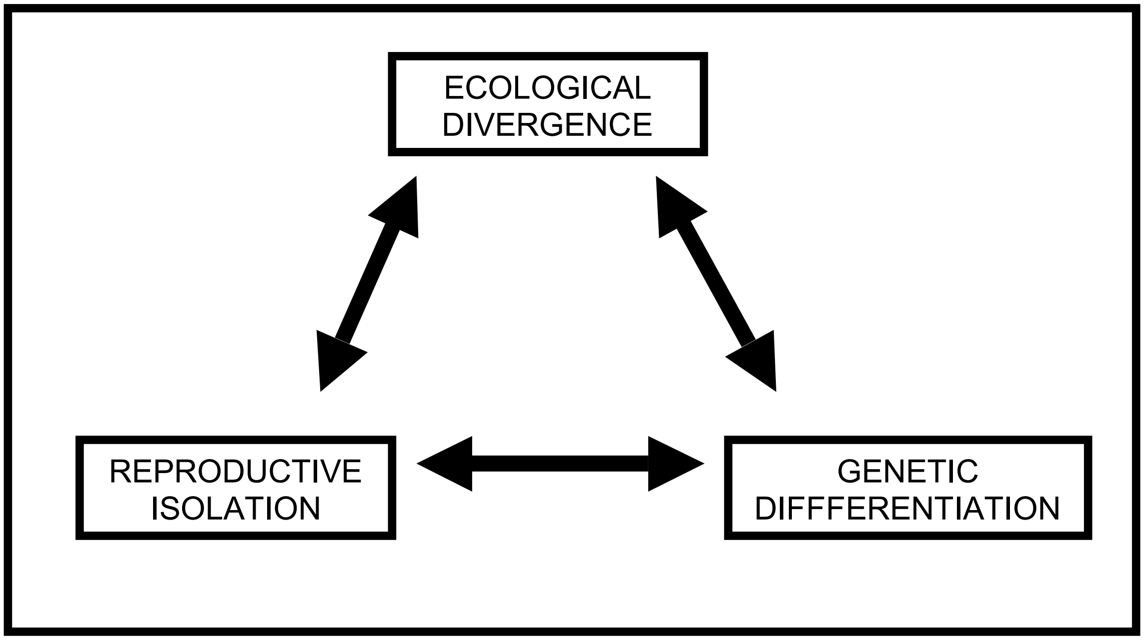

Figure 5.1. Diagram depicting the three interacting themes of the study of 'ecological speciation'. The bidirectional arrows are drawn to emphasize that changes in one aspect of the ecological speciation process reciprocally effect other aspects of the process (Funk 2009).