DIVERGENT HOST-PLANT ADAPTATION AND THE EVOLUTION OF REPRODUCTIVE ISOLATION

by

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ABSTRACT

Understanding the process of speciation requires elucidating the processes driving and constraining the evolution of reproductive isolation. For example, reproductive isolation can evolve simply as a by-product of populations adapting to different ecological environments. This process of 'ecological speciation' predicts greater levels of reproductive isolation between ecologically-divergent pairs of populations than between ecologically-similar pairs of similar age. The evolution of reproductive isolation can also be promoted by selection against hybrids (reinforcement) and can be constrained by the homogenizing effects of gene flow. This thesis examines the role of selection and gene flow in the evolution of reproductive isolation among host-associated populations of *Timema cristinae* walking-stick insects. Populations living on different host-plant species (*Ceanothus* versus *Adenostoma*) exhibit genetically-based, adaptive divergence in a suite of traits, including color, color-pattern, body size, body shape and behavior. Multiple forms of reproductive isolation were greater between populations using different hosts than between similar-aged populations using the same host. This pattern was detected for habitat isolation, immigrant inviability, sexual isolation, and cryptic postmating isolation, indicating that divergent host-plant adaptation promoted the evolution of multiple reproductive barriers. Conversely, gene flow between populations tended to erode divergence, with the exception of sexual isolation where moderate levels of gene flow promoted reinforcement. Molecular and morphological evidence suggest that the host-associated forms of *T. cristinae* are unlikely to have achieved species status such that the host forms represent either an ongoing speciation event or population divergence that has reached equilibrium. Studies of more divergent taxa in the genus are required to build up a more complete understanding of how the process of speciation unfolds, from beginning to end.

Keywords: speciation; natural selection; insects; gene flow; reinforcement

DEDICATION

This thesis is dedicated to Tom Reimchen for introducing me to evolutionary biology and for his constant support, to my parents and sister for reasons that should be obvious to them but probably are not due to my own shortcomings, and to my best friend Aspen.

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Mike Vankoeveringe, Eric Lopez, Moses Potter, Mei Zheng, Cori Paul, Kevin Johnson, Jared David, Ilona Naujokaitis-Lewis, Noah Spiegel, April Brown, and Heath Brackett. Funding was provided by the Natural Sciences and Engineering Council of Canada and the Entomological Society of Canada. I thank my numerous other friends, and apologize profusely for anyone whom I have forgotten. Thank you all.

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CHAPTER 1.
GENERAL INTRODUCTION –
THE ECOLOGY OF SPECIATION

Evolutionary processes result in two major outcomes: the evolution of adaptive traits within existing species and the formation of new species via speciation. Although adaptation proceeds by natural selection, speciation may or may not involve a role for selection and many different types of selection can be involved (Coyne and Orr 2004; Gavrilets 2004). Speciation driven by natural selection is of interest to biologists, because it provides a bridge between the study of adaptation within species and the study of macroevolutionary diversification. Interest in the role of selection in speciation dates back to Darwin (1859) and to the modern synthesis (Mayr 1947, 1963; Dobzhansky 1951), but has received renewed interest in the last decade (Funk 1998; Schluter 2000; Gavrilets 2004). This resurgence has been accompanied by a reclassification of models of speciation from geographic to process-oriented ones, although the two are certainly not independent. Thus there are now numerous books on the topic of speciation, all of which to some extent consider the role of divergent adaptation in speciation (Darwin 1859; Endler 1978; Otte and Endler 1989; Howard and Berlocher 1998; Schluter 2000, Schilthuizen 2001; Coyne and Orr 2004; Gavrilets 2004; Dieckmann et al. 2004).

1.1 Darwin and Speciation

Contrary to what is often stated, I believe that thinking about the role of selection in speciation dates back to Charles Darwin. In the Origin of Species (1859), Darwin visualized a role for natural selection in population divergence and speciation "the principle of benefit derived from divergence of character ... will generally lead to the most divergent variations ... being preserved and accumulated by natural selection ... until a sufficient amount of variation has been accumulated to form it into a well-marked variety" (pp. 117). Moreover, Darwin discussed how such divergence could be driven by disruptive selection such that "the most distinct varieties ... have the best chance of succeeding" (pp. 155). As a naturalist, Darwin knew that species could be recognized and demarcated. He did not think that species lacked boundaries, stating that "species come to be tolerably well-defined objects, and do not ... present an inextricable chaos of varying and intermediate links" (pp. 177). Instead, he preferred to view all evolution, including speciation, to arise from divergence of character in response to selection. Species were delimited from varieties, but only with respect to the magnitude of divergence, "these

forms may still be only...varieties; but we have only to suppose the steps of modification to be more numerous or greater in amount, to convert these forms into species...thus ... species are multiplied" (pp. 122). In fact the only figure in the Origin of Species is dedicated to illustrating this process of divergence and speciation via natural selection. However, Darwin was not at all explicit about the *types of changes* that were involved in speciation – in essence, adaptive divergence and speciation was seen as the same phenomenon.

1.2 Modern Synthesis

The modern evolutionary synthesis of the mid 20th century saw the emergence of the biological species concept and the idea that reproductive isolation was essential to, and a defining characteristic of, speciation (Dobzhansky 1940; Mayr 1947, 1963). Understanding speciation thus requires determining which reproductive barriers initially reduced gene flow between populations and the evolutionary forces producing them (Mayr 1963; Coyne and Orr 2004). Throughout my thesis, reproductive isolation is defined simply as barriers to gene exchange and gene establishment between diverging populations. Thus speciation can be viewed as a very continuous process, whereby populations begin as randomly mating units, progress through intermediate stages of speciation where reductions in gene flow occur and partial but incomplete reproductive isolation exists, and finally when speciation is complete the two new species can coexist with little or no gene flow between them (Fig. 1.1).

During the modern synthesis it was realized that new species could arise simply as a product of changes that occurred in allopatry, by selection, by random genetic drift, or by a combination of these factors. Interest in the exact mechanisms of speciation waned. Given geographic isolation and enough time, speciation was seen as inevitable.

Nonetheless, the founders of the modern synthesis did give some consideration to the role of ecology in speciation. For example, in the very first issue of the journal *Evolution*, Ernst Mayr published a paper entitled 'Ecological factors in speciation' (Mayr 1947). However, the focus of this paper was actually to analyze the role of ecology *versus* geography in the evolution of reproductive isolation, and Mayr concludes that

geographical separation is by far more important. During that same time period, verbal models were proposed for how ecological divergence could actually promote the speciation process (Muller 1942; Mayr 1963). For example, populations occupying distinct ecological environments might be subject to divergent natural selection; different types of ecological traits are favored in different environments and populations diverge via natural selection in these traits. If these ecological traits, or ones genetically-correlated with them, incidentally cause reproductive isolation, then speciation occurs simply as a "by-product" of ecological divergence. For example, if ecological traits also incidentally affect mating preferences or the fitness of hybrids, then reproductive isolation evolves as a by-product of divergence in the ecological traits. This 'by-product' mechanism of speciation is simple and intuitive, and was generally accepted.

1.3 Empirical Revival

Despite the acceptance of the 'by-product' mechanism of speciation, focused empirical work on the topic did not peak until the late 1990's. At this point in time, workers began to put forth explicit predictions about the patterns we expect to see in nature if divergent natural selection drives speciation. The simplest prediction is that ecologically-divergent pairs of populations will exhibit greater levels of reproductive isolation than ecologically-similar pairs of populations of similar age (Schluter and Nagel 1995; Funk 1998; Schluter 2000). Another prediction is that traits subject to divergent natural selection will often incidentally cause reproductive isolation (Schluter 2000). This process of speciation via natural selection was coined 'ecological speciation' (although past authors had used that term, e.g. Mayr 1947, it was popularized in the 1990's). One main goal at that point in time was simply to ascertain whether natural selection can promote the evolution of reproductive isolation in the wild. Can support for the predictions of ecological speciation be found in natural populations (i.e. some experimental evolution studies had already demonstrated the evolution of reproductive isolation via divergent selection; e.g. Dodd 1989, Rice and Hostert 1993 for review)? My main goal at the onset of my thesis was to test these predictions, using populations and species of herbivorous walking-stick insects (see my concluding chapter for some retrospective thoughts on these predictions).

1.4 Herbivorous Insects and the 'Different Host / Same Host' Framework

Herbivorous insects have long been thought of as exemplars of divergence and speciation via natural selection (Walsh 1864, 1867; Bush 1969; Feder et al. 1988). In more recent times, a general framework has been developed to study the ecology of speciation in herbivorous insects (Funk 1998; Funk et al. 2002). The main prediction of ecological speciation described above can be modified to apply explicitly to herbivorous insects: if ecological divergence drives speciation then pairs of populations using different host-plants should exhibit greater levels of reproductive isolation than pairs of populations on the same host-plant (Fig. 1.2; scenario 1 versus scenario 2 in all panels). This prediction assumes an 'all else equal' scenario with respect to the age of the population pairs, and levels of gene flow between them. Pairs of populations on the same host are free to diverge via environment-independent processes such as genetic drift, sexual conflict and some forms of sexual selection. Pairs of populations using different hosts may also diverge via these processes, but can also be subject to the additional diversifying effects of divergent natural selection.

If both allopatric and parapatric/sympatric taxa are studied, then the role of geography can also be analyzed. Reinforcement is a process whereby natural selection against hybrids drives the evolution of premating isolation (Dobzhansky 1951; Butlin 1995; Howard 1993; Noor 1999; Servedio and Noor 2003 for review). The key prediction of the reinforcement hypothesis is that non-allopatric (geographically contiguous or overlapping) populations will exhibit greater mating discrimination than allopatric (geographically separated) populations, because selection against hybrids only occurs in the former (Fig. 1.2; scenario 2 versus scenario 3 in panel C). This pattern is generally referred to as reproductive character displacement. Conversely, gene flow between populations erodes divergence, both in general (Felsenstein 1976; Slatkin 1987; Hendry et al. 2001) and for reinforcement specifically (Sanderson 1989; Servedio and Kirkpatrick 1997; Cain et al. 1999; Servedio 2000; Kirkpatrick 2000). Thus when gene flow constrains divergence, allopatric populations show greater divergence than parapatric / sympatric populations (the opposite pattern predicted by reinforcement; Fig. 1.2; scenario 2 versus scenario 3 in panel B).

Thus the 'different host / same host' framework can be used to simultaneously examine the role of four classes of processes in the evolution of reproductive isolation: 1) the diversifying effects of environment-independent processes, 2) the diversifying effects of ecological divergence, 3) the diversifying effects of reinforcement, and 4) the generally homogenizing effects of gene flow. I adopt this general framework throughout my thesis. There are numerous caveats and additional considerations which must be made when applying this framework (i.e. Do the differences between populations have a genetic basis? Is divergence in host-plant use a good proxy for divergent selection? Does gene flow occur in parapatry? Is there selection against hybrids to drive reinforcement?). In this section, I have outlined only the most general ideas. I present specifics in each chapter as required.

1.5 Thesis Overview and Author Contributions

My thesis is organized into three sections; 1) an overview of ecological speciation, 2) evidence for divergent host-plant adaptation among populations of *Timema cristinae* walking-stick insects and 3) studies of the evolution of various reproductive barriers in these walking-sticks, presented in the order in which they might occur throughout the life-history of an organism. This ordering of the chapters allows for a more logical flow of ideas than would occur if I presented the chapters chronologically (i.e. in the order that the experiments were implemented and the chapters written). I provide below a brief summary of each chapter, as well as information on author contributions (most of the chapters have co-authors). Because the chapters are already published, there is a lot of redundancy throughout the thesis. This redundancy concerns not the topics covered, but rather many of the references used, the description of the *Timema* study system, and some aspects of the general framework (as described in the section directly above). I apologize strongly for this redundancy. Also, the thesis is rather long and I hope that the summary of the chapters (directly below) will help readers keep track of how each chapter fits into the overall bigger picture.

Chapter 2 provides a review of the ecology of speciation. It is argued that ecological speciation can be studied using three distinct components: a source of divergent natural selection, a form of reproductive isolation, and a genetic mechanism to

link the two. Some discussion of the geography of speciation is also included. This chapter is co-authored with Dr. Howard Rundle, and we shared equally in the writing.

The next two chapters (Chapters 3 and 4) provide evidence for divergent, host-associated natural selection and for divergent adaptation between populations of *T. cristinae* using different host-plant species. The results indicate that divergent host-adaptation occurs, and thus has at least the potential to contribute to the evolution of reproductive isolation. These chapters are co-authored with my senior supervisor Dr. Bernie Crespi. I performed the experiments and collected and analyzed the data. Bernie and I shared the conceptual development. I wrote the bulk of the papers, but Bernie helped substantially with the writing.

Chapters 5 and 6 consider the evolution of divergent habitat preferences (in this case, divergent host-plant preferences). Chapter 5 considers the role of divergent host preference in reducing gene exchange between populations (i.e. by acting as a form of reproductive isolation). This chapter is co-authored with Dr. Cristina Sandoval and Dr. Bernie Crespi. I collected about 80% of the data, whereas 20% came from Cristina. I analyzed the data and wrote the bulk of the paper. Bernie and Cristina helped with the conceptual development and the writing. Chapter 6 examines the role of migration between populations in generating genetic covariance between host-preference and a fitness trait under host-associated, divergent natural selection (cryptic colour-pattern). This chapter is co-authored with Dr. Cristina Sandoval, Dr. Bernie Crespi, and Dr. Mark Kirkpatrick. I collected about 80% of the data, whereas 20% came from Cristina. Mark and I developed the concepts behind the mathematical model, but Mark derived the actual equations and analytical solutions. Mark and I co-wrote the bulk of the paper. Bernie and Cristinae helped with the conceptual development and writing.

Chapters 7 and 8 address a less-traditional form of reproductive isolation; natural selection against immigrants from divergent habitats. It is argued that this process, although synonimizing divergent selection and reproductive isolation, acts as a legitimate reproductive barrier. Chapter 7 reviews the process from a conceptual standpoint and presents data from numerous different study systems. This chapter is co-authored with Dr. Tim Vines and Dr. Dan Funk. I collected and analyzed the data. The three of us

shared the conceptual development. Tim wrote the section on hybrid zones (30% of the paper), whereas Dan and I co-wrote the rest. Chapter 8 provide an empirical demonstration of natural selection against immigrants in the *T. cristinae* system. This paper is single-authored by me.

Chapters 9, 10, and 11 consider the evolution of divergent mate preferences. Chapter 9 provides evidence that some divergence in mate preference has occurred simply as a by-product of adaptation to different hosts. Chapter 10 examines the role of reinforcement and gene flow in the evolution of divergent mate preference. Chapters 9 and 10 are co-authored with Dr. Bernie Crespi and Dr. Cristina Sandoval. I collected and analyzed the data, and wrote the bulk of the papers. Both co-authors helped with conceptual development and writing. Chapter 11 provides evidence that divergent mate preference are based upon, at least in part, pheromones and olfactory communication. This chapter also provides a general framework for studying the evolution of divergent mate preference during speciation, and some other data which help interpret the role of adaptation and reinforcement in *T. cristinae* specifically. This chapter is co-authored with Dr. Bernie Crespi, Dr. Gerhard Gries and Regine Gries. Gerhard and Regine collected the data on pheromone profiles and wrote up those results (20% of the total data in the paper). I collected and analyzed the rest of the data, including the behavioural pheromone experiments. Bernie and I shared the rest of the writing. All authors were involved in the conceptual development.

Chapter 12 examines the role of ecological divergence in the evolution of 'cryptic' or postmating, prezygotic reproductive barriers. This chapter is co-authored with Dr. Bernie Crespi. I collected and analyzed the data, and wrote the bulk of the chapter. Bernie helped with the conceptual development and writing.

Finally, at the end of the thesis I have a concluding section (Chapter 13) where I outline some unresolved issues surrounding the ecology of speciation, in *Timema* specifically and among taxa more generally.

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Figure 1.1 Schematic representation of the evolution of reproductive isolation at various 'stages' of the potentially continuous process of speciation. Populations begin as randomly mating units, progress through intermediate stages of speciation where reductions in gene flow occur and partial but incomplete reproductive isolation exists, and finally when speciation is complete the two new species can coexist with little or no gene flow between them.

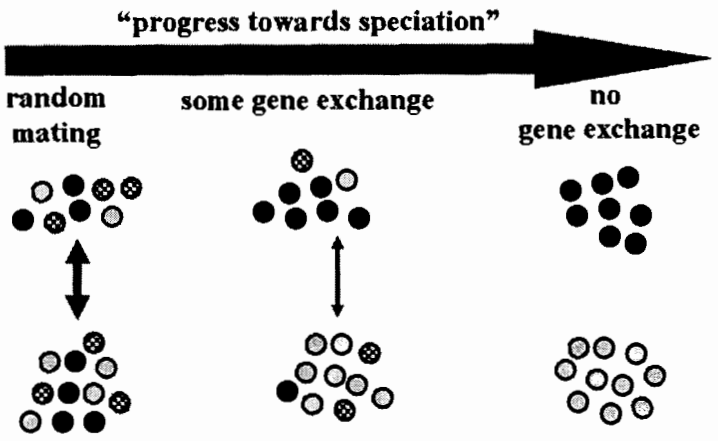
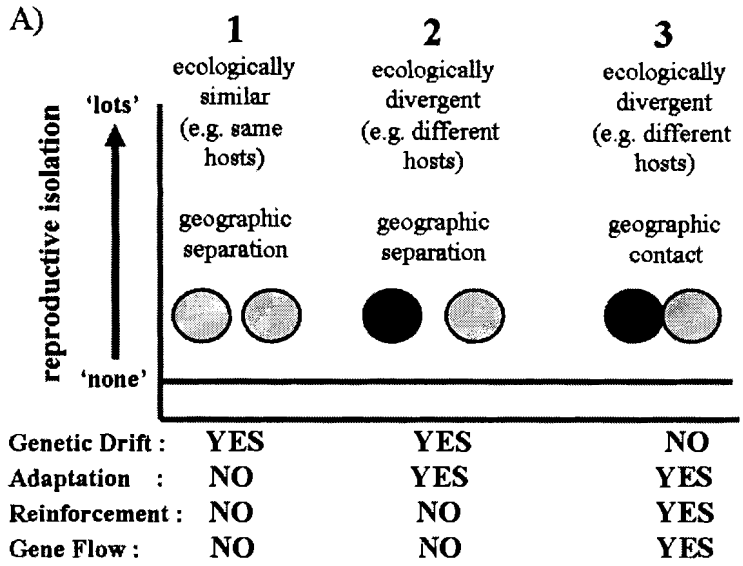


Figure 1.2 The 'Different Host / Same Host' framework for studying the evolution of reproductive isolation between populations of herbivorous insects.

Adopted from Funk (1998) and Funk et al. (2002). In all three panels (A-C), three different scenarios are presented. Scenario 1 examines reproductive isolation between geographically separated pairs of populations on the same host. Scenario 2 examines reproductive isolation between geographically-separated pairs of populations on different hosts. Scenario 3 examines reproductive isolation between geographically-contiguous (parapatric) pairs of populations on different hosts. Environment-independent processes can cause divergence under any scenario, although gene flow may negate a strong role for genetic drift specifically in Scenario 3. The likelihood of other processes is depicted below the x-axis ('adaptation' refers to ecological divergence and divergent adaptation, also shown is the likelihood of reinforcement and gene flow). A) A brief description of the scenarios. B) A hypothetical scenario where ecological divergence does not appear important for speciation. High levels of reproductive isolation are observed without ecological divergence, and reproductive isolation is not accentuated with the addition of ecological divergence. Gene flow also appears to constrain divergence. C) A hypothetical scenario where reproductive isolation evolves via the additive effects of ecological divergence and reinforcement. Little or no reproductive isolation without ecological divergence, intermediate levels with ecological divergence alone, and the highest levels when the opportunity for reinforcement is added.



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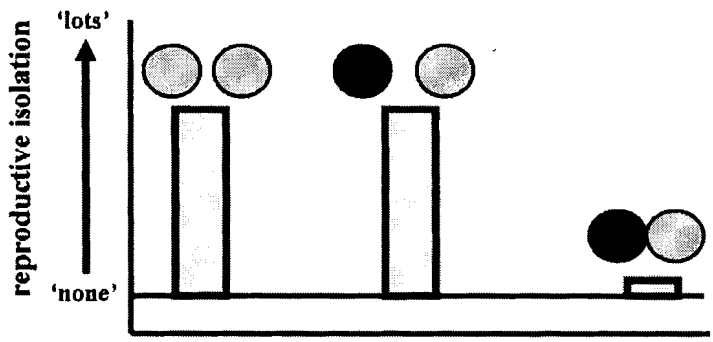
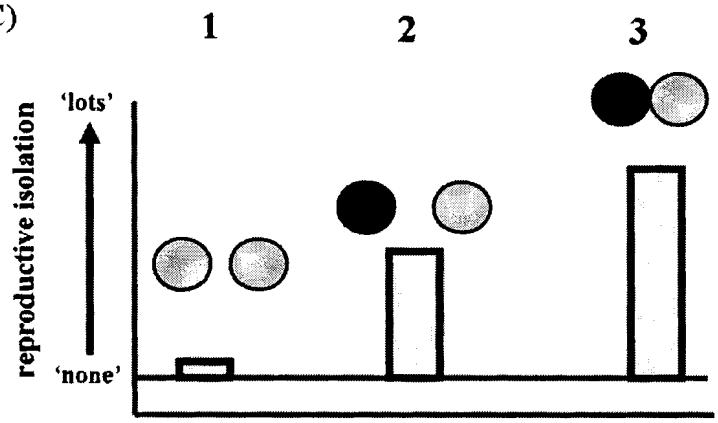


	1	2	3
Genetic Drift :	YES	YES	NO
Adaptation :	NO	YES	YES
Reinforcement :	NO	NO	YES
Gene Flow :	NO	NO	YES

C)



	1	2	3
Genetic Drift :	YES	YES	NO
Adaptation :	NO	YES	YES
Reinforcement :	NO	NO	YES
Gene Flow :	NO	NO	YES

CHAPTER 2.
ECOLOGICAL SPECIATION*

*A version of this chapter appears as Rundle, H., and Nosil, P. 2005. Ecological speciation. Ecology Letters 8: 336-352. Reprinted with permission from French National Center for Scientific Research.

2.1 Abstract

Ecological processes are central to the formation of new species when barriers to gene flow (reproductive isolation) evolve between populations as a result of ecologically-based divergent selection. Although laboratory and field studies provide evidence that 'ecological speciation' can occur, our understanding of the details of the process is incomplete. Here we review ecological speciation by considering its constituent components: an ecological source of divergent selection, a form of reproductive isolation, and a genetic mechanism linking the two. Sources of divergent selection include differences in environment or niche, certain forms of sexual selection, and the ecological interaction of populations. We explore the evidence for the contribution of each to ecological speciation. Forms of reproductive isolation are diverse and we discuss the likelihood that each may be involved in ecological speciation. Divergent selection on genes affecting ecological traits can be transmitted directly (via pleiotropy) or indirectly (via linkage disequilibrium) to genes causing reproductive isolation and we explore the consequences of both. Along with these components, we also discuss the geography and the genetic basis of ecological speciation. Throughout, we provide examples from nature, critically evaluate their quality, and highlight areas where more work is required.

2.2 Introduction

The past decade has seen a revival of the idea that the macroevolutionary phenomenon of speciation is the result of the microevolutionary process of ecologically-based divergent selection (Funk 1998; Schluter 2000, 2001). While the idea dates back at least to the modern evolutionary synthesis (e.g., Mayr 1942, 1947; Dobzhansky 1951), renewed interest in it has gone hand-in-hand with a reclassification of speciation models from a scheme of geography (i.e. sympatric vs. allopatric) to one that focuses on mechanisms for the evolution of reproductive isolation (Schluter 2000, 2001; Via 2001).

Although ecology may contribute to many mechanisms of speciation, our focus here is on the ecological model in which its contribution is fundamental. Consistent with its recent usage (Schluter 2000, 2001), we define 'ecological speciation' as the process by

which barriers to gene flow evolve between populations as a result of ecologically-based divergent selection. Selection is ecological when it arises as a consequence of the interaction of individuals with their environment during resource acquisition. Ecologically-based selection can thus arise, for example, from an individual's quest to obtain food and other nutrients, attract pollinators, or avoid predators. It can also arise from their interaction with other organisms in their attempt to achieve these goals (e.g., competition). Selection is divergent when it acts in contrasting directions in the two populations and we include here the special case in which selection favors opposite, usually extreme, phenotypes within a single population (termed disruptive selection), as occurs during sympatric speciation.

Ecological speciation is distinguished from other models of speciation in which the evolution of reproductive isolation involves key processes other than ecologically-based divergent selection. These include models in which chance events play a central role, including speciation by polyploidization, hybridization, genetic drift, and founder-events/population bottlenecks (reviewed in Coyne & Orr 2004). Non-ecological speciation also includes models in which selection is involved, but is non-ecological and/or is not divergent between environments. Examples include certain models of speciation by sexual selection (e.g., Fisher's runaway, Lande 1981; sexual conflict, Chapman *et al.* 2003) in which selection arises from the interaction of the sexes and is not divergent between environments, and models involving the fixation of different, incompatible alleles in allopatric populations experiencing similar selection (Schluter 2001).

An alternative definition of ecological speciation would restrict it to situations in which barriers to gene flow are ecological in nature. However, when the goal is to understand mechanisms of speciation (as here), it is of interest when both ecological and non-ecological forms of reproductive isolation evolve ultimately due to a specific process (i.e. ecologically-based divergent selection). Distinguishing ecological from non-ecological mechanisms, however, does not imply that the processes involved in the latter are not important to speciation, nor that they may not influence the likelihood and outcome of ecological speciation. Indeed, both possibilities are important topics and a comprehensive understanding of the role of ecologically-based divergent selection in

speciation will require careful consideration of numerous non-ecological factors (e.g., see Gavrilets 2004).

Laboratory experiments have shown that ecological speciation can occur; reproductive isolation has evolved as a by-product of adaptation to different environments in manipulative experiments (reviewed in Rice & Hostert 1993). There is also convincing evidence for its operation in nature (reviewed in Schluter 2001; Coyne & Orr 2004). For example, ecological speciation is directly implicated when traits causing reproductive isolation are under ecologically-based divergent selection (e.g., Macnair & Christie 1983; Filchak *et al.* 2000; Via *et al.* 2000; Jiggins *et al.* 2001). It is also implicated when reproductive isolation is shown to have evolved among replicate, independent populations in correlation with environment (i.e. parallel speciation; e.g., Funk 1998; Rundle *et al.* 2000; Nosil *et al.* 2002; McKinnon *et al.* 2004).

Here we review the process of ecological speciation. Because the above research demonstrates that it occurs, we focus on understanding the details of the process. To do this, we separate ecological speciation into three necessary components: an ecological source of divergent selection, a form of reproductive isolation, and a genetic mechanism to link them. This approach is based on a similar classification of theoretical models of speciation used by Kirkpatrick & Ravigné (2002). It is useful because the effects of these components can be studied, to a certain extent, in isolation of one another, and because it highlights areas that have received less attention. As we will see, our understanding of some components is good, whereas critical tests of others are lacking. We also consider two additional topics that have received less attention in previous reviews: the geography and genetic basis of ecological speciation. The literature on ecological speciation is rapidly growing and our review is by no means exhaustive. Rather, we present a selection of studies that illustrate certain points or address understudied topics.

2.3 Ecological Causes of Divergent Selection

The first component required for ecological speciation is a source of divergent selection. Three ecological causes have been recognized (Schluter 2000, 2001; Kirkpatrick & Ravigné 2002). Although they are not fully independent and distinguishing

between them may not be easy, their separate treatment is useful because it highlights the diversity of ways in which ecology may be involved and their consequences for speciation may vary. This is because the efficacy with which divergent selection is transmitted into reproductive isolation, as well as the forms of reproductive isolation that evolve, will depend on the traits under selection and how they are related genetically to those causing reproductive isolation. In this section we outline the ecological causes and consider the evidence for the contribution of each to ecological speciation. Although all three ecological causes can, in theory, generate almost any form of reproductive isolation (ecological or not), in the next section on reproductive barriers we discuss the likelihood that particular forms will evolve via specific ecological causes.

2.3.1 Environmental differences

Divergent selection can arise because of differences between populations in their environments, including, for example, habitat structure, climate, resources, and the suite of predators or competitors present (Schluter 2000). Divergent selection between environments is consistent with the classic model of allopatric speciation (e.g., Mayr 1942, 1947), although geographic separation is not a prerequisite. Divergent selection may also arise between sympatric populations occupying separate niches within a single geographic area.

The contribution of environmental differences to ecological speciation is reasonably well-understood, in part because the majority of research has focused on this mechanism. Replicated laboratory experiments have directly shown that adaptation to different environments can generate some reproductive isolation, both in sympatry (Rice & Salt 1990) and allopatry (Rice & Hostert 1993). Environmental differences also appear to be frequent sources of divergent selection in nature (reviewed in Schluter 2000). For example, reciprocal transplant experiments, the classic ecological technique for studying local adaptation of divergent forms, have shown that tradeoffs are common such that traits enhancing fitness in one environment reduce it in the other, implying divergent selection between environments. Environmental differences have also been implicated in the evolution of reproductive isolation in a few well studied cases of ecological

speciation in nature (e.g., Macnair & Christie 1983; Nagel & Schluter 1998; Via *et al.* 2000; Jiggins *et al.* 2001; Linn *et al.* 2003; see Schluter 2001).

Nevertheless, our understanding of the role of environmental differences in ecological speciation is incomplete. Most laboratory experiments, for example, have addressed the evolution of one form of reproductive isolation (sexual isolation); data on the role of environmental differences in the evolution of other forms is limited or non-existent. In addition, reproductive isolation failed to evolve in a number of these experiments (e.g., Rundle 2003; see Rice & Hostert 1993), but we have little understanding as to why. Even more remarkable, for cases in which reproductive isolation did evolve, the traits responsible were generally not even identified. Future experiments that explore how divergent selection between environments affects specific phenotypic traits causing reproductive isolation may be especially useful in addressing these gaps in our knowledge.

The prevalence in nature of divergent selection between environments is also unclear. Although reciprocal transplant experiments suggest it is common, insufficient attention has been given to the possibility of intermediate environments and, when they exist, the fitness of intermediate forms inhabiting them (Schluter 2000). If intermediate environments exist and intermediate phenotypes do well in them, then in theory it is possible for populations adapted to different environments to have diverged from one another by genetic drift alone (Schluter 2000; Gavrilets 2004). Although the end product is the same (e.g., populations that exhibit fitness tradeoffs when reciprocally transplanted), divergent selection need not have been involved in their divergence.

Environmental differences have been implicated in a number of speciation events in nature, but additional cases are needed in other systems. Of particular importance will be those that consider agents of divergent selection that have received less attention. For example, predation is ubiquitous in natural populations and adaptation to it may have important consequences for reproductive isolation. However, predator-generated divergent selection has been implicated in the evolution of reproductive isolation in only a handful of cases (e.g., Jiggins *et al.* 2001; Vamosi & Schluter 2002; Nosil 2004).

Additional tests of the role of predation, and other of enemies (e.g., parasites, pathogens), are badly needed.

Finally, we note that one of the strongest tests of the role of environmental differences has yet to be performed. If speciation is caused by adaptation to different environments, for some taxa at least we should be able to recreate the initial stages of this process in a controlled laboratory setting. In one such experiment, suggested by Schluter (2000), hybrids between divergent taxa are placed into separate environments that differ only in the aspects hypothesized to have caused their speciation. Reproductive isolation should then evolve in correlation with environment, building between populations in different environments and being absent between laboratory and natural populations from similar environments. Depending on the natural history of the taxa, a similar experiment could involve individuals from an ancestral species (e.g., the mainland ancestor of an island endemic) placed into a novel environment characteristic of a descendant. Other variants are also possible, but the key to such experiments is that they permit the ecological cause of selection, as well as the traits on which it acts, to be isolated and tested in a replicate manner.

2.3.2 Sexual selection

The second ecological source of divergent selection involves sexual selection. Because it acts on traits directly involved in mate recognition, sexual selection may be a powerful force in the evolution of reproductive isolation (Panhuis *et al.* 2001). Speciation models involving sexual selection can be classified into two types depending on whether or not differences in mate preferences evolve ultimately because of divergent selection between environments (Schluter 2000, 2001; Boughman 2002). Models involving divergent selection between environments include spatial variation in natural selection on secondary sexual traits (Lande 1982) and on mating or communication systems (Ryan & Rand 1993; Boughman 2002). Examples that do not involve divergent selection between environments, and are hence not components of ecological speciation, are models in which sexual selection arises from the interaction of the sexes. This includes Fisher's runaway (Lande 1981) and sexual conflict (Chapman *et al.* 2003). Sexual selection can thus be involved in both ecological and non-ecological speciation (Schluter 2000, 2001).

The evidence for sexual selection in ecological speciation is weaker. Although comparative studies suggest that sexual selection is associated with speciation in nature in some taxa, these tests cannot discriminate among its various causes (reviewed in Panhuis *et al.* 2001; Coyne & Orr 2004), most notably ecological vs. non-ecological. Direct tests of ecologically-based sexual selection in speciation in nature are beginning to accumulate (see Boughman 2002). For example, allopatric populations of *Anolis cristatellus* lizards from two environments (mesic and xeric) occupy distinct habitats with respect to light intensity and spectral quality, and the design of their dewlaps (a trait important in social communication, including mating) has diverged between populations in a way that increases signal detectability in each habitat (Leal & Fleishman 2004). Likewise, in freshwater limnetic and benthic threespine stickleback (*Gasterosteus aculeatus* spp.) fish, male nuptial colour and female perceptual sensitivity both vary among lakes in correlation with light environments, resulting in environment-specific signal preferences (Boughman 2001). In both cases, adaptive signal divergence appears to contribute to reproductive isolation. More studies on diverse taxa are needed, however, to provide general insights.

In contrast to accumulating evidence from natural systems, ecological models of speciation by sexual selection have never been evaluated in manipulative laboratory experiments. This is a conspicuous oversight. It is difficult to implicate any one model of sexual selection in a speciation event in nature, in part because the various models depend on parameters that are difficult to measure (Turelli *et al.* 2001). Specific predictions for some ecological models have been identified (e.g., Boughman 2002), although an alternative interpretation exists (see Coyne & Orr 2004) for even the strongest case (Boughman 2001). Laboratory experiments may be crucial in addressing such issues, allowing the feasibility of various models to be tested and providing insight into how signal traits and preferences, and hence reproductive isolation, evolve under different scenarios. Ultimately, it may be possible in some taxa to recreate speciation by ecologically-based sexual selection in the lab, thus gaining some of the strongest evidence possible.

2.3.3 Ecological interactions

Divergent selection may also arise between populations as a result of their ecological interaction with one another. Ecological interactions are distinguished from other sources of divergent selection because they occur in sympatry, although exceptions could entail allopatric populations interacting indirectly via a separate, mobile species. In addition, divergent selection arising from ecological interactions is frequency dependent because individual fitnesses depend on the frequency of the various phenotypes (Taper & Case 1992; Schluter 2000). Frequency dependent ecological interactions among individuals within a population may also generate disruptive selection that can, in theory, cause sympatric speciation (reviewed in Turelli *et al.* 2001).

At least one form of ecological interaction, interspecific competition, appears common in nature. Observational studies implicate it as the predominant source of divergent selection during ecological character displacement and, although direct tests have just begun to accumulate, they support this conclusion (Taper & Case 1992; Schluter 2000). Nevertheless, despite the apparent prevalence of character displacement, as far as we are aware there are no direct tests, from nature or the lab, linking the evolution of reproductive isolation to interspecific competition. Although divergent selection can also arise from other types of interactions (e.g., mutualism, facilitation, apparent competition; Abrams 2000; Doebeli & Dieckmann 2000; Day & Young 2004), their prevalence in nature and role in ecological speciation are also relatively unexplored. Interactions via shared predators have been shown to alter competitive interactions and affect divergent selection in a pond experiment in sticklebacks (Rundle *et al.* 2003), but the consequences for speciation are not known.

The role of ecological interactions in generating disruptive selection and causing sympatric speciation is similarly unknown. Laboratory experiments have shown that frequency-dependent competition is responsible for the sympatric, ecological diversification of single strains of asexual taxa (e.g., Friesen *et al.* 2004). Implications for ecological speciation are limited, however, because reproductive isolation does not apply. In sexual taxa, competitive interactions have also been shown to generate disruptive

selection within a single population of sticklebacks in the wild (Bolnick 2004), although in this case reproductive isolation was not examined.

The absence of direct tests of the role of ecological interactions in speciation may be explained, in part, because research has focused on the consequences of a different interaction: heterospecific matings. If heterospecific matings reduce the fitness of the individuals involved, or their hybrid offspring, selection will favour individuals that mate within their own population. This will strengthen prezygotic isolation in a process known, in the broad sense, as reinforcement (Servedio & Noor 2003). Although it features prominently in many models of speciation, reinforcement is difficult to categorize because it can complete a speciation process initiated by any mechanism, ecological or not (Schluter 2001; Rundle & Schluter 2004). If the cost to heterospecific mating originated from ecological causes (e.g., Kirkpatrick 2001), then it is tempting to consider reinforcement as a component of ecological speciation. The situation is unclear even in this case, however, because reinforcing selection need not be divergent. In classic 'one-allele' models, a single allele, causing individuals to mate with other, phenotypically similar individuals, is favored by selection in both populations (Felsenstein 1981; Servedio & Noor 2003). Therefore, whether reinforcement is a component of ecological speciation depends upon the specific circumstances.

Under this broad definition, reinforcement may not be a rare occurrence (Servedio & Noor 2003), although just how common and how often it has an ecological basis is not well understood. Ecological causes have been implicated in some cases (e.g., Rundle & Schluter 1998; Albert & Schluter 2004; Nosil *et al.* 2003). Understanding the contribution of reinforcement to ecological speciation will require careful consideration of all costs to heterospecific matings and the mechanisms (ecological or not) by which they evolved.

Finally, separating the effects on ecological speciation of reinforcement and ecological character displacement may be difficult. Both occur in sympatry from the interaction of populations and can produce the same evolutionary outcome: stronger prezygotic isolation between sympatric than allopatric populations (Servedio & Noor 2003). The extent of this problem will not be known until we determine how frequently

prezygotic isolation is strengthened as a by-product of ecological character displacement. Studies of reinforcement are beginning to consider the possibility: results of one suggest ecological character displacement was not involved (Nosil *et al.* 2003) and two others attempted to control for its contribution (Rundle & Schluter 1998; Albert & Schluter 2004). Studies that estimate the independent and combined roles of both are badly needed. The control facilitated by laboratory experiments may be especially useful in distinguishing these processes and exploring their interaction. For example, by exposing allopatric populations to experimental sympatry in the lab, reinforcement was directly implicated in the strengthening of prezygotic isolation between the Australia fruit flies *Drosophila serrata* and *D. birchii* (Higgie *et al.* 2000). In future experiments, the opportunity for reinforcing selection could be directly manipulated by housing populations sympatrically or allopatrically during mating (reinforcing selection present or absent respectively); the opportunity for competition and other ecological interactions could also be manipulated by raising the populations sympatrically or allopatrically during the rest of their life cycles (interactions permitted or prevented respectively).

2.4 Forms of Reproductive Isolation

Many forms of reproductive isolation exist that can block gene flow between populations in different ways (Coyne & Orr 2004). Below we describe seven forms: four of prezygotic isolation and three of postzygotic isolation. One is the unique product of ecologically-based divergent selection and its existence implies ecological speciation, whereas some others can be produced by any mechanism of speciation. A key question for each thus concerns the role of ecologically-based divergent selection in its evolution. We evaluate the evidence for this and highlight further types of data required. Examples from nature of each are given in Table 2.1.

2.4.1 Habitat and temporal isolation

Prezygotic isolation can arise when populations are separated in space (habitat) or time (Dres & Mallet 2002; Funk *et al.* 2002). Habitat isolation occurs when populations exhibit genetically-based preferences for separate habitats, reducing the likelihood of

heterospecific encounters (Rice & Salt 1990; Johnson *et al.* 1996). Divergent habitat preferences are most likely to cause prezygotic isolation when mating occurs in or near the preferred habitat (Johnson *et al.* 1996; Funk *et al.* 2002). For example, divergent host-plant preferences cause partial reproductive isolation between herbivorous insect populations that mate on the plant on which they feed (Table 2.1). Temporal isolation occurs when populations exhibit divergent developmental schedules such that mating occurs at different times in the populations. Importantly, both habitat and temporal isolation may be common during ecological speciation because adaptation to different environments or resources will generate selection for divergent habitat preferences or developmental schedules (e.g., individuals preferring the habitat to which they are best adapted will have higher fitness).

Although habitat and temporal isolation appear common (Table 2.1), little attention has been given to their mechanisms of evolution. Non-ecological processes, such as genetic drift, are unlikely if trait differences can be shown to be adaptive in each habitat, or if they evolve in parallel multiple times (Schluter & Nagel 1995). Different forms of ecologically-based divergent selection could be involved, however, and their relative importance is unknown. Habitat and temporal isolation may both evolve as by-products of adaptation to different environments. However, as noted above, both may also be favoured by selection if traits enhancing fitness in one environment (or when exploiting one resource) decrease it in the other. Alternatively, habitat and temporal isolation could also be favoured by selection if they altered ecological interactions between populations (e.g., reduced competition) or decreased the likelihood of heterospecific matings (i.e. by reinforcement).

2.4.2 Natural selection against immigrants (immigrant inviability)

Prezygotic isolation can arise when migrants between populations suffer reduced survival because they are poorly adapted to their non-native habitat. Although not normally considered a form of reproductive isolation, such 'immigrant inviability' can directly reduce gene flow between populations by lowering the rate of heterospecific mating encounters (Funk 1998; Via *et al.* 2000; Nosil 2004; Nosil *et al.* 2005 for review). By reducing interbreeding between populations, natural selection against immigrants

constitutes a legitimate reproductive barrier, even though it is the direct consequence of ecologically-based divergent selection. Despite being opposite sides of the same coin, the separate consideration of divergent selection and immigrant inviability is useful because the presence of the former does not guarantee that the latter was an important source of reproductive isolation during the speciation process. When speciation is allopatric, for example, 'parental' individuals may never migrate between environments and ecological speciation may occur entirely via the evolution of other forms of reproductive isolation.

Demonstrating natural selection against immigrants is consistent with ecological speciation, although as noted earlier, in theory it is possible for genetic drift to produce divergent populations that exhibit fitness tradeoffs when reciprocally transplanted (Schluter 2000; Gavrilets 2004). This alternate drift-based possibility can be ruled out if the fitness of intermediate forms (i.e. hybrids) is also reduced by ecological mechanisms (see 'ecologically-dependent postzygotic isolation' below). Quantification of the individual components of reproductive isolation in diverse taxa reveals that natural selection against migrants tends to be strong and that its relative contribution to total isolation may often be greater than that of more commonly considered forms (e.g., sexual isolation, hybrid inviability; Nosil *et al.* 2005). Our understanding of the divergent selection involved is limited, however, because data addressing the sources and phenotypic targets of selection are few (Schluter 2000). A more detailed understanding will require experiments that directly manipulate agents of selection and identify the traits involved (e.g., Nosil 2004).

2.4.3 Sexual isolation (pollinator isolation)

Prezygotic isolation can arise because individuals from different populations are less attracted to, or do not recognize, one another as potential mates. Such sexual isolation is one of the most commonly recognized forms of prezygotic isolation, but its ecological basis is unfortunately also one of the most difficult to determine. This is because sexual isolation usually involves the interaction of signal traits in one sex with preferences in the other. Differences among populations in both of these will generally arise as a by-product of mate choice evolution within populations, a process that necessarily involves sexual selection and may involve natural selection and genetic drift

as well (Kirkpatrick & Ryan 1991; Coyne & Orr 2004). An ecological basis is expected whenever sexual selection has an ecological component. As outlined earlier, this can occur when ecologically-important characters also influence mate choice, or when environmental differences generate divergent selection on mating or communication systems. Sexual isolation can also evolve by reinforcing selection within an ecological context (i.e. if the cost to heterospecific matings originated by ecological mechanisms).

Sexual isolation has received much attention in nature and a number of lines of evidence implicate ecologically-based divergent selection in its evolution. For example, pairs of populations independently adapted to different environments exhibit stronger sexual isolation than those independently adapted to similar environments (Funk 1998; Rundle *et al.* 2000; Nosil *et al.* 2002; McKinnon *et al.* 2004). In addition, traits under divergent natural selection have been shown to influence mate choice in a number of systems (e.g., Nagel & Schluter 1998; Jiggins *et al.* 2001; see Schluter 2001). Divergent selection on mating systems has also been implicated in a few cases (e.g., Boughman 2001, 2002; Leal & Fleishman 2004), and there is evidence consistent with ecologically-based reinforcement (Rundle & Schluter 1998; Albert & Schluter 2004; Nosil *et al.* 2003).

In plants, populations in different environments can be exposed to selection to adapt to different pollinators. The subsequent divergence in pollinator-related traits will generate pollinator isolation. Such pollinator isolation has been strongly implicated in monkeyflowers (Schemske & Bradshaw 1999; Bradshaw & Schemske 2003; Ramsey *et al.* 2003) and may be common in other plants (see Coyne & Orr 2004).

2.4.4 Postmating, prezygotic isolation

Postmating, prezygotic isolation exists when barriers, acting after copulation is initiated, either reduce or prevent the fertilization of eggs with heterotypic sperm. Examples include poor transfer or storage of sperm (Price *et al.* 2001), failure of fertilization when gametes come into contact (Vacquier *et al.* 1997; Palumbi 1998) and conspecific sperm or pollen preference (Howard *et al.* 1998; Rieseberg *et al.* 1995). Such barriers can evolve via numerous processes and it is not immediately apparent what their

potential role is in ecological speciation. Although reproductive proteins involved in gametic interactions often evolve rapidly via selection (Swanson & Vacquier 2002), the source of this selection is generally not known and a role for ecological causes is not required (Vacquier *et al.* 1997). Examples exist that are consistent with both ecological and non-ecological selection (see Coyne & Orr 2004). Distinguishing among the various mechanisms for the evolution of this type of barrier may require detailed knowledge of individual cases.

2.4.5 Intrinsic postzygotic isolation

Postzygotic isolation can result from genetic incompatibilities between genomes that are expressed when they are brought together in hybrids (Rice & Hostert 1993; Rundle & Whitlock 2001; Coyne & Orr 2004). These incompatibilities reduce the fitness of hybrids and, although their effects may be environment-dependent (e.g., greater consequences in a more harsh environment; see Rundle & Whitlock 2001), they do not depend on an ecological interaction between phenotype and environment. Intrinsic postzygotic isolation has received much attention in the literature, although work has focused primarily on understanding the genetic basis of two extreme forms (hybrid sterility and inviability; Wu & Ting 2004) and on exploring theoretical models for its evolution (reviewed in Coyne & Orr 2004). Its role in ecological speciation has been generally overlooked (but see Lu & Bernatchez 1998), likely in part because it can be produced by any mechanism of speciation. It is possible, however, that genetic incompatibilities evolve more rapidly under divergent selection and that they are thus an important cause of ecological speciation. Consistent with this, in all three cases where a gene causing intrinsic postzygotic isolation has been identified, there is evidence that it has evolved via positive selection (Hmr, Barbash *et al.* 2004; Nup96, Presgraves *et al.* 2003; OdsH, Ting *et al.* 1998; Wu & Ting 2004 for review). However, causes of selection (e.g., ecological or not) cannot be determined from these data alone. Sister group comparisons, similar to those used to test for a role of sexual selection in speciation (see Panhuis *et al.* 2001) may be useful in asking whether intrinsic incompatibilities evolve sooner or more frequently when divergent selection is stronger.

2.4.6 Ecologically-dependent postzygotic isolation

Postzygotic isolation can also arise when hybrid fitness is reduced because of an ecological mismatch between hybrid phenotype and their environment (Rice & Hostert 1993; Rundle & Whitlock 2001; Coyne & Orr 2004). Basically, hybrids are not well adapted to either parental environment, and in effect, fall between niches. Ecologically-dependent postmating isolation is analogous to immigrant inviability above except that divergent selection is acting against hybrids instead of parental individuals. As with immigrant inviability, ecologically-dependent postzygotic isolation and divergent selection between environments can be considered two sides of the same coin (Coyne & Orr 2004). In contrast to intrinsic postzygotic isolation, ecologically-dependent (or extrinsic) postzygotic isolation has received less attention. This is despite the fact that this form of isolation is a unique prediction of ecological speciation. To the extent that hybrid phenotypes are intermediate, ecologically-dependent postzygotic isolation is a necessary consequence of divergent selection between environments.

There are at least three techniques for demonstrating ecologically-dependent postzygotic isolation. In the first, the fitness of hybrids in the wild is compared to that in a benign environment (e.g., Hatfield & Schluter 1999). The benign environment is assumed to remove the ecological factors that reduce hybrid fitness, thus permitting an estimate of any intrinsic genetic isolation. Comparison of hybrid fitness in the wild to that in the benign environment yields an estimate of ecologically-dependent isolation. Caution is warranted, however, because non-ecological reductions in hybrid fitness may differ between environments, complicating this method (see Hatfield & Schluter 1999). In the second, backcrosses of F1 hybrids to both parental forms are used in reciprocal transplants between environments (e.g., Rundle 2002). A comparison of the fitness of the two types of backcrosses estimates a component of ecologically-dependent isolation while controlling for any genetic incompatibilities (Rundle & Whitlock 2001). In the third technique, which has never been attempted to our knowledge, parental individuals are phenotypically modified to resemble hybrids. Given proper controls for this manipulation, the fitness of these individuals in the parental environments estimates ecologically-dependent isolation alone. Such modifications may be straightforward to apply in many plants (e.g., Hodges *et al.* 2002)

Few studies have applied these above techniques and the extent of ecologically-dependent postzygotic isolation in nature is unknown. When conducting such studies, it is important to consider the possibility of intermediate environment (Schluter 2000). Average hybrid fitness may not be reduced if such an environment is accessible and hybrids perform well in it (e.g., Wang *et al.* 1997). Finally, although demonstrating ecologically-dependent isolation is an important first step, its ecological causes are also of interest. If hybrids are used, experiments designed to measure divergent selection between environments can provide important information about the ecological mechanisms of reduced hybrid fitness, such as the traits involved (e.g., Nagy 1997).

2.4.7 Sexual selection against hybrids

Finally, postzygotic isolation can also arise if hybrids, despite surviving to sexual maturity, are less likely to secure a mate. Sexual selection against hybrids, however, may or many not contain an ecological component (Schluter 2000). For example, hybrid attractiveness could be reduced as a consequence of genetic incompatibilities that accumulated from non-ecological processes. Thus, although sexual selection against hybrids appears common (Schluter 2000; Coyne & Orr 2004), the key for ecological speciation lies in understanding its origin. An ecological component is clear if hybrid sexual displays are maladapted to their environment (e.g., intermediate displays are less visible). An ecological component is also implicated if sexual display traits are condition-dependent, as theory suggests they should often be (Rowe & Houle 1996), and hybrid condition is reduced as a result of ecological mechanisms (P. Edelaar *et al.*, unpubl. manuscript). Finally, ecology is also implicated if mate preferences diverge between parental species as a consequence of ecological mechanisms and this renders hybrids unattractive because of their intermediate phenotypes. The above possibilities have received little attention, although the latter situation appears to be involved in the reduced mating success of hybrids between species of *Heliconius* butterflies. Color-patterns of these butterflies, which diverged as adaptations to mimic different model taxa, are also important traits in mate choice. Hybrid color-patterns are intermediate and fall largely outside of the range of parental mate preferences (Naisbit *et al.* 2001). Pollinator-based discrimination against hybrid plants possessing intermediate floral traits may also be a

common example of the latter scenario (e.g., Schemske & Bradshaw 1999; Emms & Arnold 2000). Additional tests of all possibilities are required.

2.4.8 Importance for ecological speciation

As we have seen above, many forms of reproductive isolation exist and they vary in the potential role of ecological processes in their evolution. Although examples exist of all types in nature (Table 2.1), the extent and relative strength of these barriers is poorly understood. This is because there are only a handful of cases in which the relative contribution of multiple barriers has been addressed in a single system (Coyne & Orr 2004). Doing so may provide important insights into the roles of ecological and non-ecological processes in speciation. For example, Ramsey *et al.* (2003) conclude that, despite multiple and substantive forms of pre- and postzygotic isolation between the two species of monkeyflower discussed earlier, ecological factors stemming for their adaptation to different environments played the central role. In whitefish ecotypes, both ecological and intrinsic genetic barriers exist, although it is not known how the latter evolved so the role of ecological selection remains unclear (Lu & Bernatchez 1998). Finally, in host-associated *Timema* walking-stick insects, natural selection against immigrants and sexual isolation contribute similarly to total prezygotic isolation and both appear to have evolved by ecological mechanisms (Nosil *et al.* 2002, 2003; Nosil 2004).

Being specific predictions of the ecological model, many studies of ecological speciation consider those forms of reproductive isolation that are likely to have been produced by ecologically-based divergent natural selection. The relative contribution of divergent selection to the evolution of those forms commonly attributed to non-ecological processes has been largely overlooked. As we noted for intrinsic postzygotic isolation, although genetic incompatibilities can evolve by drift and uniform selection, ecologically-based divergent selection may speed their accumulation. The contribution of divergent selection to the evolution of all forms of reproductive isolation requires investigation.

The barriers to gene flow important to speciation are those that evolve before reproductive isolation is yet complete. Thus when multiple barriers exist between taxa,

the temporal order of their evolution is key and may shed light on the mechanism of speciation. The relative importance of current barriers, however, may not be indicative of their historical importance (Coyne & Orr 2004). Little is known about the relative rates of evolution of various forms of reproductive isolation. Data from phytophagous insects suggest that ecological forms can evolve prior to others that may involve non-ecological process (Funk *et al.* 2002 for review). Likewise, comparative studies indicate that sexual isolation can evolve before intrinsic postzygotic isolation (Coyne & Orr 1997; Mendelson 2003). In Coyne & Orr's (1997) study of various *Drosophila* species, this result was entirely the product of sexual isolation evolving faster between sympatric than allopatric species pairs. This suggests that postzygotic isolation may often be the engine that drives the evolution of prezygotic isolation via reinforcement, although ecological interactions could also be involved. Clearly much work is needed to produce a comprehensive understanding of the temporal order of the evolution of reproductive isolation. The forms that exist between partially isolated taxa in nature are thus of great interest.

2.5 Linking Divergent Selection and Reproductive Isolation

The final component of ecological speciation is the genetic mechanism by which selection on ecological traits is transmitted to the genes causing reproductive isolation. There are two ways this can occur, distinguished by the relationship between the genes under divergent selection (i.e. those affecting ecological traits) and those causing reproductive isolation (Kirkpatrick & Ravigné 2002). In the first, these genes are one in the same. In this case, reproductive isolation evolves by direct selection because it is the pleiotropic effect of the genes under selection (Kirkpatrick & Barton 1997; termed 'single-variation' models by Rice & Hostert 1993). In the second, genes under divergent selection are physically different from those causing reproductive isolation. In this case, reproductive isolation evolves by indirect selection arising from the non-random association (linkage disequilibrium) of the genes for reproductive isolation and those for ecological traits (Kirkpatrick & Barton 1997; termed 'double-variation' models by Rice & Hostert 1993). Note that the relationship of direct and indirect selection with pleiotropy and linkage disequilibrium differs when considering selection at the genetic (as here) or phenotypic (e.g., Lynch 1985) level.

The nature of these genetic relationships is important for two reasons. First, pleiotropy and linkage disequilibrium will affect the strength of selection transmitted to the genes affecting reproductive isolation and, depending on the nature of the relationship, may facilitate or hinder speciation. Second, the genes involved will determine the form of reproductive isolation that evolves. If, for example, pleiotropy is more common between certain ecological traits and particular forms of reproductive isolation, such traits should feature prominently in ecological speciation.

2.5.1 Direct selection and pleiotropy

Speciation is facilitated when genes under divergent selection cause reproductive isolation pleiotropically. There are numerous ways this can occur. For example, habitat isolation will evolve as a direct consequence of selection on habitat preference genes if individuals mate in their preferred habitat. This is the route by which sympatric speciation is thought to be most likely (Johnson *et al.* 1996) and has been demonstrated in a laboratory experiment (Rice & Salt 1990). Sexual isolation can evolve due to changes in mate preferences that arise as a pleiotropic consequence of the adaptive divergence of mating or communication systems (Boughman 2002; Ryan & Rand 1993). Such changes in mate preferences may also cause sexual selection against hybrids as a direct consequence (Liou & Price 1994). In plants, pollinator isolation is a direct consequence of adaptation to different pollinators (e.g., Schemske & Bradshaw 1999) and temporal isolation, caused by differences in flowering time, may arise as the pleiotropic effect of adaptation to different environments (e.g., Macnair & Gardner 1998). Intrinsic postzygotic isolation can arise pleiotropically if alleles favoured by selection within each population contribute to incompatibilities between them. Finally, ecologically-based reductions in parental (i.e. immigrant inviability) and hybrid (i.e. ecologically-dependent postzygotic isolation) fitness are facilitated when genes favored by selection in one environment directly reduce fitness in the other (Via & Hawthorne 2002).

2.5.2 Indirect selection and linkage disequilibrium

Indirect selection is thought to be less effective than direct selection in the evolution of reproductive isolation (Kirkpatrick & Ryan 1991; Kirkpatrick & Barton

1997). This is because the genetic association between the genes under selection and those causing reproductive isolation (i.e. linkage disequilibrium) is not perfect, thus weakening selection on the latter (Kirkpatrick & Ravigné 2002). The amount of linkage disequilibrium that exists is affected by three factors. The first is the genetic basis of reproductive isolation. As pointed out by Felsenstein (1981), there are two distinct possibilities, termed one- and two-allele mechanisms. In a one-allele mechanism, reproductive isolation is caused by the same allele fixing in both populations (e.g., an allele causing individuals to prefer mates phenotypically similar to themselves). In a two-allele mechanism, different alleles fix in each population (e.g., a preference allele for large individuals in one population and small individuals in the other). This distinction is important when considering the effects of recombination. Recombination in a two-allele mechanism breaks down linkage disequilibrium, randomizing the association between genes under divergent selection and those causing reproductive isolation (Felsenstein 1981). In contrast, recombination creates no such problem for a one-allele mechanism and it is therefore more powerful mechanism of speciation than a two-allele.

The second is physical linkage. The maintenance of linkage disequilibrium is greatly facilitated by the physical linkage of genes on a chromosome because the likelihood of a recombination event declines with decreasing genetic map distance (Lynch & Walsh 1998). Chromosomal inversions may play a similar role in suppressing recombination and, by protecting large regions of the genome, may foster speciation by maintaining barriers to gene flow between hybridizing species (Ortíz-Barrientos *et al.* 2002). Reduced recombination, however, may decrease the chance of favorable gene combinations being brought together, interfering with the initial build up of linkage disequilibrium (Kirkpatrick *et al.* 2002). Therefore, whether reduced recombination promotes or impedes ecological speciation will depend on the relative importance of building up versus maintaining appropriate forms of linkage disequilibrium.

The third is the strength of selection. In a two-allele mechanism, linkage disequilibrium between genes affecting ecological traits and genes conferring reproductive isolation can be generated and maintained by strong selection, but may require selection to act directly on both loci to favor specific combinations of alleles (i.e. correlational selection; Diehl & Bush 1989; but see Gavrilets 2004). Although not

relevant to ecological speciation in allopatry (because reproductive isolation is a neutral trait), such conditions may exist if speciation occurs by disruptive selection in sympatry. Finally, there is one situation in which linkage disequilibrium can be high and indirect selection therefore strong. It exists when matings occur between divergent populations, as happens during reinforcement after secondary contact (Kirkpatrick & Ravigné 2002). Thus, although reinforcement relies on linkage disequilibrium between the genes that reduce fitness during heterospecific encounters and those that strengthen prezygotic isolation, it occurs under conditions that are most conducive for indirect selection (Kirkpatrick & Ravigné 2002).

2.5.3 Examples from nature

There is little evidence examining the relationships between genes under divergent selection and those causing reproductive isolation. In practice, separating pleiotropy from indirect selection facilitated by close physical linkage will be a difficult task. Linkage disequilibrium caused by tight physical linkage, however, may represent a 'fundamental' relationship similar in effect to pleiotropy (Via & Hawthorne 2002). An important question is how common pleiotropy and tight physical linkage are, and how often they are of the form that would facilitate ecological speciation.

Data are sparse. Quantitative trait locus (QTL) mapping in pea aphids identified loci with opposite effects on fecundity on the two hosts, suggesting alleles with pleiotropic effects or tight physical linkage. Such a fundamental genetic trade-off in fecundity on the two hosts could contribute to two forms of reproductive isolation: ecologically-based reductions in parental (i.e. immigrant inviability) and hybrid (i.e. ecologically-dependent postzygotic isolation) fitness during ecological speciation. A number of loci affecting performance and habitat preference also appeared to reside in similar regions of the genome, again suggesting pleiotropy or tight physical linkage (Hawthorne & Via 2001; Via & Hawthorne 2002). Such genetic correlations were also of the form that would facilitate ecological speciation. For the latter case, however, there is some doubt as to whether the experimental design actually measured two traits (Coyne & Orr 2004). Performance and host preference appear unlinked in other systems (Ortíz-Barrientos *et al.* 2002).

In monkeyflowers, hybrid sterility is either a pleiotropic effect of an allele for resistance to copper contaminated soils, or is caused by something tightly linked to it, again facilitating speciation (Christie & Macnair 1983). In two other species of monkeyflower, flower colour, an important trait contributing to pollinator isolation, is controlled in large part by a single locus (*YUP*). In the predominately bumblebee pollinated *Mimulus lewisi*, substitution of the *YUP* allele from the hummingbird pollinated *M. cardinalis* increased its attractiveness to hummingbirds and pleiotropically decreased its attraction to bumblebees, facilitating the evolution of pollinator isolation. In contrast, introgression of the *M. lewisi YUP* allele into *M. cardinalis* increased its attractiveness to bumblebees, but had little effect on its attractiveness to hummingbirds. Similarly, the genotype at a QTL locus for nectar volume significantly affected hummingbird but not bumblebee visitation (Schemske & Bradshaw 1999; Bradshaw & Schemske 2003). Accumulating evidence for reinforcement also implies that indirect selection is important. Whether reinforcement commonly involves one- or two-allele mechanism is not known (but see Servedio & Noor 2003).

Finally, in the columbines *Aquilegia formosa* and *A. pubescens*, pleiotropy or close physical linkage appears to integrate a number of floral traits that contribute to pollinator isolation (Hodges *et al.* 2002). Although pleiotropy and physical linkage of genes affecting multiple selected traits is not required for ecological speciation, it may affect its likelihood. This is because these relationships, depending on their nature, may either enhance or inhibit the response to selection of the traits involved (Barton 1995; Orr 2000; Otto 2004).

The increasing sophistication of mapping studies offers promise in exploring the genetic architecture of ecological traits and reproductive isolation. Other approaches may also be informative. Laboratory experiments, for example, could play an important role in furthering our understanding of direct and indirect selection and one-and two-allele mechanisms in ecological speciation. For example, the only laboratory test of sympatric speciation involving direct selection was successful, whereas only three of 24 involving indirect selection succeeded (Kirkpatrick & Ravigné 2002). As noted earlier, when experiments fail the reasons why may be particularly informative and more attention is needed exploring the contribution of genetic causes of such failures.

2.6 Geography of Ecological Speciation

Although ecological speciation can occur under any geographic context, geography is still important because it affects the ecological sources of divergent selection that can act, as well as the possibility of gene flow between the populations. We address both issues below.

2.6.1 The two stages of ecological speciation

A number of studies suggest that the traditional models of allopatric and sympatric speciation represent opposite extremes of the geography of speciation and may be overly simplistic (Grant & Grant 1997; Schluter 2001; Rundle & Schluter 2004). Rather, speciation in nature may often occur between these extremes and involve an allopatric and a sympatric (or parapatric) stage (Fig. 2.1). The idea is that speciation begins when populations are allopatric, with reproductive isolation accumulating as a by-product of divergent selection between their environments. The second stage is initiated upon secondary contact. Ecological interactions between the populations are added as a potential source of divergent selection and, if reproductive isolation is not yet complete, heterospecific matings may occur, adding the potential for gene flow and reinforcement as well. The amount of reproductive isolation that evolves during each stage indicates the geographic context of speciation: if reproductive isolation is complete prior to secondary contact, speciation was allopatric, whereas if little reproductive isolation existed at the time of secondary contact, speciation was essentially sympatric. The latter scenario includes in cases in which reproductive isolation evolves within a single, continuous population; it also includes the situation of parapatric speciation in which gene flow is reduced through isolation-by-distance, but is not eliminated. Key questions thus concern how often one or the other stages are absent, and when both are present, the relative importance of each to the evolution of reproductive isolation.

This two stage scenario arose, in part, from recent work on present-day sympatric limnetic and benthic threespine sticklebacks. Their speciation appears to have involved an initial allopatric and subsequent sympatric stage, with some reproductive isolation evolving during each (Rundle & Schluter 2004; Albert & Schluter 2004). Recent data

from the apple and hawthorn host-races of the apple maggot fly *Rhagoletis pomonella*, a classic case put forward in support of sympatric speciation, also suggest a more complex geographic scenario (Feder *et al.* 2003). Inversion polymorphisms, containing genetic variation affecting ecologically important diapause traits that differ between the host-races, trace their origins to allopatric populations in Mexico. Gene flow from the Mexican populations likely introduced this variation into the North American populations. It is unlikely that this introgression was responsible for any immediate reproductive isolation between populations, although it may have provided the genetic variation necessary to facilitate the subsequent host shift (Feder *et al.* 2003). Key traits that generate some prezygotic isolation between the host races, such as olfactory preferences for their respective fruits, appear to have evolved recently and in sympatry (Linn *et al.* 2003). The relative roles of divergence in allopatry and sympatry are not yet fully understood in either of these examples.

Inferring the geography of past speciation events is difficult and recent attention has focused on phylogenetic comparative methods for its reconstruction. However, the ability of these methods to test alternative hypotheses concerning the geography of speciation appears limited. This is because the key assumption of these models, that historical distributions at the time of speciation can be inferred from present-day species ranges, is generally not met (Losos & Glor 2003). Alternate population genetic and coalescent approaches hold some promise, but require simplifying assumptions of their own and their utility remains to be determined (Losos & Glor 2003). The study of ongoing speciation events, for which the geographic context can be more directly observed, is thus an important task.

2.6.2 Effects of secondary contact on speciation

Secondary contact occurs when individuals from separate populations encounter one another through migration or dispersal, or when range shifts or expansions bring formerly allopatric populations into sympatry. Gene flow between populations is possible once secondary contact is established and its occurrence is generally thought to erode their differences, hampering speciation (Servedio & Kirkpatrick 1997; Servedio & Noor 2003). However, secondary contact also permits additional sources of divergent selection,

such as ecological interactions between the populations, and it allows for the possibility of reinforcement (Fig. 2.1). Thus secondary contact can exert dual and opposing effects on the likelihood of speciation.

Consider the example of reinforcement; increased heterospecific encounter rates increases the opportunity for both reinforcement and gene flow. In theory, the magnitude of prezygotic isolation that evolves is expected to reflect a balance between these opposing forces (Kirkpatrick 2000; Servedio & Noor 2003 for review). A study of walking-stick insects demonstrates that prezygotic isolation is strongest between similar sized populations, supporting this prediction (Nosil *et al.* 2003). Furthermore, sexual isolation was found to be strongest when both divergent selection between environments and reinforcement operated. Further empirical and theoretical studies are needed that explore the interaction of gene flow with reinforcing selection and various forms of ecologically-based divergent selection (e.g., Kirkpatrick 2001).

Finally, separate from the above considerations, gene flow between species involves hybridization that can, under certain circumstances, foster speciation. For example, by recombining divergent parental genomes and generating new gene combinations, hybrid species of *Helianthus* sunflowers have undergone large and rapid adaptive transitions (Rieseberg *et al.* 2003). Although ecological divergence appears critical to the survival of the hybrid species, this does not appear to be a mechanism of ecological speciation because initial reproductive isolation appears to be the product, at least in part, of non-ecological fertility selection (Rieseberg 2000).

2.7 Genetic Basis of Ecological Speciation

Earlier we considered how pleiotropy and linkage disequilibrium transmit divergent selection into reproductive isolation. Here we are concerned with other aspects of the genetic architecture of ecological speciation including the number of genes involved, their location in the genome, the distribution of their effect sizes, and the nature of the interactions within (dominance) and among (epistasis) them. Such topics have received much attention in the study of speciation and species differences (reviewed respectively in Coyne & Orr 2004 and Orr 2001). However, as we discuss below, their

study in ecological speciation is hampered in two ways. First, empirical data specific to ecological speciation are limited. Second, the implications of such data for our understanding of how ecological speciation occurs are not clear.

What is known specifically about the genetic basis of ecological speciation? Empirical studies have shown that traits evolving via ecological selection, and that confer reproductive isolation, can be affected by few or many genes, of small or large effect, that vary in their dominance and epistatic interactions (e.g., Hatfield 1997; Peichel *et al.* 2001; Schemske & Bradshaw 1999; Bradshaw & Schemske 2003; Naisbit *et al.* 2003). Ecological speciation can proceed via divergence in just a few key genomic regions (e.g., Campbell & Berntatchez 2004; Emanuliav *et al.* 2004) and can involve a small number of traits (e.g., McKinnon *et al.* 2004; Bradshaw & Schemske 2003). Little is known regarding the contribution of mutation versus standing variation. The genetic basis of parallel evolution can determine whether independently evolved ecological traits that confer reproductive isolation involve the same or different genetic architectures, but has also received limited attention (Schluter *et al.* 2004). Different genetic architectures imply few genetic constraints on ecological speciation (e.g. Naisbit *et al.* 2003), but also suggest the possibility of non-ecological speciation of parallel evolving populations due to the fixation of incompatible alleles. Finally, ecologically-dependent reductions in hybrid fitness require phenotypes that are intermediate between parental forms. Dominance and epistasis, however, can cause departures from this. Although not specific to ecological speciation, data on the genetics of ordinary phenotypic differences between species tend to show roughly additive effects (Orr 2001).

What are the consequences for ecological speciation of such data? The hallmark of ecological speciation is adaptation to different environments, so it is tempting to use what is known about the population genetics of adaptation as a guide. For example, effect size and dominance may affect ecological speciation because they influence the probability that new mutations are fixed and thus the rate of adaptation (Turner 1981; Orr 2000). However, we lack quantitative genetic models that specifically examine the effects on ecological speciation of these aspects of genetic architecture. Such models are required because ecological speciation is concerned with the evolution of reproductive isolation, a complication absent during adaptation. Reproductive isolation is the property

of pairs of populations and the genetic basis of certain forms may differ profoundly from that of ordinary traits (Orr 2001; Coyne & Orr 2004). Until such models are considered, the genetic architecture of ecological speciation will remain a descriptive endeavor.

2.7.1 Genes causing ecologically-based reproductive isolation

The identification of individual genes conferring reproductive isolation warrants special attention because it can potentially provide unique insight into ecological speciation. For example, once such genes are identified, tests for selection at the molecular level are possible. A number of such tests have been conducted and selection has been strongly implicated in the evolution of reproductive isolation (Barbash *et al.* 2004; Presgraves *et al.* 2003; Coyne & Orr 2004; Swanson & Vacquier 2002; Wu & Ting 2004). Such tests, however, tell us little about the form of selection responsible (e.g., ecological vs. non-ecological; Vacquier *et al.* 1997). For example, although positive selection on a gene in two lineages is consistent with divergent selection, it could also be produced by uniform selection with different advantageous mutations fixing in each. Insight into the form of selection may still be possible, however, by determining the normal function of the gene in the parental populations and how it causes reproductive isolation (e.g., Sun *et al.* 2004).

2.8 Conclusions

The study of ecological speciation has come a long way in recent years. Mechanisms have been clarified, specific predictions have been recognized, and much data has been collected. Most importantly, ecologically-based divergent selection has been implicated in the evolution of reproductive isolation in a number of cases. Nevertheless, a detailed understanding of the process still eludes us, even in the best studied model systems. The reason is that the ecological speciation is complex and can encompass many different scenarios. Divergent selection can have various ecological causes, numerous forms of reproductive isolation can result, and there are different genetic mechanisms than can link them. Reinforcement may also strengthen reproductive isolation in sympatry and may itself be ecological or not. And all of this can occur under

different geographic contexts. It will be no small task to evaluate all of these possibilities to develop a general understanding of how speciation proceeds from beginning to end.

Likely many rapidly growing fields, much of the evidence is indirect, relying on observational and comparative studies. Even direct tests of specific predictions of the best understood components, such as the role of environmental differences, are in some respects qualitative. For example, sexual isolation has been shown to be stronger between populations inhabiting different, as opposed to similar, environments (e.g., Funk 1998; Rundle *et al.* 2000; Nosil *et al.* 2002, 2003). However, quantitative links between the strength of divergent selection and the magnitude of reproductive isolation are lacking. In addition, in some taxa a detailed understanding of ecological speciation should permit at least the early stages of the process to be recreated in replicate populations under controlled laboratory conditions, providing some of the strongest evidence possible.

For many topics, it is the classic ecological processes that have received the least attention. For example, we know only a little about the role of competitors and predators in the evolution of reproductive isolation, and even less concerning other possibilities such as parasites, mutualists, or facilitators. Similarly, there are few tests for ecologically-dependent postzygotic isolation in nature, although a number of techniques exist to do so. Finally, the influence of other factors on ecological speciation has yet to be considered. For example, population structure is common in nature and is known to affect many evolutionary processes. However, its effect on ecological speciation has received little attention. In addition, although the colonization of novel habitats may often involve reductions in population size, the interaction between drift and divergent selection during ecological speciation has generally been overlooked (but see Rundle 2003). The influence of shared ancestry is also not known. Closely related populations may share biases in their standing genetic variation and in their production of new variation (Schluter *et al.* 2004). How such biases affect adaptive divergence and the evolution of reproductive isolation has not been considered. Understanding the influence of these above factors will require ecological studies that integrate molecular, population and quantitative genetics, and that consider the phylogenetic history of the system (e.g., Bernatchez *et al.* 1999).

Nevertheless, we close by noting that our most general conclusion is promising. Much progress has been made in recent years and where gaps in our knowledge exist, it is often clear what needs to be done and the tools are generally available.

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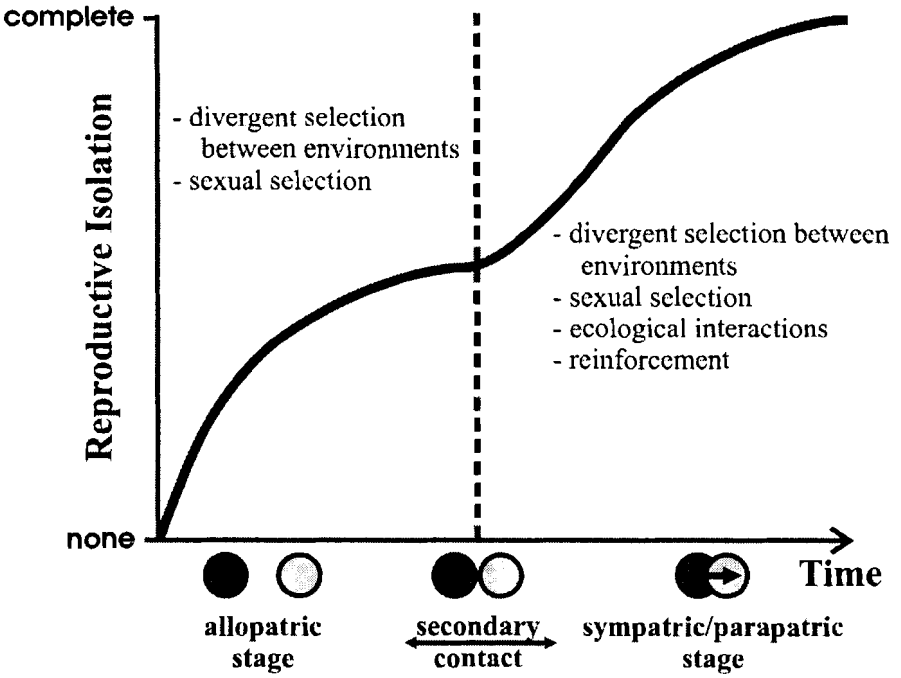
Table 2.1 Forms of reproductive isolation with examples from nature.
Also indicated is whether a particular form commonly evolves by ecologically-based divergent selection ('Ecological selection?') and the ecological causes of divergent selection that could contribute (DE = divergent environments, EI = ecological interactions, SS = sexual selection, RS = reinforcing selection).

Form of reproductive isolation	Ecological selection?	Ecological causes of selection	Examples
Habitat	probably	DE, EI, RS	leaf beetles (Funk 1998), pea aphids (Via 1999), ladybird beetles (Katakura et al. 1989), leaf-mining flies (Tavormina 1982); *Rhagoletis* fruit flies (Feder et al. 1994; Linn et al. 2003); *Eurosta* galling fly (Craig et al. 1993)
Temporal	probably	DE, EI, RS	*Enchenopa* leafhoppers (Wood & Keese 1990), *Rhagoletis* fruit flies (Feder et al. 1994; Filchak et al. 2000); *Banskia* plants (Lamont et al. 2003)
Selection against migrants	yes	DE, EI, RS	Leaf beetles (Funk 1998); *Littorina* snails (Rolan-Alvarez et al. 1997); *Bombina* toads (Kruuk & Gilchrist 1997); *Heliconius* butterflies (Mallet 1989; Mallet & Barton 1989); Pea aphids (Via et al. 2000), *Timema* walking-sticks (Nosil 2004); *Artemesia* sagebrush (Wang et al. 1997); *Gilia* plants (Nagy 1997)
Sexual	unknown (probably)	all	intertidal snails (Cruz et al. 2004); leaf beetles (Funk 1998); freshwater stickleback (Nagel & Schluter 1998; Rundle et al. 2000; Boughman 2001); *Timema* walking-sticks (Nosil et al. 2002; 2003); *Heliconius* butterflies (Jiggins et al. 2001); marine/freshwater stickleback (McKinnon et al. 2004)
Postmating, prezygotic	unknown	all	*Drosophila* (Price et al. 2001); ground crickets (Howard et al. 1998); *Helianthus* plants (Rieseberg et al. 1995)
Intrinsic	unknown	all	*Drosophila* spp. (Barbash et al. 2004; Presgraves et al. 2003; Ting et al. 1998; Wu & Ting 2004)

Form of reproductive isolation	Ecological selection?	Ecological causes of selection	Examples
Ecologically-dependent	yes	DE, EI	freshwater stickleback (Hatfield & Schluter 1999; Rundle 2002); pea aphids (Via et al. 2000); *Eurosta* galling fly (Craig et al. 1997); water lily leaf beetle (Pappers et al. 2002)
Sexual selection against hybrids	unknown	all	freshwater sticklebacks (Vamosi & Schluter 1999); *Heliconious* butterflies (Naisbit et al. 2001)

Figure 2.1 A general scenario for speciation under any geographic context. Reproductive isolation between two populations is absent at the beginning of the speciation process (at the left) and evolves to completion (at the right). Populations are initially allopatric, but secondary contact can occur at any time (dashed vertical line), commencing the second stage of the speciation process. The ecological causes of divergent selection by which reproductive isolation may evolve are listed within the panel for each stage. Depicted is an intermediate scenario in which partial reproductive isolation evolves in allopatry, but speciation is complete in sympatry.



CHAPTER 3. EXPERIMENTAL EVIDENCE THAT PREDATION PROMOTES DIVERGENCE IN ADAPTIVE RADIATION*

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3.1 Abstract

Adaptive radiation is the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage. Recent studies have identified general patterns in adaptive radiation and inferred that resource competition is a primary factor driving phenotypic divergence. The role and importance of other processes such as predation remains controversial. Here we use *Timema* stick insects to show that adaptive radiation can be driven by divergent selection from visual predators. Ecotypes using different host-plant species satisfy criteria for the early stages of adaptive radiation and differ in quantitative aspects of color, color-pattern, body size and body shape. A manipulative field experiment demonstrates that the direction and strength of divergent selection on these traits is strongly, positively correlated with the direction and magnitude of their population divergence in nature, but only when selection is estimated in the presence of predation. Our results indicate that both competition and predation may commonly serve as mechanisms of adaptive radiation.

3.2 Introduction

The 'ecological theory of adaptive radiation' states that 1) divergent natural selection drives the phenotypic divergence and speciation of lineages and 2) divergent selection itself stems from ecological differences between environments or from ecological interactions (1-3). A prediction of the theory is that the direction and magnitude of divergent selection in the wild is positively correlated with the direction and magnitude of trait divergence among natural populations (4, 5). Our understanding of adaptive radiation has been greatly increased by studies describing general patterns or documenting the process of divergent selection (6-11). However, such studies do not test the critical prediction of a correlation between selection and trait divergence. With respect to the causes of divergent selection, support for even the best-studied mechanism of interspecific competition is mostly indirect (2). The role of other processes such as predation has long been discussed (12-14), but remains controversial (2, 15-18). Part of the controversy stems from the fact that predation is notoriously difficult to study in the wild. Here we demonstrate that divergent selection and trait divergence are strongly correlated in natural populations of walking-stick insects and we elucidate predation as the source of divergent selection using a manipulative field experiment.

Timema walking-sticks are plant-feeding insects distributed throughout southwestern North America (19). The genus as a whole satisfies three of the four criteria for adaptive radiation (2); recent ancestry, environment-phenotype correlations, and rapid bursts of speciation (19, 20). Only experimental tests for trait utility at the among-species level remain to be conducted. By contrast, all four criteria are satisfied for host-associated ecotypes of *T. cristinae* adapted to feeding on two different host-plant species. Ecotypes are defined by which host-plant species they are found on, *Ceanothus* versus *Adenostoma*. Figure 3.1 depicts the insect ecotypes and Figure 3.2 depicts the host-plant species at various spatial scales. The two host-plant species differ strikingly in foliage form, with *Ceanothus* plants being relatively large, tree-like and broad-leaved, and *Adenostoma* plants being small, bush-like and exhibiting thin, needle-like leaves. For the insect ecotypes, molecular evidence supports recent ancestry (21), geographic variation in morphology is correlated with host-plant use (such that color and body shape appear

cryptically-matched to each host plant; 22, 23), and experimental evidence confirms trait utility and a higher rate of evolution of reproductive isolation when divergent host-plant adaptation occurs than when it does not (21, 23-25). Adaptive radiation in the classical sense concerns diversification among species. But by satisfying all the criteria for adaptive radiation the ecotypes of *T. cristinae*, like limnetic and benthic ecotypes of sticklebacks (15), can be considered an early stage of radiation that provides a useful model for testing outstanding questions (much like laboratory microorganisms provide useful experimental models for adaptive radiation; 3, 16). Patterns detected between the ecotypes can then be compared to patterns of among-species diversification to connect micro-evolutionary processes with macro-evolutionary patterns.

Testing the prediction that selection has driven divergence requires both quantitative estimates of trait divergence in nature and estimates of divergent selection in the wild. We quantified divergence between *T. cristinae* ecotypes in 11 quantitative traits that differ between them to varying degrees (*Ceanothus* n = 283; *Adenostoma* n = 321). These traits comprise aspects of color, color-pattern, body size and body shape. Figure 3.1 depicts the traits measured and typical differences between ecotypes (raw and size-corrected trait means each host ecotype can be found in Table 3.3). Trait divergence was estimated as mean trait value for individuals from *Ceanothus* minus mean trait value for individuals from *Adenostoma*.

A manipulative field experiment using enclosures allowed us to then estimate survival selection on these same traits on both host species in the presence versus absence of visual predation (n = 384 individuals). Specifically, we estimated standardized directional selection differentials, which measure total selection on a trait (26). We calculated 'divergent selection' as the selection differential on *Ceanothus* minus the selection differential on *Adenostoma* (see Table 3.4 for the raw selection differentials). The association between trait divergence and divergent selection was then evaluated under the two predation scenarios (present versus absent). Our results demonstrate the selection and trait divergence are strongly correlated, but only when selection is estimated in the presence of visual predation. Because we were able to manipulate the presence of predation, our findings provide a clear experimental demonstration that predation can drive phenotypic divergence during adaptive radiation.

3.3 Results and Discussion

We detected strong support for the prediction of the ecological theory of adaptive radiation: the direction and strength of divergent selection under predation was strongly, positively correlated with the direction and magnitude of trait divergence between natural populations using different hosts ($r = 0.91$, $p < 0.001$; Fig. 3.3). By contrast, there was no correlation between selection in the absence of predation and trait divergence ($r = 0.00$, $p = 1.00$). Thus there was a highly significant difference between predation scenarios (scenario x trait divergence interaction; $F_{1,24} = 35.51$, $p < 0.001$) and predation is inferred as the agent of selection driving trait divergence.

The analyses presented above are sufficient and appropriate for evaluating the association between selection and trait divergence, and for assessing the difference between predation scenarios. The results were also highly robust to different analytical methods. The pattern of a strong correlation between selection and trait divergence only in the presence of predation was detected when size-corrected trait values were considered, when non-parametric correlation was used to evaluate the association between selection and trait divergence, when selection was estimated using logistic rather than linear regression, and when analyses using uncorrelated principal components axes in the place of individual traits were conducted. Table 3.1 presents the results of all these analyses and further analytical results can be found in Table 3.6. Additionally, our estimates of selection were generally independent from which host the individuals used in the experiment originated from (i.e. ecotype) and they were unaffected by variation among replicates within treatments (i.e. different bushes in the same treatment). Specifically, we conducted selection analyses that considered not only the effect of trait values on survival, but also the effects of the interaction between trait value and host of origin and the effects of the interaction between trait value and replicate number. In these analyses, only 5 of 88 interactions were significant at $p < 0.05$, and no interaction retains significance following correction for multiple comparisons (i.e. 11 traits, supplemental materials for detailed results). Finally, because insects could disperse in both predation scenarios, differences between scenarios cannot be explained by differential dispersal.

Our findings thus provide robust evidence that phenotypic diversification has been driven by divergent selection from predators.

Further evidence for the central role of predation in population divergence is indicated by stronger absolute divergent selection in the presence versus absence of predation for 10 of 11 traits individually ($p < 0.01$, paired t-test; Fig. 3.3). Body brightness and stripe brightness exhibited particularly strong evidence for treatment-dependent selection, with selection differing significantly among treatments (-2LR = 9.45, 12.14, $p < 0.05$, 0.01 respectively). Under predation, divergent selection on these traits acted in opposite directions (Fig. 3.3). We simplified visualization and analysis of selection by considering a single 'brightness contrast' trait that represents the difference in brightness between the body and the stripe (calculated as body brightness minus stripe brightness). Selection on 'brightness contrast' varied significantly among treatments (Fig. 3.4; -2LR = 9.58, d.f. = 3, $p < 0.05$). Thus predation favored bright bodies and dull stripes on *Ceanothus* (i.e. positive 'brightness contrasts', $p = 0.06$), but the opposite combination of bright stripes and dull bodies was selected on *Adenostoma* (i.e. negative 'brightness contrasts', $p = 0.01$; combined $p < 0.05$). Conversely, selection was weak and did not approach significance on either host when predation was absent (both $p > 0.35$). Strong selection in the presence of predation apparently favors increased crypsis and has driven adaptive divergence of the ecotypes. Quantitative measures of crypsis were not employed and are not required to test the central prediction of a correlation between selection and trait divergence. However, such measurements could provide additional support for a role for crypsis and refine our understanding of color-pattern evolution in these insects (27, 28).

Five caveats warrant discussion. First, adaptive radiation requires genetically-based population divergence (2). Reciprocal-rearing experiments have shown that population divergence in the linear measurements considered here likely has a strong heritable component (23). Likewise, the presence versus absence of the striped pattern has a simple genetic basis and population divergence in this trait is unaffected by rearing environment (29). Thus the more quantitative aspects of color examined here also likely exhibit a strong genetic basis, although further studies are required to confirm this. Second, divergent selection in herbivorous insects commonly acts via trade-offs

involving physiological, rather than morphological, traits (30, 31). In *T. cristinae,* physiological trade-offs in fitness do not occur (32). Third, the traits examined are not completely independent. However, this does not affect our general conclusions because inter-trait correlations were generally modest (see Table 3.5) and associations between selection and trait divergence are predicted even if selection act on traits indirectly through selection on correlated characters (26). Even more importantly, the general patterns reported above persist when analyses are conducted using principal components (PC) axes, which are uncorrelated (see Tables 3.1 and 3.7). Fourth, estimates of selection could be biased downward in traits that are less divergent between ecotypes (due to higher measurement error in such traits). This did not occur as trait repeatabilities (i.e. error) were uncorrelated with all measures of selection (all $p > 0.35$, bivariate correlation). Fifth, adaptive radiation in a classical sense concerns divergence among species. The patterns detected between ecotypes of *T. cristinae* parallel those observed at the among-species level: in both cases trait divergence and the evolution of reproductive isolation is closely linked to host-plant use, and divergence in cryptic coloration is a central component of diversification (19).

Our results support a central prediction of adaptive radiation – a positive correlation between divergent selection and trait divergence. The findings contrast with previous studies demonstrating that the main effect of predation during adaptive radiation is to influence levels of resource competition by reducing population density through mortality (16, 17). Rather, our findings show that predators can be a critical and direct source of divergent selection during adaptive radiation. A central role for predator-driven selection in adaptive divergence had long been argued in classical studies of crypsis and mimicry (12-14, 18, 27, 28, 33-35) and our results indicate that predation may be a more general mechanism of adaptive radiation than currently appreciated, particularly for many organisms where selection stemming from interspecific competition for resources is weak.

3.4 Materials and Methods

T. cristinae were collected near Santa Barbara, California from January to June 2003 and 2004 using sweep nets. All specimens were photographed alive at standard

distance using a Canon digital camera with external flash in the same room at the University of California Santa Barbara. Each photo also included a ruler and color standards. To estimate the repeatability of all measurements, 305 individuals were photographed twice. A conservative protocol was used such that each replicate photograph was taken on a separate day and the specimens measured twice for each trait (one set of measurements taken from each separate photograph). Previous work on this species has analyzed selection in relation to the presence versus absence of a stripe pattern (22, 25). Selection on quantitative morphology and population divergence in quantitative aspects of color and color pattern was not examined in previous studies.

3.4.1 Quantitative Morphological Divergence

A total of 604 individuals were measured for quantitative morphology using the digital photographs (*Ceanothus* n = 283; *Adenostoma* n = 321). Morphological traits were chosen based upon functional considerations (i.e. color likely affects crypsis) and previous evidence of host-associated morphological divergence (23). A total of 20 measurements were taken in photoshop (Adobe Inc.), which were then collapsed into 11 traits for further analysis as described below (Fig. 3.1). All eleven traits were highly repeatable and thus all traits were retained for further analysis (see Table 3.2 for repeatability estimates).

The first set of traits represents quantitative aspects of color and color-pattern variation. The hue, saturation, and brightness of different body areas were measured using the 'eyedropper tool' set at '5 by 5 average'. We avoided sampling areas reflecting the glare of the flash or that were poorly lit. The three body color measurements of the same type but from different body areas (e.g. hue measurements from different body areas) were averaged to create three 'average' body color variables for further analysis (body hue, body saturation, body brightness). Likewise, the two stripe measurements of each type were averaged to create three stripe color variables (stripe hue, stripe saturation, stripe brightness). Additionally, the area of the stripe relative to the entire body area was estimated by outlining both the stripe and the entire body using the 'lasso' tool and then using the 'histogram' function to calculate relative areas. The second set of traits represents quantitative aspects of body size and shape. The measurements were

taken by measuring the length of the trait and of 2cm on the ruler in the photo using the 'measure tool', and then scaling to absolute length. These linear traits were chosen based upon previous work showing that the ecotypes differed primarily in three traits (overall body size, relative head width and relative femur length; 23) and by the fact that leaves of the different hosts differ markedly in width such that body width might affect crypsis (thus the fourth trait was thorax width).

Divergence in quantitative morphology was examined using both raw and size-corrected values. Size-corrected values are the residual values from a regression of each trait on a principal component (PC) axis that was a general indicator of overall body size. This PC was the first axis derived from a PC analysis that included all the linear measurements (linear measurements are *a priori* indicators of size). This PC axis exhibited high and positive loading for all linear traits (trait loadings; head width – 0.82, thorax width – 0.94, femur length – 0.75; body length – 0.89), and is thus appropriate for use in size standardization (36). Analyses using size standardization with body length alone gave congruent results. Mean divergence between ecotypes in each trait was examined after the trait was standardized to a mean zero and variance one (z-scores; 37).

3.4.2 Manipulative Field Experiment

Survival estimates stem from a previous mark-recapture experiment, where experimental details can be found (25). In summary, a replicated, random blocks design with four treatment levels was used (*Ceanothus* versus *Adenostoma* with avian predators present versus absent). A schematic figure of the experimental set-up can be found in Fig. 3.5. Avian predators were excluded using chicken-wire enclosures (3cm mesh) which were moulded to surround an entire bush. Each of the four treatments was represented twice within each of two study sites with 24 individuals released onto each bush (n = 96 individuals for each of 4 treatments; each bush previously cleared of all *Timema*). Upon release, sex ratios were equal and morph frequencies were similar among bushes. Sample bushes were separated from all other suitable host plants by a minimum distance of 5m (12m is the maximum per-generation dispersal distance; 29). Recapture surveys were conducted 3, 10, 17, and 24 days following release (no individuals recaptured on the final recapture session). At both sites, insectivorous bird species were observed foraging on or

near the experimental bushes. The previous study (25) did not quantify or analyze selection on quantitative traits in any way.

The current study uses recapture in the experiment (recaptured or not) as a proxy for survival (survived or died). This methodology is highly appropriate and effective because 1) highly congruent results regarding survival were obtained for analyses based upon raw recapture probabilities versus results obtained when recapture and survival probabilities were estimated separately (25), 2) it adds simplicity yet tends to yield results similar to more complicated survival analyses when recapture bouts are few (as in our experiment), and 3) it is common procedure in selection analyses as it allows estimation of standardized selection differentials in regression analysis (26). We note that sex had no effect on survival (25) and that sex ratios in the samples used to estimate divergence in morphology are comparable between hosts (C – 95 females and 188 males, A – 75 females and 246 males). Thus the effects of trait values, host-plant, or predation scenario on survival are not confounded by sex. Individuals used in the experiment originate from several populations on each host species (the majority from a contact zone between the host forms where variation is extreme). This approach increases the variation available for selection to act on, and thus facilitates detection of selection (15, 17). Individuals were assigned randomly to treatment, such that use of individuals from both hosts increases the power of our experiment while being highly unlikely to confound our results. Finally, we stress that the effects of host of origin on survival are considered explicitly in our selection analyses (see below).

3.4.3 Selection Analyses

Standardized linear selection differentials were estimated within each of the four treatments (survival as the binary dependent variable) using regression techniques (26). According to standard procedures, each trait was standardized to mean zero and variance one. All analyses were conducted using both linear and logistic regression and the robustness of the results to method of analysis evaluated. We note that our primary goal was parameter estimation rather than significance testing of individual differentials such that selection estimates from linear regression are likely appropriate (26, 38). Whether selection differed significantly among treatments for individual traits was evaluated using

data from all treatments and then testing the significance of interaction between trait value (continuous covariate) and treatment (categorical covariate) in a selection analysis with survival as the dependent variable. Significance levels from logistic regression analyses were assessed using the change in -2log likelihood (-2LR) when a term was removed from the model. Finally, the effects of host of origin and replicate on our selection estimates were considered as described in the results section and Table 3.6.

3.4.4 Correlation between divergent selection and trait divergence

Our central analysis tests for a correlation between the direction and magnitude of divergent selection and the direction and magnitude of trait divergence. The correlation reported in body of the paper stems from a parametric correlation analysis of the relationship between the difference in selection differentials on different hosts (i.e. divergent selection estimated using linear regression) and divergence in raw trait values between ecotypes. This correlation is sufficient and appropriate for evaluating the association between selection and trait divergence. However, to evaluate the robustness of this association, we also report this association using both raw and size-corrected trait values, selection estimates from both linear and logistic regression, and using parametric and non-parametric rank correlation of trait values against divergent selection. Results from all these analyses were congruent. Table 3.1 presents the results of all these analyses, and details can be found in the supporting materials section.

We conducted an explicit test for statistical differences between predation scenarios in the association between divergent selection and trait divergence by analyzing the interaction between predation scenario (present versus absent) and trait divergence. The analysis used repeated measures ANOVA because selection was estimated on the same sets of traits in the presence versus absence of predation such that a paired design is most appropriate (37). Divergent selection is the within-subject factor, with predation present versus absent as factor levels. Trait divergence is included as a continuous covariate.

3.4.5 Analyses on uncorrelated Principal Components Analyses

Inter-trait correlations among the eleven traits examined were relatively low (Table 3.5), but the traits are not completely independent. We thus also estimated the relationship between selection and trait divergence using principal components (PC) axes, which are completely uncorrelated. The analyses supported the analysis using individual trait values, thereby confirming a strong association between selection and trait divergence but only in the presence of predation (Tables 3.1 and 3.7 for detailed results of the PC analyses).

3.5 Acknowledgements

The authors thank D. Schluter, T.E. Reimchen and A. Mooers for comments on previous versions of the manuscript and M. Potter for technical assistance. T.E. Reimchen and J.A. Endler have provided continually useful discussion about selection from predators. Funding was provided by the Natural Sciences and Engineering Research Council of Canada.

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Figure 3.1 The traits examined depicted on representative specimens of each host ecotype.

Body color and stripe color represent three different variables each (hue, saturation and brightness). Individuals from *Adenostoma* tend to exhibit larger and brighter stripes, less-bright bodies, and shorter body size than individuals from *Ceanothus* (all traits except stripe hue and all size-corrected traits except stripe hue and thorax width differ significantly between ecotypes; $p < 0.05$, t-tests).

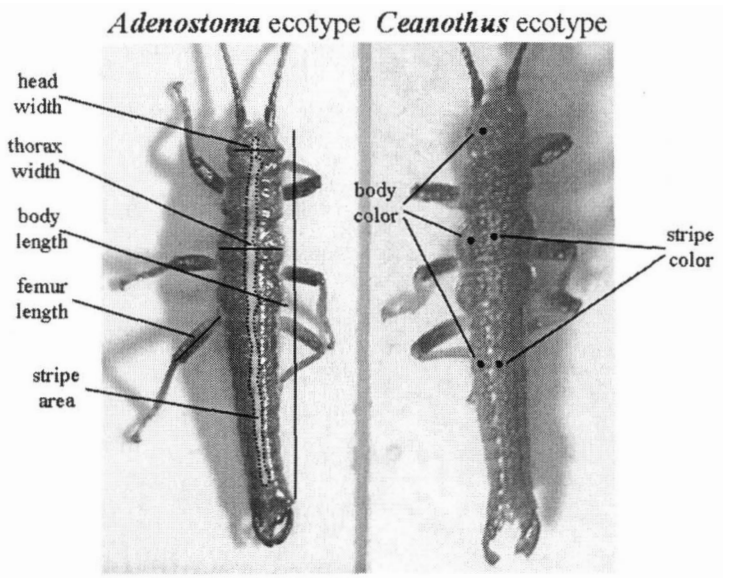

Figure 3.2 The two host-plant species used by the ecotypes of *T. cristinae* (*Ceanothus spinosus* and *Adenostoma fasciculatum*).
The hosts are depicted at three spatial scales. A) The scale of a hillside or mountain slope (roughly 20m x 20m). B) Individual plants. C) Close-ups of each host species. The close-up of *Adenostoma* was taken by C.P. Sandoval. All other photos by P. Nosil.

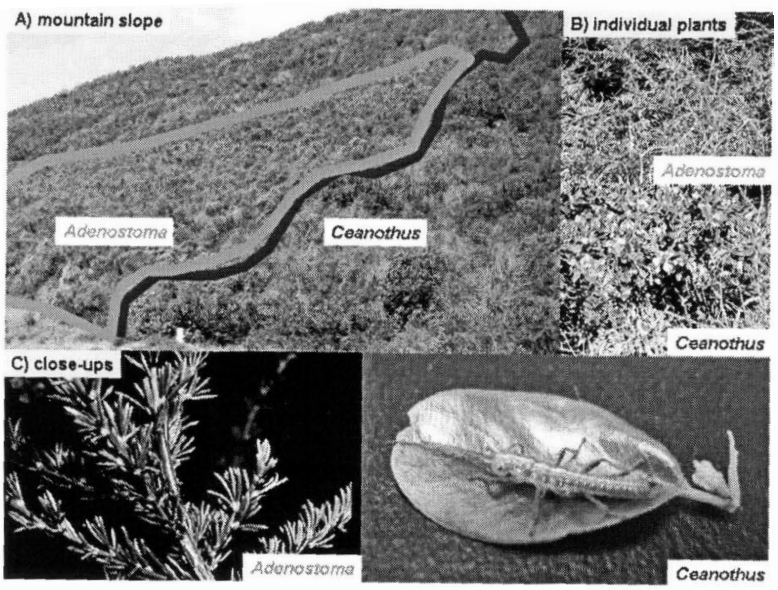

Figure 3.3 The direction and magnitude of divergent selection was positively correlated with the direction and magnitude of trait divergence observed between natural populations using different hosts, but only when selection was estimated in the presence of visual predation (predation scenario x trait divergence interaction, $p < 0.001$).
Moreover, absolute divergent selection was stronger in the presence versus absence of predation for 10 of 11 traits individually ($t_{10} = 4.14$, $p < 0.01$, paired t-test). A) predation present. B) predation absent.

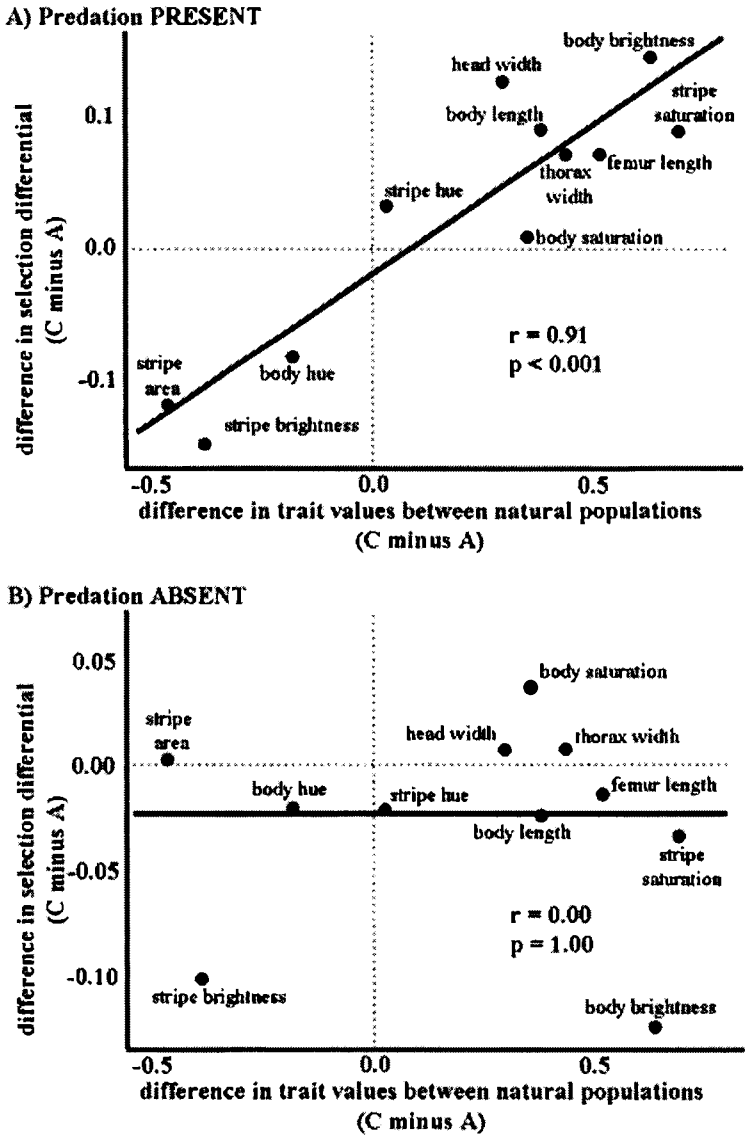

Figure 3.4 Fitness functions depicting the relationship between fitness (probability of recapture as a proxy for survival) and brightness contrast (standardized body brightness minus standardized stripe brightness). Selection on 'brightness contrast' varied significantly among treatments ($p < 0.05$). A) Predation favored bright bodies and dull stripes on *Ceanothus* (B = 0.28 (0.16), p = 0.06). B) Predation favored bright stripes and dull bodies on *Adenostoma* (B = -0.41 (0.18), p = 0.01). C), D). Selection was weak and did not approach significance on either host when predation was absent (B = 0.01 (0.15), 0.06 (0.16), on *Ceanothus* and *Adenostoma* respectively, both $p > 0.35$). The fitness functions are estimated using the cubic spline (39; dashed lines represent standard errors from 10,000 bootstrap replicates).

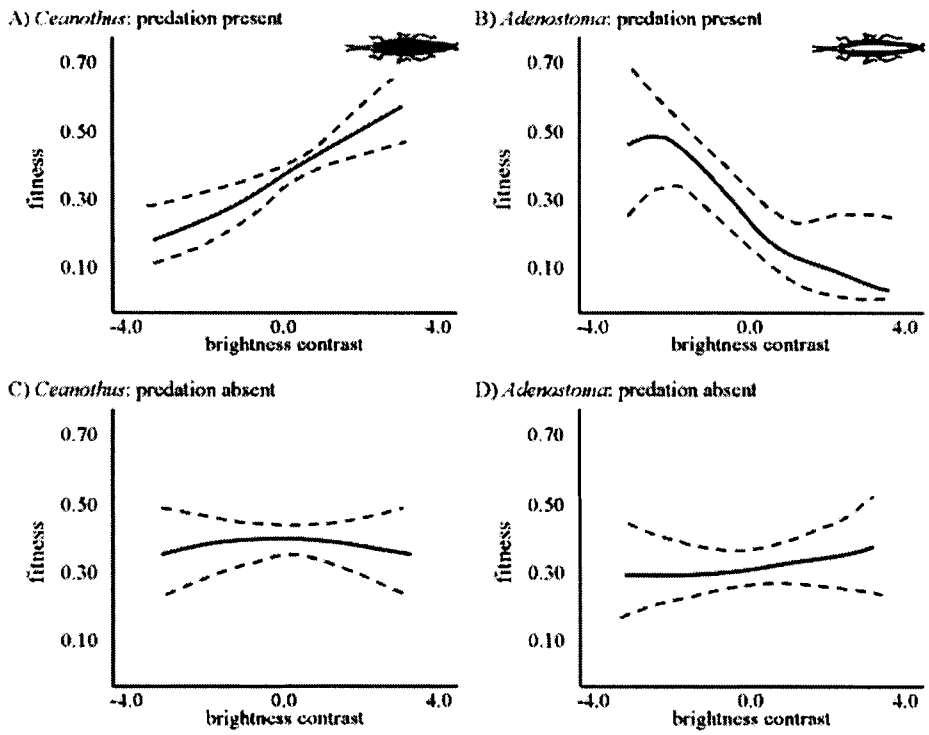

Table 3.1 The correlation between trait divergence and divergent selection in the presence versus absence of predation, estimated using different methods including: 1) estimates of selection from linear (lin) versus logistic (logit) regression, 2) the association between trait divergence and selection analyzed using either parametric (par) or nonparametric (npar) correlation, and 3) the correlation examined using individual trait values and using uncorrelated principal components (PC) axes in the place of actual trait values.

In all cases, results are shown for raw individual trait values, for size-corrected individual trait values, for the four PC axes derived using raw trait values, and for the five PC axes derived using size-corrected trait values. Four and five PC axes were used for raw versus size-corrected traits respectively because they were the number of axes required to explain over 75% of the variation. Significant results were obtained only in cases where selection was estimated in the presence of predation (denoted in bold), and r-values were consistently larger in the presence versus absence of predation.

	predation present				**predation absent**			
	Raw values		Size-corrected Values		Raw Values		Size-corrected values	
	r	p	r	p	r	p	r	p
Individual traits								
lin, par	0.91	**<0.001**	0.70	**0.016**	0.00	0.999	-0.22	0.560
lin, npar	0.75	**0.008**	0.59	**0.056**	-0.19	0.474	-0.15	0.670
logit, par	0.89	**<0.001**	0.68	**0.022**	-0.25	0.450	-0.50	0.116
logit, npar	0.74	**0.010**	0.59	**0.056**	-0.28	0.402	-0.24	0.484
PC axes								
lin, par	0.84	0.16	0.97	**0.006**	0.31	0.69	-0.05	0.94
lin, npar	0.80	0.20	1.00	**<0.001**	0.40	0.60	-0.10	0.87
logit, par	0.84	0.16	0.94	**0.017**	0.26	0.74	0.16	0.80
logit, npar	0.80	0.20	1.00	**<0.001**	0.40	0.60	0.40	0.51

3.7 Supporting Materials

3.7.1 Estimating Measurement Error

Using the 305 specimens that were measured twice, repeatability of each trait was estimated using within-individual and among-individual variance components (which represent error and biological variation respectively) (1). The results indicated that every trait exhibited highly significant among-individual variation (all $p < 0.001$), and thus all traits were retained for further analysis. Repeatability was high in most instances ($r > 0.75$ for 8 of 11 traits) and we stress that even traits exhibiting moderate repeatability exhibited much greater among-individual versus within-individual variation such that their retention for further analysis is warranted (Table 3.2).

3.7.2 Correlation between divergent selection and trait divergence

Our central analysis tests for a correlation between the direction and magnitude of divergent selection and the direction and magnitude of trait divergence. The correlation reported in body of the paper stems from a parametric correlation analysis of the relationship between the difference in selection differentials on different hosts (i.e. divergent selection estimated using linear regression) and divergence in raw trait values between ecotypes. This correlation is sufficient and appropriate for evaluating the association between selection and trait divergence (2-4). However, to evaluate the robustness of this association, we also report this association using both raw and size-corrected trait values, selection estimates from both linear and logistic regression, and using parametric and non-parametric rank correlation of trait values against divergent selection. The results of these analyses are listed in Table 3.1 of the main text (mean raw and mean size-corrected standardized trait values for individuals from each host species are presented in Table 3.3 and directional selection differentials for each treatment estimated using linear (lin) and logistic (logit) regression are presented in Table 3.4).

In the presence of predation, the association between trait divergence and divergent selection was significant for 6 of 8 statistical approaches and marginally insignificant for the other two cases ($p = 0.056$). In contrast, the association did not

approach significance in any cases when predation was absent (all $p > 0.10$). Thus the pattern of a positive association between trait divergence and divergent selection only in the presence of predation is relatively robust to different analytical techniques (note also that inter-trait correlations were generally modest such that different traits are relatively independent, particularly for size-corrected traits, Table 3.5).

We conducted an explicit test for statistical differences between predation scenarios in the association between divergent selection and trait divergence by analyzing the interaction between predation scenario (present versus absent) and trait divergence. The analysis used repeated measures ANOVA because selection was estimated on the same sets of traits in the presence versus absence of predation such that a paired design is most appropriate (5). Divergent selection is the within-subject factor, with predation present versus absent as factor levels. Trait divergence is included as a continuous covariate. The interaction between divergent selection and trait divergence was highly significant in all cases (all $p < 0.001$; raw trait values, selection differentials estimated using linear regression - $F_{1,24} = 35.51$; size-corrected trait values, selection differentials estimated using linear regression - $F_{1,24} = 32.98$; raw trait values, selection differentials estimated using logistic regression - $F_{1,24} = 63.31$; size-corrected trait values, selection differentials estimated using logistic regression - $F_{1,24} = 62.11$). The results confirm statistical differences between predation scenarios in the association between selection and trait divergence.

3.7.3 Effect of host of origin and replicate number on selection estimates

We tested whether our selection estimates were independent from which host species the individuals used in the experiment originated from (i.e. ecotype), and independent from which replicate was considered within each treatment. This was accomplished by adding two interaction terms to the selection analyses described in the main text. Specifically, we examined the trait value by host of origin interaction and the trait value by replicate interaction in ANCOVA analyses within each treatment (the model included the two interaction terms and trait value as a covariate). These interaction terms analyze whether selection on a trait is dependent on host of origin or on replicate respectively. This procedure yielded a total of 88 interactions for analysis (2 interactions

for each of 11 traits for each of 4 treatments). As reported in the main text, these analyses revealed that our estimates of selection on the 11 traits were generally independent from which host the individuals used in the experiment originated from (i.e. ecotype) and unaffected by variation among replicates within treatments. Thus only 5 of 88 interactions were significant at $p < 0.05$, and no interactions retain significance following correction for multiple comparisons (i.e. 11 traits). The full results are shown in Table 3.6.

3.7.4 Analyses on uncorrelated Principal Components Analyses

Inter-trait correlations among the eleven traits examined were relatively low, but the traits are not completely independent. We thus also estimated the relationship between selection and trait divergence using principal components (PC) axes, which are completely uncorrelated. The analyses supported the analysis using individual trait values, thereby confirming a strong association between selection and trait divergence but only in the presence of predation. These analyses use the procedures described above, but applied them to PC axes calculated using standardized trait values, rather than the individual trait values themselves (the PC axes themselves were also standardized). The PC axes were extracted using correlation matrices. PC analyses using raw and size-corrected individual trait values yielded four and five PC axes respectively (see Table 3.7 for trait loadings). Four and five PC axes were used for raw versus size-corrected traits respectively because they were the number of axes required to explain over 75% of the variation. In the presence of predation the correlation between divergent selection on PC axes and divergence between ecotypes in PC axes was strong and positive in all cases (all $r >= 0.80$; Table 3.1 in the main text), with highly significant associations for PC axes generated using size-corrected trait values (note that significance was detected despite a loss of power given these analyses are on five PC axes rather than on eleven individual traits). Conversely, in the absence of predation the correlation between selection and divergence was much weaker, negative in some instances, and never approached statistical significance. These results using PC axes confirm that the results presented in the main text are not driven solely by inter-trait correlations.

3.7.5 Supporting References

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Figure 3.5 Schematic of the experimental set-up. 24 insects were released onto each bush.

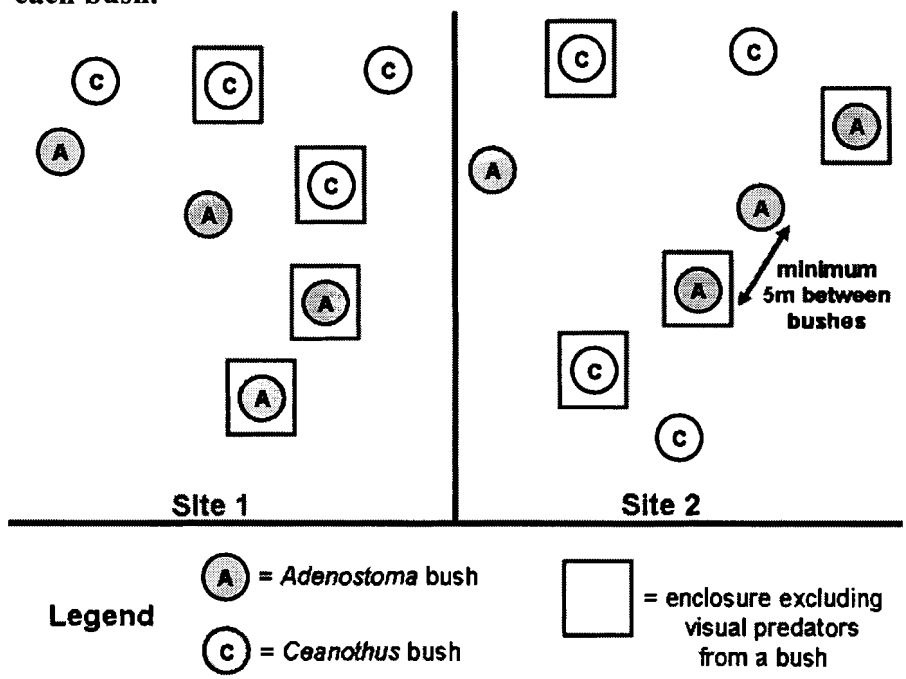

Table 3.2 Repeatabilities for the 11 morphological traits examined (n = 305 individuals).
One-way ANOVA examined within-individual versus among-individual variance components (p-values refer to the statistical significance of the latter, estimated from F-ratios). See text for details.

trait	F-ratio	p	repeatability
Body hue	6.76	<0.001	0.74
Body saturation	42.58	<0.001	0.95
Body brightness	13.29	<0.001	0.86
Stripe hue	36.73	<0.001	0.95
Stripe saturation	27.00	<0.001	0.93
Stripe brightness	10.93	<0.001	0.83
Stripe area	10.01	<0.001	0.79
Head width	2.28	<0.001	0.40
Thorax width	13.47	<0.001	0.86
Femur length	4.89	<0.001	0.66
Body length	10.07	<0.001	0.82

Table 3.3 Mean raw and mean size-corrected standardized trait values for individuals from each host species.

Trait	*Ceanothus* raw mean	*Adenostoma* raw mean	*Ceanothus* size-corrected mean	*Adenostoma* size-corrected mean
Bod hue	-0.099	0.088	-0.155	0.136
Bod sat	0.189	-0.167	0.094	-0.083
Bod brig	0.337	-0.297	0.271	-0.239
Stripe hue	0.014	-0.012	-0.044	0.039
Stripe sat	0.368	-0.324	0.281	-0.248
Stripe brig	-0.208	0.183	-0.189	0.166
Stripe area	-0.250	0.221	-0.202	0.178
Head width	0.157	-0.139	-0.085	0.075
Thorax width	0.233	-0.205	-0.012	0.011
Femur length	0.276	-0.243	0.131	-0.115
Body length	0.201	-0.178	-0.052	0.046

Table 3.4 Directional selection differentials for each treatment estimated using linear (lin) and logistic (logit) regression.
C = Ceanothus, A = Adenostoma

	C: predation		A: predation		C: no predation		A: no predation	
	lin	logit	lin	logit	lin	logit	lin	logit
Body hue	-0.094	-0.467	-0.012	-0.062	-0.012	-0.051	0.007	0.031
Body saturation	0.012	0.052	0.005	0.024	-0.001	-0.002	-0.038	-0.176
Body brightness	0.119	0.541	-0.024	-0.123	-0.085	-0.368	0.038	0.176
Stripe hue	0.017	0.075	-0.015	-0.080	0.015	0.064	0.036	0.165
Stripe saturation	0.001	0.006	-0.085	-0.446	-0.016	-0.670	0.017	0.077
Stripe brightness	-0.014	-0.059	0.135	0.744	-0.087	-0.378	0.013	0.059
Stripe area	-0.060	-0.256	0.057	0.306	-0.001	-0.004	-0.005	-0.023
Head width	0.021	0.091	-0.104	-0.596	0.022	0.094	0.014	0.063
Thorax width	0.013	0.057	-0.057	-0.308	0.008	0.033	0.000	-0.002
Femur length	0.060	0.259	-0.009	-0.046	-0.026	-0.112	-0.013	-0.059
Body length	0.051	0.218	-0.037	-0.198	0.030	0.128	0.053	0.241

Table 3.5 Inter-trait correlations (r-values from bivariate correlations) were generally weak such that different traits are relatively independent, particularly upon size-correction.

Upper-diagonal shows correlations for raw traits and lower diagonal shows correlations for size-corrected traits (codes: body hue – 1; body saturation – 2; body brightness – 3; stripe hue – 4; stripe saturation – 5; stripe brightness – 6; stripe area – 7; headwid – 8; thorax width – 9; femur length – 10; body length – 11).

	1	2	3	4	5	6	7	8	9	10	11
1	X	0.30	0.19	0.86	0.11	-0.09	-0.37	0.06	0.29	0.13	0.21
2	0.24	X	0.15	0.21	0.79	0.08	-0.15	0.22	0.47	0.43	0.27
3	0.13	0.03	X	0.26	0.27	0.21	-0.38	0.24	0.32	0.26	0.25
4	0.86	0.14	0.21	X	0.03	-0.15	-0.47	0.09	0.28	0.15	0.23
5	0.02	0.74	0.14	-0.10	X	-0.18	-0.15	0.27	0.55	0.48	0.36
6	-0.07	0.12	0.25	0.13	-0.17	X	0.32	-0.02	-0.08	-0.05	-0.11
7	-0.34	-0.07	-0.34	-0.44	-0.06	0.31	X	-0.17	-0.22	-0.13	-0.19
8	-0.18	-0.22	-0.03	-0.16	-0.25	0.08	-0.01	X	0.68	0.47	0.63
9	0.27	0.29	0.08	0.19	0.30	-0.03	-0.06	-0.42	X	0.63	0.85
10	-0.04	0.21	0.04	-0.03	0.19	0.01	0.05	-0.37	-0.33	X	0.51
11	0.05	-0.23	-0.07	0.07	-0.18	-0.09	-0.01	-0.37	0.10	-0.53	X

Table 3.6 The interaction between trait value and host of origin (ecotype) or the interaction between trait value and replicate number was significant for only 5 of 88 cases.

Degrees of freedom are $F_{1,96}$ for the host of origin interactions and $F_{3,96}$ for the replicate interactions.

trait	interaction	C: predation		A: predation		C: no predation		A: no predation	
		F	p	F	p	F	p	F	p
Body hue	host	2.90	0.09	0.05	0.83	0.76	0.38	1.17	0.28
Body hue	replicate	0.99	0.40	3.22	0.03	0.66	0.58	0.79	0.51
Body sat	host	0.66	0.42	0.20	0.66	0.56	0.46	0.27	0.61
Body sat	replicate	0.66	0.58	1.62	0.19	1.06	0.37	2.12	0.10
Body brig	host	0.56	0.46	2.38	0.13	1.62	0.21	0.52	0.47
Body brig	replicate	0.41	0.75	0.13	0.94	0.46	0.71	2.36	0.08
Strip hue	host	3.45	0.07	1.46	0.23	1.25	0.27	1.29	0.26
Strip hue	replicate	0.36	0.78	3.18	0.03	0.40	0.76	1.46	0.23
Stripe sat	host	0.10	0.75	0.13	0.72	0.33	0.57	1.50	0.22
Stripe sat	replicate	0.93	0.43	0.30	0.82	0.70	0.55	0.57	0.64
Strip brig	host	0.10	0.75	2.87	0.09	0.03	0.86	0.04	0.84
Strip brig	replicate	1.79	0.16	2.24	0.09	0.41	0.75	0.33	0.81
Strip are	host	5.33	0.02	2.50	0.12	1.08	0.30	0.06	0.81
Strip are	replicate	2.29	0.08	1.52	0.22	0.43	0.73	0.08	0.97
Head wid	host	0.01	0.91	0.04	0.85	0.00	0.96	0.81	0.37
Head wid	replicate	3.49	0.02	0.29	0.84	1.05	0.37	0.81	0.49
Thor wid	host	1.29	0.26	0.84	0.36	0.09	0.76	1.59	0.21
Thor wid	replicate	3.04	0.03	0.11	0.95	0.46	0.71	0.97	0.41
Fem leng	host	1.18	0.28	0.97	0.33	1.65	0.20	1.80	0.18
Fem leng	replicate	1.51	0.22	0.59	0.63	0.30	0.82	1.11	0.35
Bod leng	host	0.39	0.54	1.31	0.26	0.10	0.75	0.81	0.37
Bod leng	replicate	2.70	0.05	0.21	0.89	1.52	0.22	0.31	0.82

Table 3.7 Trait loadings for principal components (PC) axes from PC analyses using raw and size-corrected trait values (which yielded four and five PC axes respectively).
Shown in brackets is the percent of variance explained by that axis.

	PC on raw trait values				PC on size-corrected trait values				
	1 (37)	2 (18)	3 (11)	4 (11)	1 (24)	2 (19)	3 (14)	4 (12)	5 (10)
Body hue	0.49	-0.73	0.15	0.09	0.77	-0.34	-0.22	0.02	0.16
Body saturation	0.64	0.14	0.68	-0.11	0.57	0.63	0.10	0.14	-0.19
Body brightness	0.47	-0.13	0.03	0.56	0.32	-0.02	-0.35	0.54	0.04
Stripe hue	0.49	-0.79	0.01	0.10	0.73	-0.44	-0.32	-0.04	0.17
Stripe saturation	0.67	0.30	0.51	-0.30	0.47	0.68	0.23	-0.03	-0.37
Stripe brightness	-0.14	0.24	0.28	0.87	-0.18	0.17	-0.07	0.82	0.38
Stripe area	-0.45	0.55	0.14	0.12	-0.52	0.30	0.40	0.16	0.31
Head width	0.65	0.32	-0.45	0.11	-0.49	-0.17	-0.50	0.22	-0.60
Thorax width	0.89	0.23	-0.19	0.02	0.55	-0.01	0.55	0.26	-0.15
Femur length	0.71	0.30	0.00	-0.02	0.07	0.64	-0.40	-0.36	0.53
Body length	0.77	0.22	-0.40	0.02	0.04	-0.58	0.63	-0.02	0.17

CHAPTER 4. DOES GENE FLOW CONSTRAIN TRAIT DIVERGENCE OR VICE-VERSA? A TEST USING ECOMORPHOLOGY AND SEXUAL ISOLATION IN *TIMEMA CRISTINAE* WALKING-STICKS*

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4.1 Abstract

Population differentiation often reflects a balance between divergent natural selection and the opportunity for homogenizing gene flow to erode the effects of selection. However, during 'ecological speciation' trait divergence results in reproductive isolation and becomes a cause, rather than a consequence, of reductions in gene flow. In order to assess both the causes and the reproductive consequences of morphological differentiation, we examined morphological divergence and sexual isolation among seventeen populations of *Timema cristinae* walking-sticks. Individuals from populations adapted to using *Adenostoma* as a host plant tended to exhibit smaller overall body size, wide heads and short legs relative to individuals using *Ceanothus* as a host. However, there was also significant variation in morphology among populations within host-plant species. Mean trait values for each single population could be reliably predicted based upon host-plant used and the potential for homogenizing gene flow, inferred from (1) the size of the neighboring population using the alternate host and (2) mtDNA estimates of gene flow. Morphology did not influence the probability of copulation in between-population mating trials. Thus morphological divergence is facilitated by reductions in gene flow, but does not cause reductions in gene flow via the evolution of sexual isolation. Combined with rearing data indicating that size and shape have a partial genetic basis, evidence for parallel origins of the host-associated forms, and inferences from functional morphology, these results indicate that morphological divergence in *T. cristinae* reflects a balance between the effects of host-specific natural selection and gene flow. Our findings illustrate how data on mating preferences can help determine the causal associations between trait divergence and levels of gene flow.

4.2 Introduction

Both natural selection and gene flow can influence the degree of population differentiation observed in nature (Endler 1977). When populations use different habitats, divergent natural selection can cause differentiation in ecologically important characters (Schluter 2000 for review). Conversely, gene flow between divergent populations acts as a homogenizing force, eroding population differentiation (Slatkin 1987). Determining the degree to which selection and gene flow affect population divergence has received renewed theoretical and empirical attention (Crespi 2000; Schluter 2000; Hendry et al. 2001, 2002; Lenormand 2002; Saint-Laurent et al. 2003). In particular, inverse associations between levels of gene flow between populations and the degree of adaptive differentiation in morphological or behavioral traits have been reported in a wide range of taxa, including fish (Lu and Bernatchez 1999; Hendry et al. 2002), amphibians (Storfer and Sih 1998, Storfer et al. 1999), birds (Dhondt et al. 1990; Smith et al. 1997), reptiles (King and Lawson 1995) and arthropods (Sandoval 1994a; Ross and Keller 1995; Riechert 1993; Riechert and Hall 2000; Riechert et al. 2001).

Levels of gene flow often reflect geographic separation and the degree of dispersal between populations, with greater gene flow resulting in reduced trait divergence. However, levels of gene flow can also indicate the degree of reproductive isolation between populations, independent of any geographic barriers to gene flow. Indeed, cause and effect are reversed if trait divergence causes reproductive isolation, thus lowering gene flow between diverging taxa (i.e. 'ecological speciation'; Schluter 1998; Lu and Bernatchez 1999). Thus, associations between trait divergence and reductions in gene flow can arise via two non-exclusive, but opposing, mechanisms. As noted by Hendry et al. (2001, 2002), these two processes could generate a positive feedback loop whereby low gene flow allows adaptive divergence, which in turn further reduces gene flow by increasing reproductive isolation. Despite the appeal of linking trait divergence to the evolution of reproductive isolation (e.g. Tregenza et al. 2000a,b), studies of selection / gene flow balance generally have not elucidated the causal associations between trait divergence, gene flow and reproductive isolation. For example, divergence in traits that act as proximate mating cues will cause reductions in gene flow

via the evolution of sexual isolation. Likewise, when hybrids exhibit intermediate trait values and an intermediate niche is unavailable, divergence in traits important for foraging or anti-predator defense can result in ecologically-dependent postmating isolation (Rundle and Whitlock 2001, Rundle 2002). Without information on whether the traits studied influence reproductive isolation, it is difficult to infer whether trait divergence is a cause or consequence of reductions in gene flow, or both.

In this study, we used a combination of morphological, molecular and behavioral data to analyze the causes and the reproductive consequences of geographic variation in morphology in a phytophagous insect. Because this study system has been the subject of related work on divergent natural selection and speciation (Nosil et al. 2002, 2003), it allows us to draw parallels between the causes of trait divergence and the degree of reproductive isolation. Moreover, we assess whether morphological divergence causes the evolution of sexual isolation, providing a direct test of whether morphological divergence is more likely to be a cause or an effect of reductions in gene flow.

Timema walking-sticks are wingless, phytophagous insects that inhabit the chaparral of California, other areas of the western United States, and northern Mexico (Vickery 1993; Crespi and Sandoval 2000). *T. cristinae* exhibits two genetically-determined color-pattern morphs, with an unstriped morph more common on *Ceanothus spinosus* (Rhamnaceae) and a striped morph more common on *Adenostoma fasciculatum* (Rosaceae) (Sandoval 1993, 1994a,b). These two host-plant species are very structurally different, with *Ceanothus* plants being relatively large, tree-like and broad-leaved, and *Adenostoma* plants being small, bush-like and exhibiting thin, needle-like leaves. *T. cristinae* is heavily preyed upon by visual predators such as lizards and birds, and each morph is more cryptic on the host plant on which it is more common (Sandoval 1994a,b). Patches of these different hosts grow in a mosaic patchwork and local color-pattern morph frequencies in *T. cristinae* are determined by a gene flow – selection balance between patches exhibiting the different selective regimes (Sandoval 1994a).

A balance between natural selection and gene flow also affects the evolution of reproductive isolation in *T. cristinae*. Levels of sexual isolation are greater between pairs of populations using different host plants than between similar-aged populations using

the same host, where mitochondrial and nuclear DNA-sequence divergence is used as a proxy for time since divergence (Nosil et al. 2002). Moreover, sexual isolation has been enhanced in geographic areas where populations using different hosts interbreed and produce offspring with reduced fitness (i.e. 'reinforcement'; Nosil et al. 2003). High levels of gene flow counteract the effects of host-adaptation and reinforcement on the evolution of reproductive isolation. Thus the magnitude of reproductive isolation between populations is greatest when migration rates between populations adapted to alternate host plants are sufficiently high to facilitate reinforcement, but low enough that gene flow does not erode adaptive divergence in mate choice (Nosil et al. 2003).

In this study, we test two hypotheses for the causes of geographic variation in morphology among populations of *T. cristinae*. First, if host-specific natural selection is a primary cause of morphological differentiation, then populations using different host plants should exhibit greater differentiation than populations using the same host plant, and the traits examined should have a genetic basis. However, gene flow into a population, from an adjacent population using the alternate host, may cause the study population to become (1) more similar to other populations using the alternate host and (2) more differentiated from other populations using the same host that are not incurring gene flow. Under this type of a scenario, morphological differentiation evolves under a balance between natural selection and gene flow, with gene flow acting as both a homogenizing and a diversifying force, depending on its context (Slatkin 1987; Ross and Keller 1995).

Second, if divergence in body size and shape has directly driven a reduction in gene flow, via the evolution of sexual isolation, then such morphological traits should influence the probability of copulation in between-population mating trials. This latter hypothesis is of particular interest because color-pattern has diverged among populations of this species (Sandoval 1994a), but does not influence the probability of copulation in between-population mating trials (Nosil et al. 2002). Thus the sexual isolation that has evolved between populations adapted to alternate hosts is independent of colour-pattern (although colour-pattern might influence within-population mate choice; Nosil *et al.* 2002) and the traits causing the substantive levels of sexual isolation observed among populations of this species have yet to be determined. Collectively, our results provide

new insights into the causal associations between trait divergence, gene flow and the evolution of reproductive isolation.

4.3 Materials and Methods

4.3.1 Study sites, Collection and Rearing

First-instar *T. cristinae* were collected from 17 study sites in the Santa Ynez Mountains, California in February 2001 and 2002 using sweep nets. Other species of *Timema* do not occur in sympatry with populations from these sites. Patches of the two host plant species used by *T. cristinae* are usually distributed in parapatric patches of varying size, forming a mosaic at the scale of a mountain slope. However, some host patches are geographically separated from all other host patches by regions lacking suitable hosts. We define a 'population' of walking-sticks as all of the insects collected within a homogenous patch of a single host-plant species. 'Parapatric' insect populations are in contact with a population of insects adapted to the alternative host (i.e. they have a 'neighboring' population), whereas 'allopatric' populations are separated from all other populations adapted to the alternative host by distances > 50 times the per-generation gene flow distance (12m is the mean per-generation dispersal distance and allopatric populations were separated from populations of the alternate host by 1-3km). Parapatric sites were chosen such that each population had only one neighboring population of the alternate host. Individuals were collected from nine populations that utilize *C. spinosus* as a host and from eight populations that use *A. fasciculatum* as a host (Table 4.1).

Walking-sticks were maintained in glass jars at the University of California at Santa Barbara (20 degrees C) with 10-15 individuals per jar. Individuals from different populations and the sexes were kept separate. Animals were reared to maturity (4-6 weeks of rearing) on the foliage of either their native host plant or the alternative host plant and then preserved in 80% ethanol.

4.3.2 Morphometrics

Seven linear measurements were taken on 1004 walking sticks (n = 497 males, 507 females); these measures included head width, left hind-leg femur length, left hind-

leg tarsal length, abdominal length, genital length on females and genital width on males, length of the subgenital plate, and thorax width. Measurements were taken with a digital micrometer under a binocular microscope at 10 to 40 X magnification. Each trait was measured twice and measurement error was low for all traits (all repeatabilities > 0.90, $p < 0.001$). The average of the two measurements was used in all statistical analyses. We also recorded the color-pattern of each individual (striped or unstriped; Sandoval 1994a,b for details). To reduce potential bias, all measurements were done blind to population of origin and were carried out by one individual (P. Nosil).

4.3.3 DNA Sequencing

Migration rates between populations using different host-plant species were inferred using 107 previously published mtDNA (cytochrome oxidase I) sequences collected from the seventeen study populations and from two populations that were each adjacent to one of the study populations, but were not used in morphometrics (mean number of individuals per population = 6.0, range = 3-11; sequences were collected in two previous studies (Nosil et. al 2002, 2003).

4.3.4 Measures of Morphological Divergence

We derived multivariate indices of morphology using principal components (PC1; using correlation matrices) and canonical variate analyses (discriminant analyses). The latter method ordinates *a priori* groups (in this case, population of origin) so that it maximizes the between-group variation in relation to the within-group variation (unlike principal component analysis which ordinates independently of trait contribution to between-group and within-group variation). Discriminant analysis is a powerful technique for the analysis of size-related characters as it overcomes the problem of information redundancy in the character set by taking into account the within-group covariation between characters. There was no evidence for host-associated divergence in the second or third axis (and these axes explained only up to 15% of the variance in morphology); thus, we focus our analysis of shape variation on the first canonical variate axes from these discriminant analyses (CV1 hereafter).

Second, we examined variation in single, size-corrected morphological traits using the residual values from a regression of each trait on PC1, as PC1 was a general index of body size (see Results). We report results using pooled among-group slopes for all cases except thorax width, where we report results using separate within-groups slopes (the relationship between trait size and PC1 tended to be homogeneous among study sites; $p > 0.10$ for the trait size x population interaction in all ANCOVA analyses except for thorax width where $p < 0.05$).

We assessed multivariate population differentiation in morphology using nested ANOVA (PC1, CV1, single size-corrected traits) and nested MANOVA (all size-adjusted univariate traits) analyses. This method allowed us to estimate the amount of morphological variation attributable to variation between hosts, variation among populations within hosts, and variation within populations (error). In *T. cristinae*, the sexes are highly dimorphic in quantitative morphology and thus were treated separately in all cases.

Finally, we also tested whether interpopulation divergence in quantitative morphology is correlated with divergence in color-pattern morph frequency, a trait for which population differentiation has been previously shown to be under a balance between host-specific selection and gene flow (Sandoval 1994a,b). The 17 populations tested yielded 136 pairwise comparisons of divergence in color-pattern morph frequency and quantitative morphology. We tested for associations between populations distance matrices using the Mantel test, a nonparametric method that evaluates the strength of associations between matrices using randomization (Manly 1997). Significance levels were estimated using 10,000 randomizations. The Mantel program was designed by B. Manly and is commercially available through Western Ecosystems Technology Inc.

4.3.5 Relative Population Sizes and the Geographic Potential for Gene Flow

Previous work in *T. cristinae* has shown that host-plant patch size and walking-stick population size are strongly and positively correlated ($r = 0.79$ and 0.73 for *Ceanothus* and *Adenostoma* patches respectively, $n = 13$ patches of each host, data from Sandoval 1994a). In additions, levels of mtDNA gene flow into a study population, from

its neighbouring population, increase with increasing relative size of the host-plant patch used by the neighboring population ($r = 0.86, 0.62, 0.92$, $p < 0.01, 0.05, 0.01$ for the effective number of migrants (Nm), migration rate estimated using effective population sizes (m), and the migration parameter M ($M = m$ / mutation rate) respectively, $n = 8$; data from Nosil et al. 2003, obtained using the coalescent-based methods of Beerli and Felsenstein 2001). Consequently, the size of the host plant patch occupied by a study population, relative to the size of the patch occupied by its neighboring population using the alternate host, reflects the 'geographic potential for gene flow' into a study population. Patch sizes were estimated from aerial photographs (as in Sandoval 1994a; Nosil et al. 2003).

4.3.6 Predicting Mean Trait Values Using a Selection – Gene Flow Balance

We tested whether mean trait values for each single population reflect the effects of a balance between selection and gene flow using two, independent measures of gene flow : (1) geographic potential for gene flow, calculated as the relative sizes of the study populations and their neighboring population of the alternate host and, (2) coalescent-based estimates of migration rates between adjacent populations, calculated using mtDNA sequence variation.

Under the first method, when populations use *Ceanothus* as a host plant, the size of the neighbouring population of *Adenostoma* serves as the index of the opportunity for gene flow to erode local adaptation to *Ceanothus*. Conversely, when populations use *Adenostoma* as a host plant, the size of the neighbouring population of *Ceanothus* serves as an index of the opportunity for genes conferring adaptation to *Ceanothus* to be introduced into the population. Consequently, single *Adenostoma* populations were assigned values of [size of neighbouring patch / (size of study patch + size of neighbouring patch)]. Patches of *Ceanothus* represent a divergent selective regime and were assigned values of [1- [size of neighbouring patch / (size of study patch + size of neighbouring patch)]]. Thus for each study population the value assigned to it simply represents the proportion of the total area (study population area plus neighboring population area) occupied by *Ceanothus*.

Under the second, DNA-based methods, we used two independent approaches to derive indices of the balance between selection and gene flow. The first approach is based on estimating the proportion of individuals within a population that were derived from a *Ceanothus* population, using estimates of N_e and Nm. For populations using *Adenostoma* as a host plant, this value is represented by the proportion of individuals in the population that are estimated to be migrants from the neighbouring population of the alternate host (m). Conversely, for populations using *Ceanothus* as a host plant, this value is represented by the proportion of individuals in the population that are not migrants ($1 - m$). We used our mtDNA sequence data to obtain estimates of m into each parapatric study population, from their neighbouring population using the alternate host (with m assumed to be zero for allopatric populations). To begin, we estimated Nm using the methods of Beerli and Felsenstein (2001), which are tailored for estimating asymmetric migration rates between pairs of population and have less restrictive assumptions than F_{st}-based methods (see Whitlock and McCauley 1999 for discussion). Second, we estimated m from Nm by calculating total population size (using previously published regression equations for patch size versus population size; Sandoval 1994a), and dividing this number by 0.5 to obtain female population size (mtDNA is maternally inherited). We note that although N is unlikely to be equal to N_e (Frankham 1995), our analyses depend only on variation in relative migration rates and are thus unaffected by N_e/N ratios (i.e. scaling N to N_e changes only the absolute estimates of m).

Under the second DNA-based approach, we used the migration parameter M ($M = m$ / mutation rate), obtained from MIGRATE (Beerli and Felsenstein 2001), to derive an index of the balance between selection and gene flow. The relative combined area of a study population and its neighboring population occupied by *Ceanothus* was set to zero for allopatric *Adenostoma* populations and (arbitrarily) to 500 for allopatric *Ceanothus* populations. The value of M obtained from MIGRATE was assigned to parapatric *Adenostoma* populations while parapatric *Ceanothus* populations were assigned a value of 500 – M (maximum M was 345). We note that because we conducted analyses of selection – gene flow balance using a nonparametric test (Spearman Rank Correlation; see below) changing the scaling factor (i.e. the value 500) does not affect our results. We

stress that our analyses of the relationship between gene flow and neighbouring population size depend only on relative (rather than absolute) migration rates.

Spearman rank correlations were used to test whether population mean values for PC1, CV1 and each size-adjusted single trait were correlated with our three indices of selection – gene flow balance. Due to the *a priori* expectation that gene flow would erode host-associated divergence, we report significance levels from one-tailed tests.

4.3.7 Rearing Environment and Morphological Variability

For a subset of the populations studied (n = 6 populations), we raised some of the individuals on their native host and some on the alternative host (Table 4.1). We used two-way ANOVAs to test whether morphological variation among individuals from these populations was influenced by genotype (native population), rearing environment (host reared on) and a genotype by environment interaction. We attained congruent results when we used native host, rather than native population, as our 'genotype' term.

4.3.8 Morphological Divergence and Reproductive Isolation

We assessed whether morphological divergence contributes directly to the evolution of premating isolation by testing for a morphological basis to sexual isolation among populations of *T. cristinae.* Sexual isolation was estimated in a previous study, using no-choice mating trials. One male and one female were placed in a 10cm petri dish and at the end of one hour we scored whether the male and female were paired (male on female without genital contact) or not, and copulating or not (Nosil et al. 2002 for details).

In the current study, we restrict our analyses to between-population mating trials, as we are interested in the potential influence of morphological divergence between populations on the probability of interbreeding between populations. First, we used logistic regression to test if the difference in trait values between a male / female pair influenced the probability of copulation (significance tested using likelihood ratio tests, all d.f. = 1). Adding population-pair as a factor (see below) in the logistic regression

yielded no significant main effects or interaction terms and thus is excluded from the analyses presented.

Second, we tested for assortative mating by morphology in the between-population mating trials that resulted in copulation. Among copulating pairs, ANCOVA's were used to determine whether male trait values were correlated with female trait values and to test whether such a relationship differed among the 28 different pairs of populations examined (i.e. test for homogeneity of slopes); in Nosil et al. (2002), sexual isolation was estimated for all pairwise comparisons between eight of the populations in the current study, yielding a total of 28 pairwise comparisons between populations that pertain to the current study. We report results from analyses using both single traits and our multivariate indices of size and shape. All statistical analyses were conducted using SPSS (v.10.1).

4.4 Results

4.4.1 Morphological Divergence Between Hosts and Among Populations

Populations of *T. cristinae* from *Adenostoma* differed significantly in multivariate morphology from those on *Ceanothus* (Table 4.2). Although populations using the same host tended to be morphologically similar, a significant proportion of the morphological variation was also partitioned among populations using the same host (e.g. significant variation between hosts and among populations within hosts; Table 4.2). For example, for both PC1 and CV1 comparable proportions of morphological variation were partitioned between hosts and among populations within hosts (Table 4.2; see below for description of these indices of morphological variation). Graphical analyses confirmed that the study populations exhibit both host-specific and population-specific variation in morphology: while populations using the same host tend to cluster together in morphospace, two *Adenostoma* populations were situated near the bulk of the *Ceanothus* populations and three *Ceanothus* populations were situated near the bulk of the *Adenostoma* populations (Fig. 4.1). Analyses of single, size-adjusted trait values were congruent with multivariate results; for most traits, we observed both host-specific and population-specific morphological variation (Table 4.2).

4.4.2 Indices of Morphological Divergence

In a principal components analysis, all the measured traits exhibited high and positive loadings for PC1, indicating that PC1 largely reflects variation in general body size (Table 4.3). In a discriminant analysis, the first canonical variate axis (CV1) explained 58% of the variance in male morphology. CV1 exhibited high positive loadings for femur and tarsal lengths but a strong negative loading for head width, indicating that walking-sticks with high scores for CV1 are characterized by narrow heads and long legs (Table 4.3). For females, the first canonical variate axis in the discriminant analysis explained 55% of the variance. As in males, CV1 exhibited a high positive loading for femur length but a high negative loading for head width, indicating that the same traits contribute to between-population shape variation in both sexes (Table 4.3; correlation between group centroids in females and group centroids in males, $r = 0.93$, $p < 0.001$).

We detected host-specific morphological divergence in body size (PC1) and shape (CV1) in both males and females. Specifically, PC1 scores were higher for individuals from populations using *Ceanothus* than for individuals from populations using *Adenostoma* (Table 4.2). Likewise, CV1 scores were higher for individuals from populations using *Ceanothus* than for individuals from population using *Adenostoma*. Thus individuals from populations using *Ceanothus* tended to be larger in overall body size and have relatively small heads and long legs.

Analyses of single, size-adjusted traits were congruent with multivariate analyses ; individuals from populations using *Ceanothus* tended to exhibit smaller, size-adjusted head widths and larger, size-adjusted femur lengths (Table 4.2).

4.4.3 Predicting Mean Trait Values Using a Selection – Gene Flow Balance

Mean trait values for each population could be predicted by host-plant used and the opportunity for homogenizing gene flow. Thus, population means for PC1 (body size) and CV1 (body shape) were significantly correlated with both the geographic (% total area occupied by *Ceanothus*) and DNA-based indices of the balance between selection and gene flow (males and females, all $p < 0.05$, Table 4.4). Moreover, population means for the two traits that contribute most to CV1 were often correlated with our indices of

the balance between selection and gene flow, even following size-adjustment (size-adjusted head width and size-adjusted femur length, Table 4.4; all other size-adjusted traits $p > 0.05$ in all cases). Thus homogenizing gene flow from neighboring populations of the alternate host accounts for the morphology of populations that exhibit size and shape variation indicative of populations using the alternate host (Fig. 4.1).

Because mean PC1 and CV1 values for each population were correlated with one another ($r = 0.79, 0.71$ for males and females respectively, both $p < 0.01$), we assessed the effects of selection - gene flow balance on each morphological variable independently in multivariate analyses (separate analyses for each sex; PC1 and CV1 treated as independent variables). Multiple regression models including both PC1 and CV1 were significant overall for both the geographic and the DNA-based indices of selection - gene flow balance (all $r > 0.60$, all $p < 0.05$). We were able to statistically distinguish the independent contributions of PC1 and CV1 to the overall model only for the geographic selection - gene flow index in males (PC1, $B = 41.63$, S.E. $B = 19.79$, $p < 0.05$; CV1, $B = 8.97$, S.E. $B = 14.66$, $p = 0.55$) and for the DNA-based selection - gene flow index in females (PC1, $B = 5.11$, S.E. $B = 17.09$, $p = 0.77$; CV1, $B = 38.21$, S.E. $B = 17.33$, $p < 0.05$; individual contribution of PC1 and CV1 non-significant in the other two regression analyses, all partial $r < 0.10$, all $p > 0.10$). Thus both size and shape variation among populations appears to be influenced by a balance between selection and gene flow.

Finally, population differentiation in color-pattern morph frequency was significantly correlated with population differentiation in PC1 (Mantel test, $r = 0.37, 0.29$ for males and females respectively, both $p < 0.001$) and CV1 ($r = 0.52, 0.38$ for males and females respectively, both $p < 0.001$). We note that mean PC1 and CV1 scores do not differ between color-pattern morphs within populations for males (all $p > 0.10$, nested ANOVA) but do differ between color-pattern morphs within populations for females (mean PC1 = 0.31, -0.33, mean CV1 = 0.36, -0.40 for unstriped and striped morphs respectively, $F_{15,425} = 1.98, 172$ for PC1 and CV1 respectively, both $p < 0.05$; nested ANOVA). Thus quantitative morphology is likely to have diverged among populations via direct selection on size and shape, selection on a correlated trait (i.e. color-pattern), or some combination of these processes.

4.4.4 Rearing Environment and Morphological Variability

The results of the common garden experiment revealed that size and shape variation apparently have a partial genetic basis (highly significant 'genotype' term for all variables; Table 4.5; Fig. 4.2). Environmental effects, when detected, were interactive with genotype or much weaker than the effects of genotype. For example, for PC1 in both males and females, the effects of genotype (native population) were interactive with the effects of environment (host reared on). Individuals from *Ceanothus* populations tended to grow larger when reared on their native host than when reared on the alternate host, whereas the morphology of individuals from *Adenostoma* was unaffected by rearing environment (Fig. 4.2).

4.4.5 Morphological Divergence and Reproductive Isolation

There was no evidence that population divergence in morphology contributed to the evolution of reproductive isolation. When the sexes were from different populations, the probability of copulation was not influenced by the difference between them in body size (likelihood-ratio (LR) tests from logistic regression analyses; same-host pairs, $LR = 1.69$, $p = 0.19$, $n = 416$; different-host pairs, $LR = 0.01$, $p = 0.99$, $n = 480$) or body shape (same-host pairs, $LR = 1.90$, $p = 0.17$; different-host pairs, $LR = 0.06$, $p = 0.81$; $p > 0.05$ for all single traits as well). Moreover, in the between-population mating trials that did result in copulation, there was no evidence for assortative mating by morphology; for all the variables examined, the relationship between male trait values and female trait values did not differ among the 28 pairs of populations tested (all $p > 0.05$, ANCOVA test for homogeneity of slopes) and it was not significant overall in any case (all $r < 0.10$, all $p > 0.05$).

4.5 Discussion

We detected host-specific morphological divergence in *T. cristinae*: on average, individuals from populations using *Adenostoma* as a host plant exhibited smaller overall body size and shorter legs and wider heads than individuals from populations using *Ceanothus* as a host. However, there was also significant variation in morphology among populations within host-plant species, which was associated with variability in levels of

gene flow between populations using alternate hosts. Thus the degree of morphological differentiation observed among populations of *T. cristinae* reflects a balance between host-specific selection and homogenizing gene flow.

4.5.1 Natural Selection and Host-Associated Morphological Divergence

Multiple lines of evidence implicate natural selection as the cause of morphological differentiation in *T. cristinae*. First, morphometric differences between allopatric populations using different hosts were always in the same direction; genetic drift is highly unlikely to cause different populations in similar environments (i.e. hosts) to converge on similar morphologies. A role for selection is strengthened if similar traits have evolved independently, via parallel evolution, in multiple populations that inhabit similar environments (Schluter and Nagel 1995). Phylogenetic analysis of mitochondrial and nuclear DNA (Nosil et al. 2002), coupled with population-genetic and nested-cladistic analyses (Nosil and Crespi, unpublished data), indicate that divergence in host-plant use among these *T. cristinae* populations has occurred multiple times. Thus, morphological divergence may have occurred repeatedly and in parallel with divergence in host plant use, strongly implicating selection as the cause of evolution.

Second, phylogeographic and molecular-genetic data also indicate that differentiation via genetic drift is unlikely to have caused the greater morphological divergence observed between pairs of populations using different versus the same host plant. Pairs of populations using different host plants are not more differentiated in mtDNA or at a nuclear locus (ITS-2) than are pairs of populations using the same host plant (Nosil et al. 2002). Moreover, levels of gene flow between adjacent pairs of *T. cristinae* populations are generally too high to allow differentiation via genetic drift (e.g. $Nm > 1$; Nosil et al. 2003). For example, previous work on these *T. cristinae* populations has shown that adjacent pairs of populations using different host plants are weakly or not differentiated at mtDNA, while geographically-separated populations are strongly differentiated (mean Fst = 0.07, 0.31 respectively; Nosil et al. 2003), indicative of substantial gene flow between neighboring populations. Finally, consideration of functional design suggests that host-specific differences in size and shape represent adaptations to divergent predation regimes, with small body size being important for

crypsis when resting against the thin, needle-like leaves of *Adenostoma* but not against the broad leaves of *Ceanothus*. The importance of small size for crypsis on *Adenostoma* is supported by divergence in color-pattern between populations of *T. cristinae* using different hosts. Thus, populations using *Adenostoma* exhibit a much higher frequency of the striped color-pattern morph than do *Ceanothus* populations and the striped morph is much more cryptic on *Adenostoma* than is the unstriped morph (Sandoval 1994a,b). The thin, white, longitudinal stripe along the dorsal surface of this morph apparently functions as disruptive coloration, breaking up the insect's body into smaller segments and improving crypsis against the thin leaves of *Adenostoma*. Such disruptive coloration is expected and common among other cryptic animals that live in heterogeneous environments (Endler 1990; Merilaita et al. 1999).

Adenostoma and *Ceanothus* are also very structurally different, with *Ceanothus* plants being larger, woodier and more tree-like than the small, bush-like *Adenostoma* plants. Host-specific morphological differences could also be related to differences in the types of morphology that facilitate efficient movement and manoeuvring on structurally-different plants (e.g. Moran 1986; Bernays 1991). Under either of the above scenarios, fecundity selection for larger size in females (Leather 1988) could be offset by host-specific selection for smaller size on *Adenostoma*, exerted either by visual predators or by host plant surfaces. Notably, host-specific natural selection, rather than genetic drift, has caused the evolution of reproductive isolation (Nosil et al. 2002) and possibly physiological divergence, as shown by the genotype by environment interactions detected in this study.

The results of our common garden experiment suggest that the traits examined have a genetic basis. Because each test animal was born in the wild and spent a brief period on its native host prior to capture in the first instar, some of the morphological variation observed might be attributable to maternal effects (Mosseau and Dingle 1991) or environmental induction (e.g. Gillham and Claridge 1994). However, size and shape are likely to be under at least partial genetic control because : (1) the time spent on the native host in the field is negligible relative to the time spent being reared in the lab, (2) the magnitude of the genotype effects was large and highly significant (this result is independent of environmental, but not maternal, effects), (3) environmental effects on

morphology were either nonsignificant, interactive with genotype, or much weaker than the effects of genotype, (4) the results of common garden experiment were highly consistent across the six populations tested, (5) previous studies have consistently revealed a genetic basis to morphological variation in other insects (e.g. Carroll and Boyd 1992; Arnqvist and Thornhill 1998) and (6) if size and shape in *T. cristinae* did not have a genetic basis, it is exceedingly unlikely that the pattern of variation in natural populations would conform to that expected under a balance between selection and gene flow. Due to relatively low absolute migration rates (Sandoval 1993, Nosil et al. 2003), most individual *T. cristinae* spend their entire development on the same host plant species; environmental or maternal effects on morphology cannot account for why (as a result of gene flow) some populations using *Adenostoma* exhibit the morphology typical of *Ceanothus* populations, and visa versa.

Collectively, our results implicate host-specific natural selection as the cause of morphological divergence in *T. cristinae*. Measurements of selection will allow a direct test of this hypothesis (Lande and Arnold 1983; Endler 1986), estimation of the degree to which gene flow prevents populations from attaining optimal trait values (cf. Hendry et al. 2001), and an assessment of whether host-associated divergence in quantitative morphology represents a correlated response to divergent selection on color-pattern within populations, given that female morphology differed between color-pattern morphs within populations). Studies of phytophagous insects have traditionally focused on detecting evidence for physiological adaptations to the use of different host plants (Rausher 1982; Via 1989; Sheck and Gould 1993; Craig et al. 1997). Our results suggest that host-specific selection on insect morphology may also be common (see also Moran 1986; Bernays 1991; Carroll and Boyd 1992).

4.5.2 Parallels with Previous Studies of Selection - Gene Flow Balance

Previous work on a wide range of taxa has demonstrated inverse associations between trait divergence and levels of gene flow (e.g. fish, Lu and Bernatchez 1999; Hendry et al. 2002; Saint-Laurent et al. 2003; amphibians, Storfer and Sih 1998; Storfer et al. 1999; birds, Dhondt et al. 1990; Smith et al. 1997; reptiles, King and Lawson 1995; insects, Sandoval 1994a; Ross and Keller 1995; Riechert 1993; Riechert and Hall 2000;

Riechert et al. 2001). Moreover, such associations have been documented for morphology (e.g. Sandoval 1994a; Lu and Bernatchez 1999; Hendry et al. 2002; Saint-Laurent et al. 2003), behavior (e.g. Riechert 1993; Storfer and Sih 1998; Storfer et al. 1999; Riechert et al. 2001) and life-history traits (Dhondt et al. 1990), and the traits examined span a wide range of functions (e.g. antipredator defense, Sandoval 1994; Storfer and Sih 1998; Storfer et al. 1999; foraging ability, Hendry et al. 2002; flight, Smith et al. 1997; agonistic behavior, Riechert 1993; Riechert and Hall 2000; Riechert et al. 2001). Inverse associations between gene flow and trait divergence thus appear to be common and widespread in nature, suggesting that trait divergence often reflects a balance between selection and gene flow.

Our study expands on previous studies of selection - gene flow balance by testing whether the traits examined also influence mate choice, making it possible to infer the degree to which trait divergence is a cause versus a consequence of reduction in gene flow. We detected no evidence that body size or shape influences the probability of copulation in between-population mating trials, indicating that morphological divergence among populations does not reduce gene flow via the evolution of sexual isolation. Thus morphological divergence is more likely to be a consequence than a cause of reductions in gene flow. Given the ability of our mating data to elucidate the causes of variation in levels of reproductive isolation (see Nosil et al. 2002, 2003) and given the large size of our samples, this result is unlikely to stem from a lack of statistical power. This same causal association exists for color-pattern morph frequency in *T. cristinae*, where the frequency of the more cryptic morph within a patch is inversely related to the potential for gene flow from adjacent patches of the alternate host (Sandoval 1994a) and color-pattern is also not used in between-population mate choice (Nosil et al. 2002). Although morphological divergence does not reduce gene flow via premating isolation, the effects of morphological divergence on ecologically-dependent postmating isolation (i.e. reduced 'hybrid' fitness; Rundle 2000; Rundle and Whitlock 2001) are unknown and offer promising avenues of further research.

Finally, we note that a large proportion of geographic variation in morphology remained unexplained by our indices of the balance between selection and gene flow. Several processes could account for this unexplained variation, including variation in the

strength of selection among populations of the same host, inaccuracy in our estimates of gene flow, and rare, episodic instances of gene flow into the currently 'allopatric' populations.

4.5.3 Conclusions

Our results have broad implications for studies of natural selection and the causes of speciation. Although assortative mating by size is common among insects (Crespi 1989) and despite high levels of reproductive isolation among populations of *T. cristinae* (Nosil et al. 2002, 2003), we did not detect size or shape-assortative mating within this species. Moreover, previous studies have shown that the traits used in within-populations mate choice do not always contribute to between-population mating discrimination or to species recognition (e.g. Claridge and Morgan 1993; Boake et al. 1997; Nosil et al. 2002). Collectively, these findings indicate that data on between-population mating preferences are required to determine if and how inter-population trait differentiation is causally related to the evolution of premating isolation.

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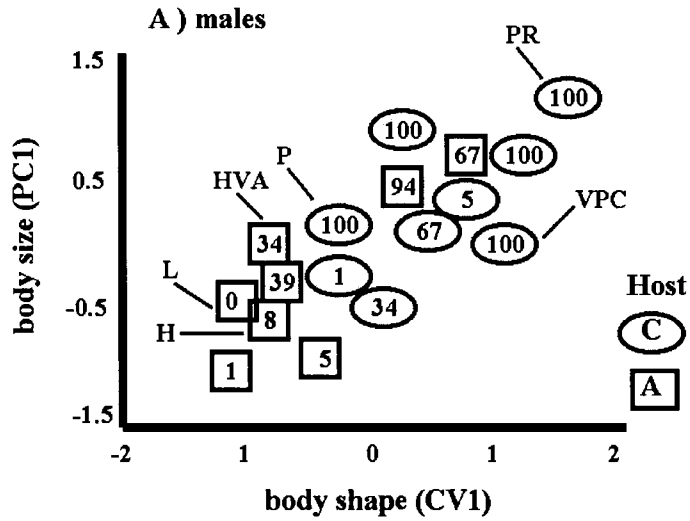
Figure 4.1 Inter-population divergence in morphology for 17 host-associated populations of *T. cristinae*.

Values shown are population means for PC1 (an index of overall body size) and group centroids for CV1 (an index of shape, see Table 4.3 for trait loadings). Marker depicts which host a population utilizes. Ovals - *C. spinosus*. Squares - *A. fasciculatum*. Some populations have a neighboring population of the alternative host (parapatric sites), while others do not (allopatric sites). The proportion of the combined area of a study population and its neighbor that is occupied by *Ceanothus* is shown within each maker (e.g. 0 and 100 for allopatric *Adenostoma* and *Ceanothus* populations respectively). Population codes are shown for the six populations used in the common garden experiment. A) males. B) females.



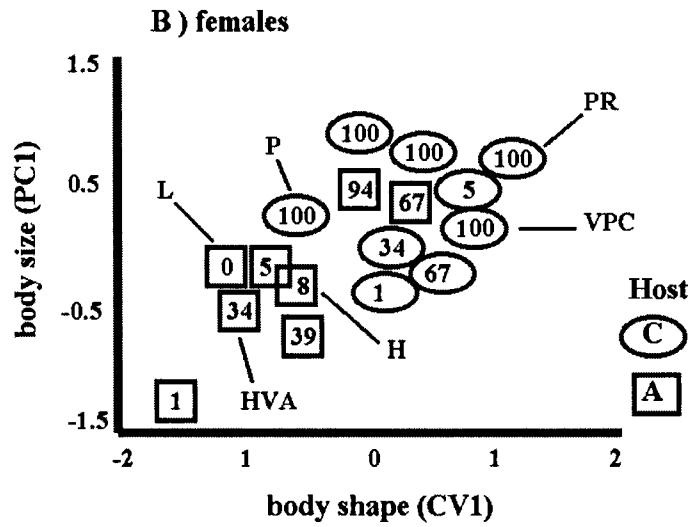
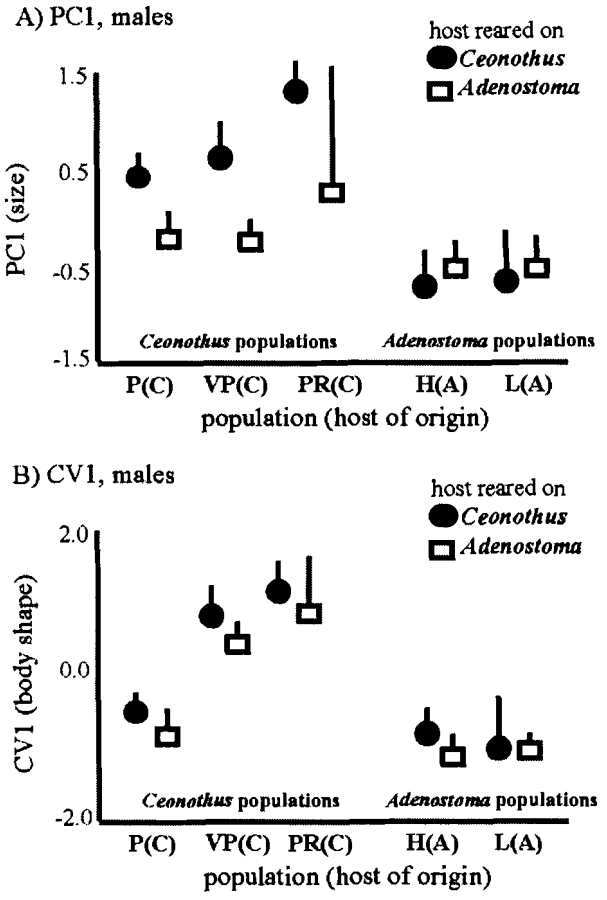
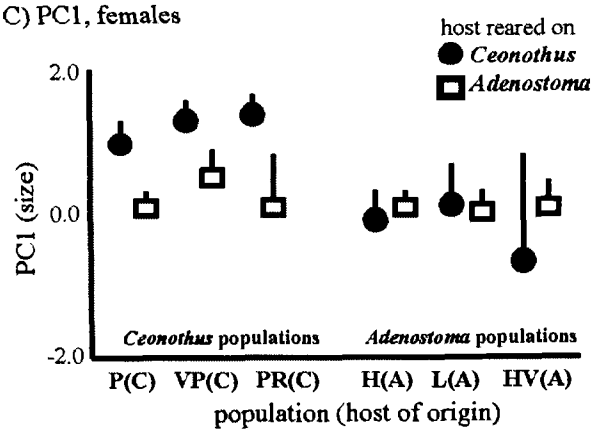


Figure 4.2 Mean body size (PC1) and body shape (CV1) for walking-sticks collected from *Ceanothus* versus *Adenostoma*, and reared in the laboratory on either their native or the alternate host.

Mean trait values (± 95% C.I.) are shown for each population, and statistical results are presented in Table 4.5. Males from HV(A) are not shown, as only two individuals were reared on *Ceanothus* successfully.




C) PC1, females
host reared on
Ceonothus
Adenostoma
2.0
0.0
-2.0
PC1 (size)
Ceonothus populations
Adenostoma populations
P(C) VP(C) PR(C) H(A) L(A) HV(A)
population (host of origin)

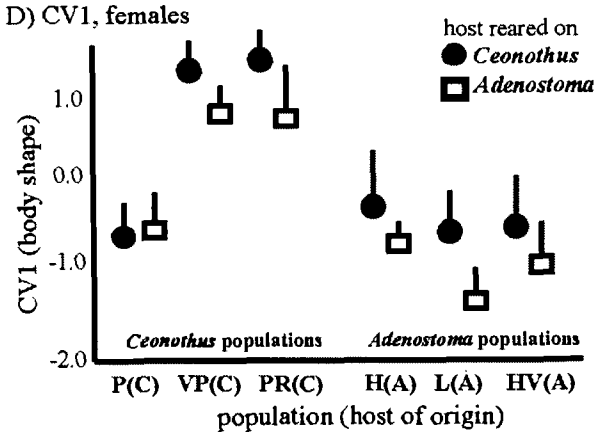

D) CV1, females
host reared on
Ceonothus
Adenostoma
1.0
0.0
-1.0
-2.0
CV1 (body shape)
Ceonothus populations
Adenostoma populations
P(C) VP(C) PR(C) H(A) L(A) HV(A)
population (host of origin)

Table 4.1 Numbers of male and female walking stick insects measured from each of the seventeen study sites (C = *Ceanothus* sites; A = *Adenostoma* sites). Also shown for each study population is the relative size (geographic area) of the neighbouring patch of the alternative host plant (zero for allopatric populations) and the proportion of individuals reared on the alternative (versus native) host plant. For cases where the population used in the current study was also examined in Sandoval (1994a), the number in brackets beside the population name refers to the site number in Sandoval (1994a).

Population	Host	Relative Size of Neighbor	N (males)	% reared on alternate host	N (females)	% reared on alternate host
P (17)	C	0	58	40	78	44
HVC	C	0.66	15	0	10	0
HVA (15)	A	0.34	32	6	35	20
M (8)	A	0.39	38	0	25	0
L (13)	A	0	43	35	61	44
VPC	C	0	62	53	57	53
VPA (2)	A	0.94	26	0	28	0
OUTA	A	0.67	27	0	28	0
PR (1)	C	0	34	15	48	19
MBOXC (10)	C	0.95	31	0	26	0
OGC (12)	C	0.99	25	0	30	0
H (5)	A	0.08	52	33	48	25
MBOXA (10)	A	0.05	4	0	3	0
OGA (12)	A	0.01	15	0	7	0
SC	C	0	17	0	7	0
OUTC	C	0.33	13	0	10	0
PE	C	0	5	0	6	0

Table 4.2 Results of nested ANOVA (PC1, CV1, single size-correct traits) and nested MANOVA (all size-adjusted traits) analyses estimating the proportion of morphological variation attributable to variation between hosts and variation among populations within hosts.
Also shown are mean (s.d.) trait values for populations using each host plant.

Trait	F-ratio Hosts	% Hosts	F-ratio Populations	% Pops	Mean (s.d.) *Ceanothus* populations	Mean (s.d.) *Adenostoma* populations
Males						
PC1	6.57*	14.5	8.35***	17.8	0.25 (0.99)	-0.27 (0.77)
CV1	5.92*	18.4	15.94***	28.4	0.47 (1.21)	-0.52 (1.20)
MANOVA (sc)	11.46***	-	4.30***	-	-	-
ANOVA (sc)						
head width	0.99	2.5	3.39***	8.5	1.69 (0.05)	1.71 (0.05)
femur length	24.27***	9.7	8.34***	18.8	2.97 (0.10)	2.90 (0.09)
tarsal length	5.88*	2.4	3.04***	6.8	0.73 (0.05)	0.74 (0.04)
abdominal length	4.26*	2.4	5.87***	14.8	8.80 (0.80)	9.04 (0.85)
genitalia	1.18	0.0	2.38**	4.8	0.50 (0.04)	0.50 (0.04)
subgenital plate	0.01	0.0	1.10	4.3	1.07 (0.07)	1.08 (0.06)
thorax width	0.02	0.1	2.23**	3.8	2.85 (0.13)	2.85 (0.12)
Females						
PC1	11.08**	23.3	7.64***	14.8	0.25 (0.93)	-0.29 (1.00)
CV1	8.62**	25.0	13.99***	23.9	0.44 (1.22)	-0.51 (1.12)
MANOVA (sc)	11.66***	-	4.05***	-	-	-

Trait	F-ratio Hosts	% Hosts	F-ratio Populations	% Pops	Mean (s.d.) *Ceanothus* populations	Mean (s.d.) *Adenostoma* populations
ANOVA (sc)						
head width	46.94***	23.5	7.89***	15.2	3.42 (0.80)	3.91 (0.88)
femur length	11.05**	3.3	11.39***	26.3	3.81 (0.12)	3.75 (0.11)
tarsal length	0.34	0.0	1.18	0.6	0.95 (0.05)	0.95 (0.05)
abdominal length	4.84*	1.6	4.64***	11.6	13.70 (1.51)	14.15 (1.42)
genitalia	3.55	2.3	0.96	0.0	2.89 (0.13)	2.91 (0.13)
subgenital plate	0.32	1.2	2.45**	5.0	2.64 (0.21)	2.68 (0.19)
thorax width	10.78**	7.2	2.54**	4.9	4.97 (0.22)	4.89 (0.25)

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Table 4.3 Principal component scores for the first principal component axis (PC1) and standardized canonical discriminant function coefficients for the first canonical variate axis (CV1) (from principal components and discriminant analyses respectively).
In both sexes, high scores for CV1 characterize walking-sticks with narrow heads and long legs.

Trait	Males PC1	Females PC1	Males CV1	Females CV1
head width	0.79	0.86	-0.47	-0.51
femur length	0.82	0.88	1.17	1.39
tarsal length	0.65	0.78	0.20	0.06
abdominal length	0.45	0.48	-0.03	-0.37
genitalia*	0.36	0.78	-0.16	-0.13
subgenital plate	0.63	0.65	-0.11	-0.26
thorax width	0.72	0.76	0.03	0.12

*genital length for females, genital width for males

Table 4.4 Population means for PC1 and CV1 are correlated with geographic and molecular-genetic indices of the opportunity for divergence, under a balance between natural selection and gene flow (shown are r-values from Spearman Rank Correlation analyses).
The opportunity for divergence was calculated from the size of adjacent populations using the alternate host (geographic method) and DNA estimates of migration rates between adjacent patches (DNA-based methods; see methods for derivation of these indices). Also shown are results for the two size-corrected (sc), univariate traits where a significant relationship was detected between morphological divergence and indices of the balance between selection and gene flow.

comparison	PC1	CV1	HW (sc)	FL (sc)
Males – Selection / Gene Flow Balance				
Geographic method	0.74***	0.65**	-0.43**	0.39
DNA-based method 1 (Ne)	0.66**	0.69**	-0.41**	0.54*
DNA-based method 2 (M)	0.64**	0.72**	-0.42**	0.58**
Females – Selection / Gene Flow Balance				
Geographic method	0.69**	0.52*	-0.72**	0.22
DNA-based method 1 (Ne)	0.79***	0.66**	-0.81***	0.25
DNA-based method 2 (M)	0.78***	0.62**	-0.80***	0.23

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Table 4.5 Results of ANOVA analyses of common-garden rearing experiment testing for the effects of genotype (population of origin), environment (host reared on) and the genotype by environment interaction term (population x host reared on) on morphological variation in *T. cristinae*
Figure 4.2 depicts mean trait values for each multivariate index of morphology for the six study populations, where some individuals were reared to maturity on their native host and some individuals were reared on the alternate host.

Population	Males PC1	CV1		Females PC1	CV1
population of origin	10.80****	33.77***		9.26***	32.39***
host reared on	3.68	0.30		13.23***	11.44**
population x host reared on	8.03****	0.99		7.61***	1.30

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

CHAPTER 5.
THE EVOLUTION OF HOST PREFERENCE IN ALLOPATRIC VERSUS PARAPATRIC POPULATIONS OF *TIMEMA CRISTINAE* WALKING-STICKS*

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5.1. Abstract

Divergent habitat preferences can contribute to speciation, as has been observed for host-plant preferences in phytophagous insects. Geographic variation in host preference can provide insight into the causes of preference evolution. For example, selection against maladaptive host-switching occurs only when multiple hosts are available in the local environment and can result in greater divergence in regions with multiple versus a single host. Conversely, costs of finding a suitable host can select for preference even in populations using a single host. Some populations of *Timema cristinae* occur in regions with only one host-plant species present (in allopatry, surrounded by unsuitable hosts) whereas others occur in regions with two host-plant species adjacent to one another (in parapatry). Here we use host choice and reciprocal-rearing experiments to document genetic divergence in host preference among 33 populations of *T. cristinae*. Populations feeding on *Ceanothus* exhibited a stronger preference for *Ceanothus* than did populations feeding on *Adenostoma*. Both allopatric and parapatric pairs of populations using different hosts exhibited divergent host preferences, but the degree of divergence tended to be greater between allopatric pairs. Thus gene flow between parapatric populations apparently constrains divergence. Host preferences led to levels of premating isolation between populations using alternate hosts that were comparable in magnitude to previously documented premating isolation caused by natural and sexual selection against migrants between hosts. Our findings demonstrate how gene flow and different forms of selection interact to determine the magnitude of reproductive isolation observed in nature.

5.2 Introduction

Natural selection plays a role in speciation when it causes the evolution of reproductive isolation (Funk, 1998; Schluter, 2000; Kirkpatrick & Ravigne, 2002; Coyne & Orr, 2004). Divergent habitat preferences cause premating isolation when they reduce encounters, and thus matings, between individuals from different populations (i.e. 'habitat isolation', Tavormina, 1982, Rice & Salt, 1988; Stanhope et al., 1992; Craig et al., 1993; Feder et al., 1994; Duffy, 1996; Via, 1999; Linn et al., 2003; Coyne & Orr, 2004). Host-plant preferences in phytophagous insects can cause habitat isolation because many such insects feed, mate and oviposit exclusively on their hosts (Bush, 1969; Hawthorne & Via, 2001; Berlocher & Feder, 2002; Funk et al., 2002). Although host preferences are common in phytophagous insects, geographic variation in preference and its causes are rarely described (but see Jaenike & Grimaldi, 1983; Forister, 2004). Also, some studies testing for divergent preferences do not detect them (e.g. Jiggins et al., 1997; Poore & Steinberg, 2001). Thus the factors both driving and constraining the evolution of host preference require further study (Jaenike & Holt, 1991; Carriere, 1998).

Here we examine the role of three evolutionary processes in structuring geographic variation in host preference among populations of walking-stick insects; (1) selection for reduced search costs and efficient host finding, (2) selection against maladaptive host-switching, and (3) gene flow between populations. We refer to host-plant preferences of herbivores throughout but stress that the hypotheses and implications apply to the habitat preferences of many organisms.

Host preferences can diverge both with and without selection against switching between different, utilized hosts (we use the term 'utilized' to refer to host species that an insect species uses; other host species that the insect species cannot or does not use may exist in the environment as well). There is no selection against switching between utilized hosts when only one host is utilized in the local environment. Under this scenario, search and efficiency costs can favor increased preference for the single, utilized host because individuals without strong preferences accrue lower fitness, but for reasons other than switching to an alternate host (Jaenike, 1990, 1991; Bernays & Wcislo, 1994; Janz &

Nylin, 1997; Carriere, 1998; Bernays & Funk, 1999). For example, such individuals might take longer to locate or to decide whether to feed on the utilized host, thereby wasting time and energy while increasing predation risk. Alternatively, individuals may suffer low fitness because they attempt to use a 'non-utilizable' host. When preference evolution is driven by such selection, populations in habitats where only a single host is utilized still evolve preference for that host.

When switching between utilized hosts is maladaptive (i.e. when local adaptation results in fitness trade-offs between hosts), host preferences can also diverge via selection against individuals that switch between hosts (Balkau & Feldman, 1973; Kawecki 2004). Under this scenario, preference for one host is favored because individuals choosing another host suffer reduced fitness (but see Fry, 1996; Kawecki, 1996, 1997). This form of selection only acts in populations where there is the opportunity for switching between utilized hosts (i.e. when more than one utilized host is available in the environment) and forms the cornerstone of many verbal and mathematical models of sympatric speciation (Bush, 1969; Johnson et al., 1996; Berlocher & Feder, 2002; Dres & Mallet, 2002; Kawecki, 2004 for review). Although selection in this scenario actively favors reduced host-switching, it often acts on host preference loci indirectly via their genetic association with loci conferring host-specific fitness (c.f. Kirkpatrick & Barton, 1997; see also Coyne & Orr, 2004). One possible outcome of this process is greater preference divergence in geographic regions where multiple hosts are utilized (in sympatry or parapatry) than between geographically-isolated populations that use a single, yet different, host (allopatry). We will refer to this pattern as 'character displacement' of host preference.

In contrast to the forms of selection described above, gene flow often erodes population differentiation (Slatkin, 1987; Hendry et al., 2001, 2002; Hendry & Taylor, 2004). When gene flow constrains divergence, populations exchanging genes in sympatry or parapatry exhibit weaker divergence than do geographically-isolated, allopatric populations. The scenarios outlined above consider each of the evolutionary forces in isolation, but evolution in nature will reflect a balance between these different processes (Table 5.1).

In this study, we examine host preferences of *Timema* walking-sticks feeding on one of two distinct host-plant species (*Ceanothus spinosus* or *Adenostoma fasciculatum*) under allopatry (one host available in the local environment) and parapatry (both hosts available in the local environment). The study has four main goals: 1) to test whether host preferences differ between populations using different hosts, 2) to assess whether population divergence has a genetic basis, 3) to estimate the importance of host preference relative to other premating barriers, and 4) to ascertain which evolutionary processes explain among-population variation in host preference. With respect to evolutionary processes, explicit predictions can be made. Preference evolution driven by search costs predicts divergence even between allopatric populations that use a single (but different) host each. If selection against maladaptive host-switching is important, then 'character displacement' of host preference is expected. Finally, if gene flow constrains differentiation, then preference divergence will be weaker when migration between utilizable hosts occurs (in parapatry) occurs than when it does not (in allopatry). In addition, we provide an estimate of total premating isolation between the host-associated forms of *T. cristinae* by combining the results on host preference with previous estimates of premating isolation caused by natural and sexual selection against between-host migrants (i.e. 'immigrant inviability' and 'sexual isolation' respectively; Nosil *et al.*, 2002, 2003; Nosil, 2004; Nosil *et al.*, 2005). Collectively, the results help explain the processes driving and constraining the evolution of reproductive barriers.

5.2.1 Study system

Timema walking-sticks are wingless insects inhabiting the chaparral of Southwestern North America (Vickery, 1993; Crespi & Sandoval, 2000). Individuals feed and mate exclusively on the hosts upon which they rest and thus host preference can result in premating isolation. Patches of the two host species used by *T. cristinae* are usually distributed in parapatric patches of varying size. However, some host patches are geographically-separated from all others by regions lacking suitable hosts (Fig. 5.1). Each 'sample site' is contiguous area of one or both hosts that is separated from all other sample sites by regions without suitable hosts. We focus on divergence between populations, where a 'population' of walking-sticks is defined as all of the insects

collected within a homogenous patch of a single host-plant species (as in Nosil *et al.*, 2002, 2003). Thus 'Parapatric' insect populations are in contact with a population of insects adapted to the alternative host (i.e. they have a 'neighboring', adjacent population using the alternative host), whereas 'allopatric' populations are separated from all other populations adapted to the alternative host by distances > 50 times the 12m per-generation gene flow distance (Sandoval, 1993). Sample sites with both hosts were chosen such that there was only one population on each host species.

Previously published studies of *T. cristinae* examined a number of factors other than host preference. These studies documented adaptive morphological divergence (Sandoval, 1994a; Nosil & Crespi, 2004) and reproductive isolation between populations using different hosts caused by immigrant inviability and sexual isolation (Nosil *et al.*, 2002, 2003; Nosil, 2004; Nosil *et al.*, 2005). We have now examined host preference in this system in two distinct contexts. A related study has shown that the genetic covariance between color-pattern and host preference within populations occurs only in parapatric populations (Nosil *et al.* 2006; see Discussion for summary). Divergence in mean host preference between populations is the topic of the current study and has not been examined previously.

The conditions for the evolutionary processes outlined above to contribute to preference evolution are met. First, selection for specialization due to search and efficiency costs can occur. For example, *Timema* are heavily preyed upon by visual predators (birds, lizards; Sandoval, 1994a,b; Nosil, 2004) and time spent searching for or deciding whether to rest upon a host could increase predation risk. Second, selection against maladaptive host-switching can occur in parapatric populations. Each of two main color-pattern morphs in *T. cristinae* has higher survival on the host-plant on which it is more common, due to differential visual predation (Sandoval, 1994a,b; Nosil, 2004). Thus divergent selection acts on color-pattern and, on average, switching hosts results in low survival (Nosil, 2004). Selection can act indirectly on host preference loci within parapatric populations via the positive genetic covariance between color-pattern and host preference (Nosil *et al.*, 2006). Finally, both morphological and mitochondrial DNA sequence divergence is consistently lower between adjacent, parapatric pairs of populations than between geographically-separated pairs of populations. This pattern

indicates gene flow between parapatric populations (Sandoval, 1994a; Nosil *et al.*, 2003; Nosil & Crespi, 2004).

Populations of *T. cristinae* exist in a geographic mosaic. It is unlikely that every population represents an entirely independent evolutionary replicate because evolution within each population may have a different starting point, depending on colonization history (i.e. on the preference of ancestral populations). Current day preference represents a combination of the retention of ancestral preference and evolution towards or away from it. We do not claim that differences between populations represent 'divergence' in the sense that they evolved totally in situ. Rather, we examine general trends across multiple populations on different hosts and try to ascertain what processes account for the variation in host preference. We focus throughout on comparing data combined from multiple populations that are similar in host use or geography to data combined from multiple populations that are dissimilar, yielding large sample sizes for most of our analyses. We note the few cases where we do examine individual populations are warranted because there is evidence for independent evolution within populations occupying different geographic regions (Nosil *et al.*, 2002, 2003).

5.3 Materials and Materials

5.3.1 Field collecting and insect maintenance

T. cristinae were collected from 33 study sites in the Santa Ynez Mountains, California between January and June in 1992, 1996, 2001-2004 using sweep nets. Other species of *Timema* do not occur in sympatry with *T. cristinae*. Walking-sticks were maintained in glass jars at the University of California at Santa Barbara (20 degrees C) with 10-15 individuals per jar. Individuals from different populations and the sexes were kept separate. Animals were fed the foliage of *Ceanothus*, except in the case of the reciprocal-rearing experiment (see below). Table 5.4 provides a description of each population and population-specific sample size sizes for each experiment.

5.3.2 Experiment #1 (field-caught individuals, single insect per replicate)

All the experiments were a choice situation because individuals of *T. cristinae* will accept their non-native host if given no choice and can be reared successfully on either host. Host-preference tests were performed using randomly-collected insects. Individual walking-sticks (total n = 1426) were placed in the bottom of a 500ml plastic cup (height, 15cm) with one 12cm host cutting from each host-plant species in the cup. The bottom end of each host cutting was placed in a plastic aqua-pic filled with water which held the cutting upright and kept it fresh. The top of each container was covered with wire netting secured by elastic bands. Assays were initiated in the evening and test animals were left in darkness overnight. In the morning, we recorded which host species each individual was resting on. For assays where the test individual did not choose a host (i.e. they were resting on the container, < 5% of trials), the container was left overnight until a host was chosen (for up to two nights). Each individual was used only once. All scoring was done blind to population of origin by P. Nosil.

5.3.3 Experiment #2 (field-caught individuals, multiple insects per replicate)

The second experiment simulated a scenario where multiple individuals might simultaneously be picking a host, as might occur in nature. Preference tests were conducted on walking-sticks collected in 1992 and 1996. In 1992, we offered insects one 30 cm high branch of each host species (branches kept 10 cm apart and out of contact in a Styrofoam sheet floating in a container with water, thereby keeping the plant fresh and preventing insects from escaping). Approximately 10 insects from the same population were placed on the styrofoam, midway between the two branches, and left overnight. The following morning the number of insects on each branch was counted by C.P. Sandoval. The choice test was replicated for each population based on insect availability. Individuals that did not choose a host (< 5%) were excluded from analysis and each insect was used only once. Due to a shortage of insects in 1996, each replicate had only one insect and the procedure was modified. The two branches of the host plants were placed inside of a 0.5 liter plastic cup covered with netting. The plants were kept fresh using water-filled aqua-pics. This slight methodological modification in 1996 is very unlikely to affect our conclusions because it involves only four populations and our

conclusions were well-supported by the other experiments presented in this study (Table 5.4 for populations affected). Branches were obtained from the same site as the individuals were collected from. Different plant individuals were used for each replicate and the pair of branches within each replicate collected from adjacent plants in the field. Mean preference from each replicate (% of individuals picking *Ceanothus*) was used as a single data point in all statistical analyses.

5.3.4 Experiment #3 (genetic crosses)

The third experiment provides the same general information as the first two, but additionally represents a common-garden experiment. Individuals from within 20 populations were crossed with one another in 2003 and 2004 ('within-population crosses' - both parents always from the same population). All the individuals used in the crosses were sexually-immature instars captured in the field that were reared to sexual maturity in the absence of the opposite sex on *Ceanothus* cuttings. A small number of between-population crosses were also conducted (see below). A single virgin male and a single virgin female were housed together in a petri dish until copulation was observed and then fed *Ceanothus* cuttings until the female died (females lay eggs single and daily). The following spring (after the eggs overwintered) offspring were scored for host preference within a few days of emergence using the same protocol as experiment #1. Each family mean was used as a single data point in statistical analyses.

A portion of these same data (64 of 145 families and 428 of 988 individuals, all from 2003) come from an experiment designed to measure the genetic covariance between host preference and color-pattern (Nosil *et al.*, 2006). In this experiment, non-random mating was imposed such that both parents were always the same color-pattern morph. This does not qualitatively affect our conclusions in any way because the subset of the crosses where parents were mated randomly with respect to color-pattern includes all 20 populations and yields the same result as the full database (e.g. mean population preference using only the crosses with random mating is highly correlated with mean population preference using full database, $r = 0.71$, $p < 0.001$).

5.3.5 Statistical analyses

All statistical analyses were conducted using SPSS (v.12). Trends from the three experiments were always in the same direction. In cases where all three experiments did not yield significant results individually, we also report Fisher's combined probability values (Sokal & Rohlf, 1995).

5.3.6 Geography and population divergence in host-plant preferences

We first tested whether individuals derived from populations feeding on *Ceanothus* exhibit different host preferences than individuals derived from populations feeding on *Adenostoma*. For this analysis we used chi-square tests (for Experiment #1, as the preference data was categorical) and t-tests (for Experiments #2 and #3, as the preference data was continuous). In addition, for the largest experiment (#1) we examined whether pairs of populations using different host-plants exhibited greater divergence in host preference than pairs of populations using the same host plant using a Mantel t-test (Manly 1997; this analysis considers all pairwise comparisons among populations). These analyses use all the data and test for divergence independent of geography.

If search costs contribute to evolution, host preference should diverge even between individuals from allopatric populations where there is no opportunity for selection against host-switching. To test this prediction we repeated the chi-square and t-test analyses, but restricted them to individuals from allopatric populations. We then examined whether difference in host preference occurs in parapatry alone by repeating the analyses using only individuals from parapatric populations.

We assessed whether host preference differed between geographic scenarios (allopatry / parapatry) by testing for an interaction between host-plant used and geography in logistic regression (experiment 1) and ANOVA analyses (experiments 2 and 3). The interactions term test for an effect of geography, but do not explicitly examine the direction of differences. Strengthening of preferences in response to selection against maladaptive host-switching is expected to leave two directional patterns: 1) greater preference for the native host in parapatric versus allopatric populations and 2)

greater divergence in preference between allopatric versus parapatric pairs of populations using alternate hosts.

We examined whether preference for the native host (i.e. the host of the population from which an individual is derived) is greater for individuals derived from allopatric populations than those derived from parapatric populations (Experiment #1 – chi-square tests; Experiments #2 and #3 – t-tests). To account for asymmetry in the host preference of populations using different hosts and to avoid confounding difference between hosts with variability among populations within hosts, we conducted separate analyses for populations from each host species. The tests above doe not account for population-specific variation or examine divergence between population pairs per se. Thus we also compared divergence in host preference between allopatric versus parapatric pairs of populations that use different hosts. In this analysis, pairs of populations, rather than individuals, become the unit of replication and the difference between population pairs is compared between the two geographic comparisons (allopatric pairs versus parapatric pairs) using a t-test. Allopatric populations were paired randomly into different-host pairs but our results are unaffected by alternative pairings because allopatric populations of the same host tend to have similar host preferences. Parapatric populations were always paired with the adjacent population on the alternative host. Each population was used in only a single pairwise comparison.

5.3.7 Selection-gene flow balance

We assessed whether mean trait values for each single population reflect the effects of a balance between selection and gene flow using a quantitative index of this balance. When populations use *Ceanothus* as a host plant, the size of the adjacent population of *Adenostoma* serves as the index of the opportunity for gene flow to erode local adaptation to *Ceanothus* (population sizes inferred from host-plant patch sizes, see below). Conversely, when populations use *Adenostoma* as a host plant, the size of the adjacent population of *Ceanothus* serves as an index of the opportunity for alleles conferring adaptation to *Ceanothus* to be introduced into the population. Thus for each study population the value assigned to it simply represents the proportion of the total area (area of the study population plus the area of the adjacent population using the alternate

host) occupied by *Ceanothus*. Allopatric populations (which do not have an adjacent population) apparently undergo little or no gene flow (Nosil *et al.*, 2003), and are assigned values of zero (for *Adenostoma* populations) or 100 (for *Ceanothus* populations). Parapatric populations are assigned values between zero and 100, based upon the relative abundance of *Ceanothus*.

Previous work indicates that this index accurately estimates the geographic potential for gene flow as 1) field sampling has shown that patch size and population size are strongly, positively correlated (Sandoval, 1994a) and 2) the relative size of the population using the alternative host that is adjacent to a focal population is strongly correlated with the migration rate from the adjacent population into the focal population (Nosil *et al.*, 2003; Nosil & Crespi, 2004; migration estimated from mtDNA sequence data and the coalescent-based methods of Beerli & Felsenstein, 2001). In particular, we refer readers to Nosil & Crespi (2004) for a detailed validation.

Patch areas were calculated from aerial photographs and ground-truthing (as in Sandoval 1994; Nosil *et al.* 2003; Nosil and Crespi 2004). Spearman rank correlation was used to test whether mean population preference for *Ceanothus* was correlated with the proportion of the total area occupied by *Ceanothus* (i.e. the index of selection / gene flow balance). To avoid conflating differences between hosts with differences among populations within hosts, analyses were run separately for populations using each host species (these analyses were conducted for experiments 1 and 3 only, due to lack of replication among populations using the same host in experiment 2).

5.3.8 Genetic basis of host preference

Four different lines of evidence were used to assess whether population divergence in host preference has a genetic basis. First, congruence in population divergence between the results from field-caught and genetic cross data suggests a genetic basis to population divergence. Second, the genetic crosses represent a common-garden experiment such that differences among populations in experiment #3 are likely to have a genetic basis. Third, for a subset of the populations studied (n = 6) in Experiment #1, we raised some of the individuals on their native host and some on the alternative

host (from first instar until sexual maturity comprising approximately 4-6 weeks of rearing, Fig. 5.3 for sample sizes). We used logistic regression analyses to test whether host picked (*Ceanothus* or *Adenostoma*) in these populations was influenced by genotype, rearing environment (host reared on) or a genotype by environment interaction (assessing significance using likelihood ratio tests (LR)). We conducted two analyses, one using population of origin as the genotype term and one using host of origin as the genotype term. We report the results from a full model that included both factors and the interaction as well as the results from a reduced regression model derived using backward elimination (the reduced model removes all terms for which the significance of –2 log LR was > 0.10 in the full model). Fourth, some genetic crosses were also conducted between individuals from different populations using alternate hosts (n = 26 families). The preference of the F1 'hybrids' emerging from such crosses (n = 70 individuals) was assayed using the protocols in experiment #1. Hybrid preferences were then compared to the preferences of nymphs emerging from within-population crosses (using only the populations for which both within-population and between-population crosses were conducted).

5.3.9 Components of premating isolation

We estimated total premating isolation caused by the combined effects of host preference, selection against immigrants (immigrant inviability), and divergent mate preferences (sexual isolation), as well as the relative contribution of each of these three individual components to total isolation (see Ramsey et al., 2003 for details of the estimation procedure).

Individual components of reproductive isolation (RI) specify the magnitude of reproductive isolation caused by a given barrier to gene flow when it acts alone. The individual contribution of host preference (RI_h) was estimated as the absolute value of the [% difference between a population pair in mean preference for *Ceanothus*], immigrant inviability (RI_m) was estimated as [1 – (immigrant survival / resident survival)] and the individual contribution of sexual isolation (RI_s) as [1 – (heterotypic mating frequency / homotypic mating frequency)]. Where relevant, the two values from a population pair were always averaged. Total reproductive isolation is computed as multiplicative

function of the individual components at sequential stages in the life history, but a given component of reproductive isolation can only eliminate gene flow that has not been eliminated by a previous component. Host preference acts before selection against migrants which in turn acts before sexual isolation. Thus the absolute contribution of host preference is ($ACh = RI_h$), the absolute contribution of selection against migrants is ($AC_m = RI_m (1 - AC_h)$), the absolute contribution of sexual isolation is $AC_s = RI_s [(1 - (AC_h + AC_m)]$ and total isolation is ($AC_h + AC_m + AC_s$). The relative contribution of any component is simply the absolute contribution divided by total isolation.

We estimated components of reproductive isolation between pairs of populations under three major eco-geographical scenarios: 1) allopatric pairs of populations using the same host species, 2) allopatric pairs of populations using different host species, and 3) parapatric pairs of populations using alternate host species. Analyses have already been conducted for immigrant inviability and sexual isolation and estimates of these barriers are taken directly from Nosil (2004). For host preference, we used all the populations for which $n > 5$ for both populations in a population pair in experiment #1. Allopatric populations were paired randomly into same-host or different-host pairs, using each population in only a single pairwise comparison. Our results are unaffected by alternative pairings or the use of populations with smaller sample sizes because allopatric populations of the same host tend to have similar host preferences (same-host pair reported – VPC x PE; different-host pairs PR x L and PE x LRN). Parapatric populations were always paired with the adjacent population on the alternative host. When multiple population pairs comprised a single eco-geographic comparison, the overall mean of the different population means was used.

5.4 Results

5.4.1 Divergence in host-plant preferences

T. cristinae from populations feeding on *Ceanothus* differed significantly in host preference from those feeding on *Adenostoma*. In both experiments using field-caught insects, individuals from populations feeding on *Ceanothus* exhibited a stronger preference for *Ceanothus* than did individuals from populations feeding on *Adenostoma*

(both $p < 0.01$, Table 5.2). Likewise, laboratory-emerged nymphs whose parents were from populations feeding on *Ceanothus* showed a greater preference for *Ceanothus* than did nymphs whose parents were derived from populations using *Adenostoma* ($p < 0.01$, Table 5.2). Thus pairs of populations using different host-plant species exhibited significantly greater divergence in host preference than did pairs of populations using the same host species ($n = 175$ and 176 pairwise comparisons respectively from experiment #1, Mantel $t = 4.60$, $p < 0.001$).

5.4.2 Divergence in allopatry – 'search costs'

The results above demonstrate that host preference has diverged between populations using different hosts, but do not test which processes contribute to divergence. To test whether search costs in allopatry contribute to evolution we repeated the t-test and chi-square analyses reported above, but restricted them to individuals from allopatric populations. Consistent with the divergence of allopatric populations, individuals from allopatric populations feeding on *Ceanothus* exhibited a stronger preference for *Ceanothus* than did individuals from allopatric populations feeding on *Adenostoma* ($p < 0.01$ in all three experiments, Table 5.2).

5.4.3 Divergence in parapatry – 'character displacement'

Divergence also occurred in parapatry. Individuals from parapatric populations feeding on *Ceanothus* exhibited a stronger preference for *Ceanothus* than did individuals from parapatric populations feeding on *Adenostoma* ($p < 0.01$, 0.05, 0.52 for experiments 1-3 respectively, combined probability $p = 0.0011$, Table 5.2). Host preferences tended to differ for individuals from allopatric versus parapatric populations, as indicated by host-use x geography interactions in logistic regression ($LR = 18.22$, $p < 0.001$, experiment 1) and ANOVA analyses ($F_{1,143} = 1.65$, $p = 0.20$; $F_{1,145} = 5.93$, $p < 0.05$, experiments 2 and 3 respectively; combined probability among experiments $p = 0.001$).

We conducted two explicit tests for character displacement of host preference, both of which yielded no evidence for its occurrence. First, preference for the native host (i.e. the host that the population an individual is derived from uses) tended to be significantly greater for individuals derived from allopatric populations than for

individuals derived from parapatric populations, particularly for *Ceanothus* populations (differences were always greater for allopatric populations, even if not statistically so; combined across hosts and experiment $p = 0.0001$; Table 5.3).

Second, comparing population divergence of allopatric versus parapatric pairs of populations provides the most explicit test for character displacement (where divergence for each individual population pair is calculated as percent of individuals from the *Ceanothus* population preferring *Ceanothus* minus percent of individuals from the *Adenostoma* population preferring *Ceanothus*). Such an analysis of population divergence revealed that, if anything, allopatric pairs show greater mean divergence than parapatric pairs (Experiment #1, mean of differences for 6 parapatric pairs = 14%, s.d. = 21, mean of differences for 3 allopatric pairs = 45%, s.d. = 43, $t_7 = 1.49$, $p = 0.18$; Experiment #3, mean of differences for 4 parapatric pairs = -7%, s.d. = 14, mean of differences for 2 allopatric pairs = 22%, s.d. = 10, $t_4 = 2.56$, $p = 0.063$; t-tests; combined $p < 0.05$). Thus analyses of mean preference show that parapatric populations do show divergence, but provide no evidence that divergence has been strengthened in parapatry. In fact, it appears that parapatric populations show weaker divergence than allopatric populations.

5.4.4 Selection-gene flow balance

We tested whether mean preference for each single population could be predicted by host-plant used and the opportunity for homogenizing gene flow. The results provide some support for this hypothesis, dependent on the host species and experiment considered. For populations using *Ceanothus*, mean population preference for *Ceanothus* was significantly correlated with our index of the balance between selection and gene flow for experiment #1 ($rho = 0.88$, $p < 0.001$) and was marginally insignificant for experiment #3 ($rho = 0.49$, $p = 0.065$). These results held up reasonably well when populations with small sample sizes were excluded ($n > 9$ individuals for experiment 1 and $n > 4$ families for experiment 3; $rho = 0.92, 0.52$, $p < 0.001$, $p = 0.15$ respectively). For populations using *Adenostoma*, the trends were much weaker (using all the populations, $rho = 0.18, 0.54$, $p = 0.30, 0.066$ for experiments 1 and 3 respectively, combined $p = 0.098$; excluding small samples, $rho = 0.24, 0.48$, $p = 0.30, 0.14$ respectively). We return to this variability between hosts in the discussion.

5.4.5 Genetic basis for population divergence

Four different lines of evidence suggest that population divergence in host preference has a strong genetic basis. First, results from both field-caught and laboratory-emerged insects tend to be congruent; in both cases populations from *Ceanothus* exhibited greater preference for that host (Tables 5.2, 5.3; the correlation between population means for experiments 1 and 2 was rho = 0.47, $p = 0.051$). Second, the results from the genetic crosses represent a common-garden experiment and thus differences between populations likely represent genetic divergence (Table 5.2). Third, logistic regression analysis of the reciprocal-rearing experiment revealed no evidence that environmental effects (i.e. host species reared upon) influence host preference and strong evidence that genotypic effects do affect host preference (Fig. 5.2). This strong effect of genotype occurred when host of origin was used as the genotype term (full model, host of origin -2LR = 4.10, d.f. = 1, $p < 0.05$, host reared upon -2LR = 0.21, d.f. = 1, $p = 0.65$, interaction -2LR = 1.13, d.f. = 1, $p = 0.29$; reduced model, host of origin -2LR = 12.832, d.f. = 1, $p < 0.001$, other terms removed) and when population of origin was used as the genotype term (full model, population of origin -2LR = 8.01, d.f. = 5, $p = 0.15$, host reared upon -2LR = 0.61, d.f. = 1, $p = 0.44$, interaction term -2LR = 2.86, d.f. = 5, $p = 0.72$; reduced model, population of origin -2LR = 16.90, d.f. = 5, $p < 0.01$, other terms removed). Fourth, F1 'hybrids' between the host forms exhibit intermediate preferences, indicative of genetic differences between the host forms (Fig. 5.3).

5.4.6 Components of premating isolation

Total premating isolation is non-existent for allopatric pairs using the same host, strongest for allopatric pairs using alternate hosts, and intermediate for parapatric pairs using alternate hosts (total isolation = -0.04, 0.67 and 0.51 respectively, Fig. 5.4). Within each 'eco-geographic' comparison, the individual components of isolation caused by host preference, immigrant inviability and sexual isolation are roughly similar. This similarity among components is even greater for the absolute contribution to total isolation because host preference acts earliest in the life history. For populations using different hosts, roughly similar levels of total premating isolation are observed under allopatry and parapatry but arise via different individual components of reproductive isolation.

Specifically, host preference and immigrant inviability contribute strongly under allopatry whereas sexual isolation contributes strongly under parapatry.

5.5 Discussion

5.5.1 Causes of host preference evolution

We examined the effects of three evolutionary processes on host preference evolution in *T. cristinae* walking-stick insects; 1) selection for reduced search costs and efficient host finding, 2) selection against maladaptive host-switching and 3) between-population gene flow. There is no direct evidence to support the 'search costs' hypothesis in *Timema* (i.e. search costs have not been measured), but nonetheless, allopatric populations clearly show differentiation in host preference. Habitat fidelity in the absence of fitness trade-offs between hosts has been detected in other systems (Futuyma *et al.*, 1984) and among allopatric populations (Funk, 1998; Forister, 2004). These observations, coupled with the results of this study, show that active selection again maladaptive host-switching is not required for preference evolution.

Selection against maladaptive host-switching can also contribute to host preference evolution. Local adaptation, via divergent natural selection, results in performance trade-offs between alternative habitats. This process favours the evolution of divergent host preferences because individuals switching hosts are selected against. Fitness trade-offs between different habitat have been detected in a number of taxa (Schluter, 2000 for review), including host-associated insects (Blau & Feeny, 1983; Katakura *et al.*, 1989; Craig *et al.*, 1997; Carroll *et al.*, 1997; Filchak et al., 2000; Via *et al.*, 2000), molluscs (Giesel, 1970; Rolan-Alvarez *et al.*, 1997), amphibians (Storfer & Sih, 1998; Storfer *et al.*, 1999) and fish (Schluter, 2000). In several of these cases, forms adapted to alternative habitats exhibit a preference for their native habitat, and thus are partially reproductively isolated (e.g. ladybird beetles, Katakura *et al.*, 1989; *Eurosta solidaginis*, Craig *et al.*, 1993; pea aphids, Via, 1999; *Rhagoletis* flies, Feder et al., 1994). These results suggest that fitness trade-offs commonly drive the evolution of divergent habitat preferences. However, explicit tests of this hypothesis are lacking – that is, there

are few tests of host preferences in sympatric versus allopatric populations (but see Forister 2004).

Selection against host-switching is a process, and one predicted outcome of this process is increased divergence in sympatric versus allopatric populations. However, this process need not always result in such 'character displacement' of host preference (e.g. Lemmon *et al.,* 2004). For example, selection against host-switching almost certainly occurs in *T. cristinae* (Nosil 2004) and likely contributes to preference evolution because parapatric populations exhibit divergence in host preference in the face of gene flow and mean levels of divergence that are not drastically (nor always) lower than those observed for allopatric populations. Most likely, selection against switching between *Adenostoma* and *Ceanothus* contributes to preference evolution but greater relative divergence is not observed in parapatric populations because of gene flow in parapatry (which decreases parapatric divergence) and strong direct selection on preference in allopatry (which increases divergence in allopatry). Notably, character displacement of mate preferences has occurred in *T. cristinae* (Nosil *et al.,* 2003), perhaps because direct selection for mate preference is weak in allopatry and because insects can move whereas plants cannot (such that mating decisions occur more commonly than host picking decisions).

We found some evidence for an inverse association between population divergence and gene flow. The results were likely not stronger because selection against host-switching in parapatry counters the homogenizing effects of gene flow. Our results indicate host preference evolution can indeed occur in the face of gene flow (see also Forister, 2004; Emelianov *et al.,* 2004), but that divergence might be somewhat constrained. The standard interpretation of an inverse association between gene flow and population divergence is that gene flow constrains divergence (Slatkin, 1987). However, causality can be reversed because adaptive trait divergence itself may reduce gene flow (i.e. 'ecological speciation' Schluter, 2000; Lu & Bernatchez, 1999; Hendry *et al.,* 2002, 2003; Hendry & Taylor, 2004; Hendry, 2004). In *T. cristinae*, both processes likely act. To some extent, gene flow must constrain divergence because divergence is greatest between allopatric populations, yet the adaptive divergence of allopatric populations cannot reduce contemporary gene flow between them (i.e. as they are geographically-separated). In the parapatric scenario, host preference is likely to itself reduce gene flow

(see section on reproductive isolation below). Thus in *T. cristinae*, these two processes might be involved in a positive feedback loop whereby low gene flow allows adaptive divergence, which in turn further reduces gene flow by increasing reproductive isolation (Hendry, 2004). An outstanding question is why this feedback has not resulted in greater divergence.

Multiple lines of evidence indicate that the population divergence in host preference detected in this study has a partial genetic basis. While our experiments do not unequivocally rule out maternal effects (Mousseau & Dingle, 1991), all of the available evidence indicates that genetic divergence has occurred such that there has been progress towards genetically-differentiated host forms (rather than the evolution of plasticity).

5.5.2 Asymmetry in host preferences

The divergent host preferences detected in this study were atypical in that they were relative, not absolute. Thus, individuals from populations using either host plant often preferred to rest on *Ceanothus*, with walking-sticks collected from *Ceanothus* exhibited a much stronger preference for *Ceanothus* than those from *Adenostoma.* In parapatric populations where host choice is possible, this pattern might reflect the outcome of directional fecundity selection, which can counteract selection to prefer *Adenostoma.* Selection on color-pattern is divergent and can indirectly cause the evolution of divergent host preference via the positive genetic association between color-pattern and preference (Nosil *et al.,* 2006). In contrast, fecundity selection favors preference for *Ceanothus* independent of color-pattern because females from both hosts exhibit higher fecundity on *Ceanothus* (Sandoval & Nosil, 2005). Thus fecundity selection might constrain the evolution of strong preference for *Adenostoma.*

Additionally, the ancestral host of *T. cristinae* is not unequivocally known but it is possible that evolution away from an ancestral preference for *Ceanothus* is ongoing (Crespi & Sandoval, 2000). Thus allopatric populations of *Ceanothus* may simply retain the ancestral preference and exhibit strong preference for their native host. Conversely, allopatric *Adenostoma* populations would not exhibit a strong preference for their native host as they are in the process of evolving away from the ancestral preference. This

process could also explain the stronger association between gene flow and trait divergence detected in the *Ceanothus* versus *Adenostoma* populations. Allopatric *Adenostoma* populations would not have evolved away from the ancestral preference and thus exhibit similar preferences to that of parapatric populations.

5.5.3 Evolution of means versus genetic covariances

This study focused on divergence in population means. The evolution of trait means in response to natural selection depends on the genetic covariance between traits within populations (Lande, 1979; Arnold, 1992; Schluter, 1996). Thus to better understand host preference evolution, genetic covariance between host preference and color-pattern (a trait known to be under host-specific selection) has also been examined in a subset of the populations studied here (Nosil *et al.*,2006). At parapatric sites, divergent selection results in differentiation between adjacent populations on different hosts in both traits (Sandoval, 1994a,b; Nosil, 2004). Migration between hosts occurs and generates non-random associations between alleles at color-pattern and host preference loci (linkage disequilibrium; Nei & Li 1973; Kirkpatrick *et al.* 2002), resulting in strong genetic covariance between color-pattern and host preference. In allopatry, divergent selection and migration between hosts does not occur and genetic covariance is absent.

Collectively, these studies shed additional light on the mechanisms of population divergence because they show that genetic covariance need not result in greater population-level divergence. In *T. cristinae*, parapatric populations using different hosts show weaker population-level differentiation in both host preference and color-pattern than do allopatric populations, despite stronger genetic covariance within the former. This result indicates that host preference can evolve in parapatric populations via indirect selection (i.e. due to direct selection on color-pattern), but it also suggests that such indirect selection acting thru imperfect genetic associations is a weak diversifying force compared to direct selection (Felsenstein 1981,; Kirkpatrick & Barton, 1997). Heterogeneous environments might promote divergence and sympatric speciation by favoring genetic covariance (Lande, 1979; Kawecki, 2004), but hamper speciation by exposing populations to gene flow (Felsenstein, 1981; Slatkin, 1987).

5.5.4 Components of premating isolation

Coyne & Orr state that "the central problem of speciation is understanding the origin of those isolating barriers that actually or potentially prevent gene flow in sympatry" (2004, p. 57). They note that this involves two major tasks; determining which reproductive barriers were involved in the initial reduction in gene flow between populations and then understanding which evolutionary forces produced these barriers. Our results shed light onto both these issues. The observed divergence in host preference will cause partial (albeit relatively weak) premating isolation even though it is asymmetric because individuals from populations using different hosts should encounter one another less frequently than individuals from within the same host (Coyne & Orr, 2004). The host-associated forms of *T. cristinae* represent conspecific populations and thus three major forms of premating isolation (habitat isolation, immigrant inviability and sexual isolation) are involved in the initial divergence between populations. With respect to evolutionary forces, our studies show that selection facilitates divergence in all three forms of premating isolation examined, whereas gene flow constrains it (see also Nosil *et al.* 2002, 2003; Nosil, 2004). Moreover, both host-specific selection for local adaptation and selection to avoid maladaptive hybridization / host-switching facilitate divergence, indicating that multiple forms of selection are involved in the evolution of reproductive barriers.

The host-associated forms of *T. cristinae* are unlikely to have achieved species status by any criterion, as indicated by only a 60% barrier to gene flow at the premating level (Fig. 5.4) and a general lack of neutral mtDNA differentiation between adjacent populations on different hosts due to ongoing gene flow (Nosil *et al.*, 2003). Thus these host forms represent either an ongoing speciation event or population divergence that has reached equilibrium. Further studies of more divergent species within this genus may shed light onto the factors driving the transition from a host race or ecotype to a species. Clearly though, selection is central to divergence, indicating that the population-genetic processes acting within contemporary populations can also influence the formation of new species (Charlesworth *et al.*, 1982).

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Figure 5.1 Schematic representation of the different types of populations examined. A 'sample site' is defined as a contiguous area of one or both hosts that is separated from all other sample sites by regions without suitable hosts.

A 'population' of walking-sticks is defined as all the insects captured within a homogeneous patch of a single host-plant species (light boxes = *Adenostoma* populations; dark boxes = *Ceanothus* populations). Thus parapatric populations have an adjacent population that uses the alternate host whereas allopatric populations do not (therefore six populations are depicted below). Also shown is the proportion of the total area of a sample site that is occupied by *Ceanothus* (C). Letters within the boxes denote striped (S) versus unstriped (U) color-pattern morphs within populations. The current study focuses on divergence in mean host preference between populations, whereas a related study examined genetic covariance between color-pattern and host preference within populations (Nosil *et al.*, 2006).

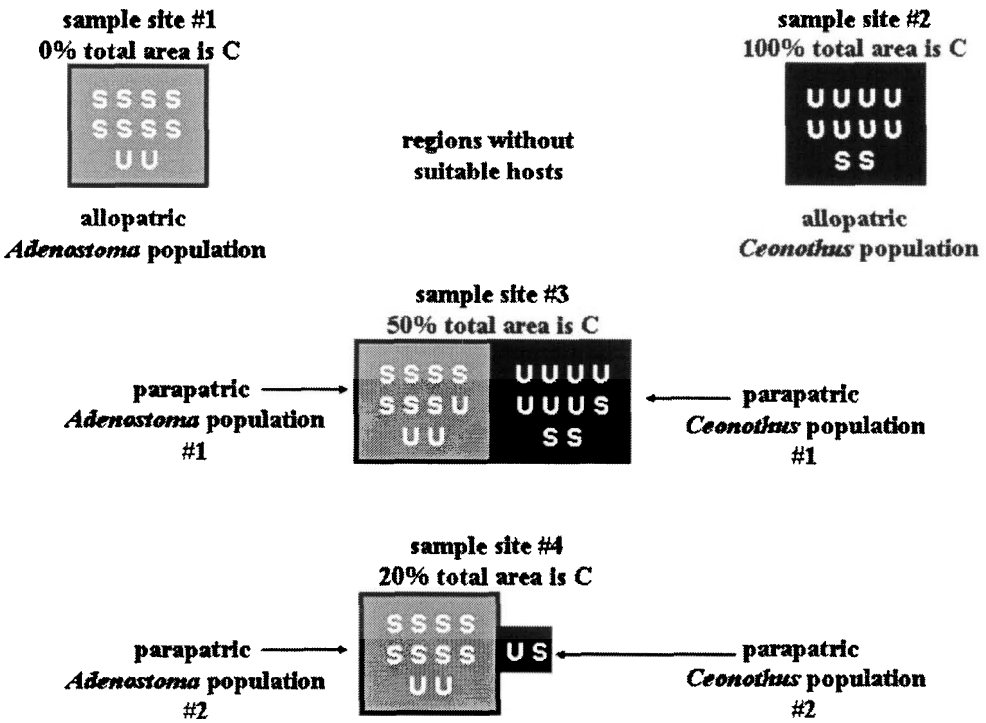

Figure 5.2 In a reciprocal-rearing experiment the effects of rearing were insignificant whereas genotype effects (population and host of origin) were significant (Results for statistics).

Shown is host preference (mean % picking *Ceanothus* ± 95% C.I) of field-caught first instars reared until sexual maturity on *Ceanothus* (C) versus *Adenostoma* (A) (about 4-6 weeks of rearing). Populations adapted to *Adenostoma* are depicted on the left, populations adapted to *Ceanothus* in the center and means pooled for individuals from multiple populations of the same host on the right (for each host species). Numbers above the x-axis refer to the number of individuals tested.

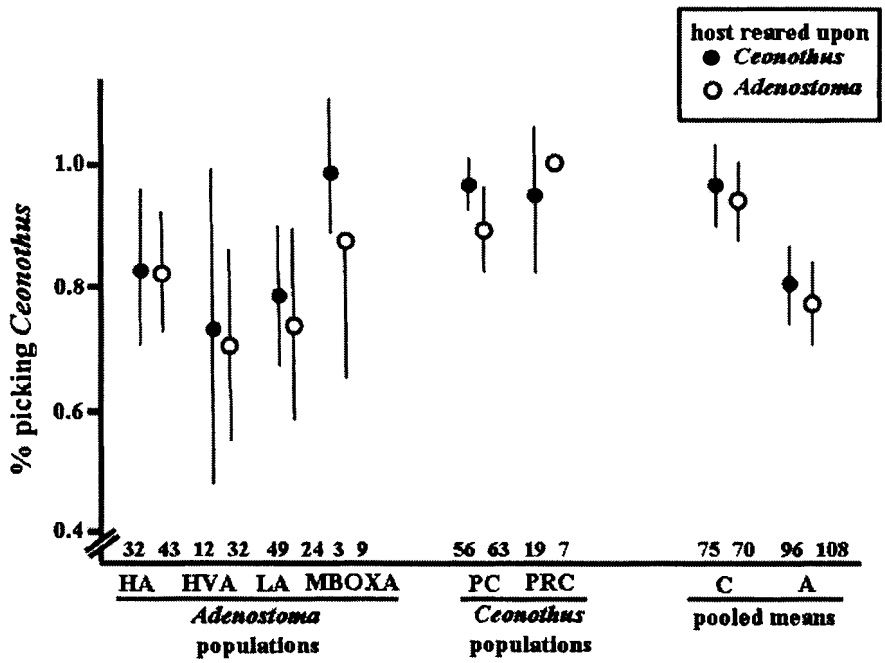

Figure 5.3 F1 'hybrids' between host forms show intermediate host preferences. Host preference (mean % picking *Ceanothus* ± 95% C.I.) of laboratory-emerged nymphs from crosses within versus between divergent host forms (C x C – both parents from the same population of *Ceanothus*; C x A or A x C – parents from different populations with one parent from each host; A x A – both parents from the same population of *Adenostoma*). Number of individuals for each cross type is shown above the x-axis (with number of families in brackets to the right). Individual means are shown but trends with family means are congruent.

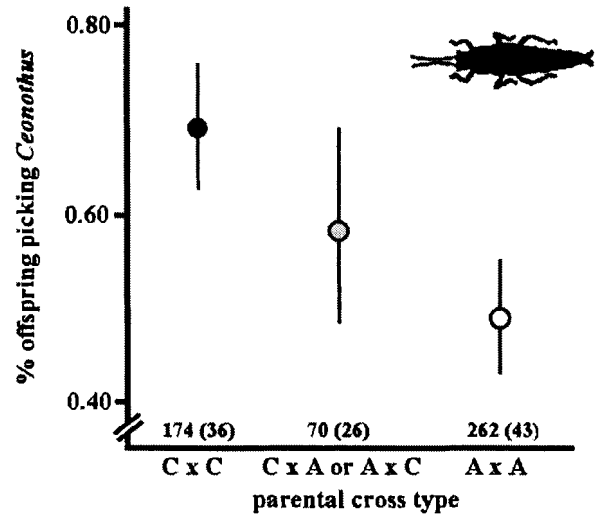

Figure 5.4 Components of reproductive isolation under different ecological and geographic scenarios.

Pairs of populations using the same host show very little reproductive isolation. For populations using different hosts, roughly similar levels of total premating isolation are observed under allopatry and parapatry but arise via different individual components of reproductive isolation. Shown graphically are the absolute contributions of host preference, immigrant inviability and sexual isolation to total premating isolation. The relative contribution of each component is simply its absolute contribution divided by total isolation. Individual components (strength of the barrier acting in isolation) are labelled to right of the bar for each barrier.

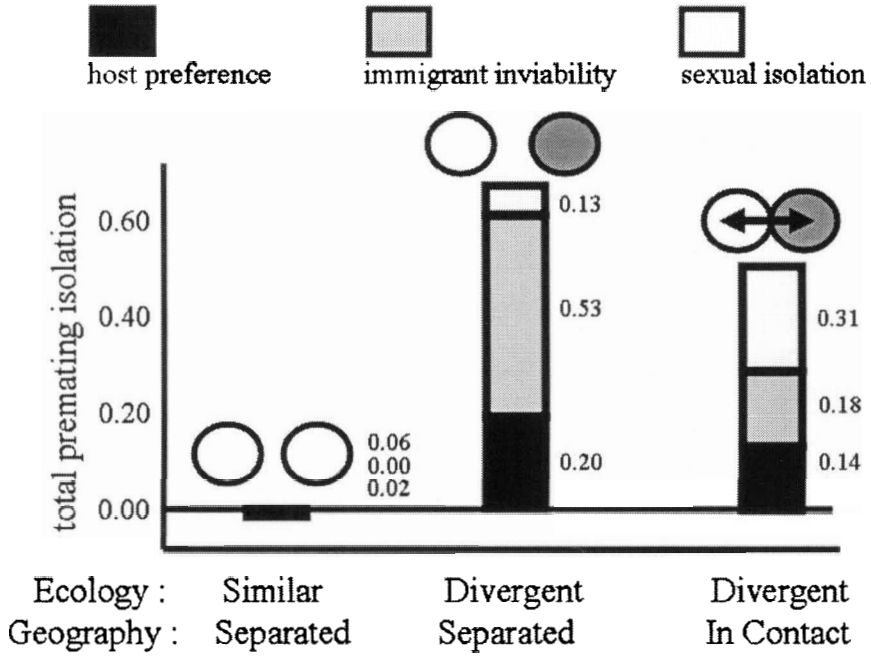

Table 5.1 Hypotheses and predictions for the evolution of habitat preferences.

Evolutionary mechanism	Geographic context	Predictions regarding geographic variation
Selection to reduce search costs and to increase efficiency; can act directly on preference loci	Can act in populations where a single or multiple hosts are available	Divergence between populations using alternate hosts, even when they each use a single (but different) host
Selection against habitat-switching; often acts indirectly on preference loci via their genetic association with fitness loci	Acts only in populations where multiple habitats exists (i.e. where there is the opportunity for habitat-switching)	Greater divergence between parapatric / sympatric populations than between allopatric populations ('character displacement')
Gene flow	Acts when gene flow can occur between populations using alternate hosts, most likely in parapatry / sympatry	Greater divergence between allopatric populations than between parapatric/sympatric populations
Balance between selection and gene flow	N/A	Variable, depending on the relative strength of different forms of selection and levels of gene flow

Table 5.2 Mean host preference (% picking *Ceanothus*) for individuals from populations of *T. cristinae* feeding on two different host-plant species (A – *Adenostoma*, C – *Ceanothus*).
Experiment #1 uses field-captured insects with each individual considered a replicate and a chi-square test was used to determine whether host species picked is dependent on host of origin. Experiment #2 uses field-captured insects with multiple individuals from the same host per replicate. A t-test is used to test whether mean preference differs for replicates with individuals from *Ceanothus* versus *Adenostoma*. Experiment #3 assesses the host preferences of F1 laboratory emerged nymphs derived from genetic crosses (both parents from the same host and population). Each family is considered a replicate and a t-test on family means is used to test for differences between offspring derived from parents from populations using *Ceanothus* versus *Adenostoma*. Pops = populations. Allop. = Allopatric. Parap. = Parapatric.

experiment	mean (s.d.) C pops	mean (s.d.) A pops	test statistic	df	# replicates	# individuals
Pooled						
Experiment #1	90 (30)	72 (45)	79.03***	1	n/a	1426
Experiment #2	75 (36)	56 (35)	2.89**	141	143	710
Experiment #3	67(32)	50 (34)	2.96**	143	145	988
Allop. only						
Experiment #1	93 (25)	63 (48)	82.30***	1	n/a	615
Experiment #2	78 (15)	30 (12)	5.06***	12	14	117
Experiment #3	72 (25)	40 (35)	4.25***	60	62	480
Parap. only						
Experiment #1	85 (36)	74 (44)	10.57**	1	n/a	811
Experiment #2	73 (42)	56 (35)	2.18*	127	129	598
Experiment #3	61 (37)	56 (30)	0.65	81	83	508

*$p < 0.05$, **$p<0.01$, ***$p<0.001$

Table 5.3 Tests for whether preference for the native host (the host that the population that an individual is derived from uses) differs between individuals from allopatric versus parapatric populations.
Differences between groups were tested using a chi-square test (Experiment #1) and t-tests (Experiment #2, #3). Table 5.2 provides mean preferences.

experiment	test-statistic	d.f.	p-value
***Adenostoma* populations**			
Experiment #1	6.47	1	<0.05
Experiment #2	1.30	102	0.20
Experiment #3	1.91	72	0.06
***Ceanothus* populations**			
Experiment #1	12.76	1	<0.001
Experiment #2	0.35	37	0.73
Experiment #3	1.52	69	0.13

Table 5.4 Host preference of *T. cristinae* walking-sticks collected from populations using *C. spinosus* and *A. fasciculatum* as host-plants.
Abbreviations are ind. = individuals, reps.= replicates, fam. = families, pop. = population, C = *Ceanothus,* A = *Adenostoma.* %C refers to the percent of individuals picking *Ceanothus*

			Experiment #1			Experiment #2			Experiment #3		
Host	Pop.	% area C	# ind.		% C	# reps.	# ind.	% C	# fam.	# ind.	% C
C	p	100	149		93	-	-	-	10	77	57
C	hvc	34	29		79	-	-	-	6	10	25
C	vpc	100	174		91	-	-	-	1	2	50
C	pr	100	68		93	11	89	78	-	-	-
C	outc	67	21		90	-	-	-	-	-	-
C	gibr	100	34		94	-	-	-	-	-	-
C	pe	100	33		100	-	-	-	12	132	79
C	r12c	70	70		90	10	10	90	14	97	73
C	r6c	100	1		100	-	-	-	3	11	71
C	mboxc	5	52		87	-	-	-	12	50	67
C	vpwc	100	3		100	-	-	-	-	-	-
C	outwc	100	4		100	-	-	-	-	-	-
C	ogc	1	41		76	-	-	-	-	-	-
C	sc	100	1		100	-	-	-	4	16	83
C	wcc	100	3		100	-	-	-	6	43	82
C	r9c	90	-		-	11	11	73	-	-	-
C	ptc	54	-		-	7	24	51	-	-	-
C	vpac	94	-		-	-	-	-	1	6	67
C	mc	39	16		81	-	-	-	2	15	46
total			699		-	39	144	-	71	459	
A	hva	34	98		78	7	75	66	17	78	40
A	ma	39	52		62	24	196	46	10	80	63

			Experiment #1			Experiment #2			Experiment #3		
Host	**Pop.**	**% area C**	**# ind.**		**% C**	**# reps.**	**# ind.**	**% C**	**# fam.**	**# ind.**	**% C**
A	la	0	139		63	3	28	30	13	135	42
A	vpa	94	103		84	17	135	61	-	-	-
A	ha	8	111		81	-	-	-	-	-	-
A	outa	67	84		86	-	-	-	1	1	100
A	r12a	70	57		44	20	20	60	11	125	60
A	r23a	0	1		0	-	-	-	7	28	54
A	mboxa	5	68		60	7	58	66	7	30	74
A	oga	1	9		89	2	19	44	-	-	-
A	lrn	0	5		80	-	-	-	6	36	21
A	r21a	10	-		-	18	18	61	-	-	-
A	pta	54	-		-	6	27	43	-	-	-
A	loga	15	-		-	-	-	-	2	16	46
total			727		-	104	566	-	74	529	-

CHAPTER 6.
MIGRATION AND THE GENETIC COVARIANCE BETWEEN HABITAT PREFERENCE AND PERFORMANCE*

*A version of this chapter appears as Nosil, P., Crespi, B.J., Sandoval, C.P., and Kirkpatrick, M. 2006. Migration and the genetic covariance between habitat preference and performance. American Naturalist 167: E66-E78. Reprinted with permission from the American Society for Naturalists.

6.1 Abstract

Studies of the genetic covariance between habitat preference and performance have reported conflicting outcomes, ranging from no covariance to strong covariance. The causes of this variability remain unclear. Here we show that variation in the magnitude of genetic covariance can result from variability in migration regimes. Using data from walking-stick insects and a mathematical model, we find that genetic covariance within populations between host-plant preference and a trait affecting performance on different hosts (cryptic color-pattern) varies in magnitude predictably among populations according to migration regimes. Specifically, genetic covariance within populations is high in heterogeneous habitats where migration between populations locally-adapted to different host plants generates non-random associations (i.e. linkage disequilibrium) between alleles at color-pattern and host preference loci. Conversely, genetic covariance is low in homogeneous habitats where a single host exists and migration between hosts does not occur. Our results show that habitat structure and patterns of migration can strongly affect the evolution and variability of genetic covariance within populations.

6.2 Introduction

A fundamental equation in evolutionary genetics states that between-generation evolutionary change in trait means, $\Delta\bar{\mathbf{z}}$, is a function of the matrix of genetic variances and covariances, **G**, and the vector of selection gradients, β : $\Delta\bar{\mathbf{z}} = \mathbf{G}\beta$ (Lande 1979). Thus genetic covariance between traits is a central topic in evolutionary biology because it may either constrain or facilitate both adaptation (Lande 1979; Brodie 1989; Arnold 1992; Barton 1995; Schluter 1996; Hodges et al. 2002; Sinervo and Svensson 2002; Otto 2004) and speciation (Felsenstein 1981; Diehl and Bush 1989; Hawthorne and Via 2001; Kirkpatrick and Ravigné 2002; Blows and Higgie 2003; Coyne and Orr 2004; Kawecki 2004).

In contrast to general agreement as to how genetic covariances affect the evolution of trait means, the degree to which genetic covariances themselves evolve, and how they evolve, remain controversial (Gould 1977; Lande 1979, 1980; Turelli 1988; Arnold 1992; Roff 2000; Begin and Roff 2004). Of particular interest is the evolution of genetic covariance within populations, because covariance among populations can arise simply from natural selection causing correlated change in a suite of traits among populations (Armbruster and Schwaegerle 1996; Sinervo and Svensson 2002). Thus we focus on the evolution of genetic covariance within populations.

By one mechanism, genetic covariance within populations may simply reflect underlying developmental constraints (that is, patterns of pleiotropy) that remain relatively unchanged through space and time (Gould 1977; Arnold 1992). Physical linkage might also remain relatively constant and help maintain associations between alleles at different loci (linkage disequilibrium), thereby facilitating constant genetic covariance (Hawthorne and Via 2001). Another mechanism that generates genetic covariance within populations is migration between genetically-differentiated populations. Theory clearly demonstrates that migration produces associations between alleles at different loci (linkage disequilibrium), even when they are physically-unlinked (Kimura 1956; Nei and Li 1973; Kirkpatrick et al. 2002). Natural selection can play a role in this latter process by causing the required population divergence.

In this paper, we test for genetic covariance within populations generated by migration between populations. We predict greater genetic covariance in habitats where migration between divergent populations occurs. Empirical examples of genetic covariance generated by migration are lacking and are of interest on two levels. In a general sense, we would like to know if migration plays an important role in determining how covariances evolve, or whether pleiotropy is the dominant factor. In a more specific context, we are interested in understanding the causes of variation in the genetic covariance between habitat preference and traits conferring fitness in different habitats. This question has been the focus of substantial empirical work and has lead to conflicting conclusions. Thompson (1988) and Jaenike and Holt (1991, Tables 2 and 6) reviewed these studies and found reports of no covariance, weak covariance, and strong covariance (see also below for more recent references). The causes of this variability are unclear.

Research on genetic covariance between habitat preference and traits linked to habitat-specific performance has focused on two scenarios. One body of work is focused on background color-matching via cryptic coloration, a classic example of adaptation via natural selection (Cott 1940; Kettlewell 1973; Endler 1984). Because cryptic organisms are subject to strong selection to prefer backgrounds upon which they are well-hidden, covariance is expected between substrate preference and body color. While some studies detect the expected covariance (Kettlewell 1955; Gillis 1982), others do not (e.g. Steward 1985; Grant and Howlett 1988). A second situation involves host preference and traits conferring adaptation to hosts in phytophagous insects (Thompson 1988; Diehl and Bush 1989; Hawthorne and Via 2001; Kawecki 2004). This scenario is well-studied because genetic covariance between host preference and performance has implications for speciation via host shifting. Again, some studies have detected the expected covariance between these two traits (e.g. Via 1986; Singer and Thomas 1998; Hawthorne and Via 2001; Bossart 2003; Forister 2004), while others have not (Futuyma and Moreno 1988; Jaenike and Holt 1991; Fox 1993; Fry 1996; Poore and Steinberg 2001).

Populations of phytophagous insects differ in the ecological and spatial structure of the habitats they occupy. This diversity provides the opportunity to examine whether variation in regimes of migration contributes to variation in genetic covariance. In this study, we document genetic covariance between host-plant preference and host plant-

specific performance (represented here by cryptic coloration). We show that the magnitude of this genetic covariance within populations varies positively with the opportunity for migration between divergent populations. Thus genetic covariance is strongly affected by habitat structure. We also estimate genetic variance and show that it does not vary systematically with the opportunity for migration. Thus the low genetic covariance observed when there is no migration between populations does not appear to stem solely from a lack of genetic variation. Our findings indicate that patterns of migration can strongly affect the evolution and variability of genetic covariance within populations. This effect may at least partly explain why previous studies of phytophagous insects have sometimes found a correlation between preference and performance, and sometimes not.

We begin by describing the biology of the walking-stick populations studied here. We then present a simple model for the evolution of the genetic covariance due to migration, which is based on the biology of our system but also applies more generally. Next, we test the predictions of the model using both field data and a quantitative genetic experiment. Finally, we discuss the implications of our results for explaining the evolution of genetic covariance between preferences and performance.

6.3 Study System

Timema cristinae walking-sticks feed on one of two host-plant species; *Ceanothus spinosus* or *Adenostoma fasciculatum*. A "population" is defined as all the insects collected within a patch of a single host species (as in Nosil et al. 2002, 2003; Nosil and Crespi 2004; Nosil 2004). We focus here on nine populations from two distinct types of study "sites" that are separated from all other sites by regions without suitable hosts (that is, a site is a contiguous area of one or both hosts). "Homogeneous" sites contain only one of the host-plant species and thus only one population of walking-sticks. "Heterogeneous" sites contained both hosts and thus two populations of walking-sticks, one feeding on each host species.

We studied walking-sticks in three homogeneous and three heterogeneous sites. Homogeneous sites 1 and 3 contained only *Ceanothus*, while homogeneous site 2

contained only *Adenostoma*. In heterogeneous sites 4 to 6, the frequency of *Ceanothus* varied from 5% to 70% (Table 6.1). Our study focuses on genetic covariance within populations. Host patches are sufficiently large and distinct relative to the mobility of these insects that each population at a heterogeneous site receives only limited migration from the other population using the alternate host (Sandoval 1993; Nosil et al. 2003; see below for details).

Genetic covariance was assessed between two traits that are involved in adaptation and reproductive isolation. The first trait is cryptic color-pattern. This trait is determined by a single diallelic locus, with the allele for an unstriped morph dominant over the allele for a striped morph (Sandoval 1993). Both morphs occur within populations on each host, but the unstriped morph is more common on *Ceanothus* (mean frequency = 81%) while the striped morph is more common on *Adenostoma* (mean frequency = 72%; Sandoval 1994a; Nosil et al. 2002; Nosil 2004). Population divergence has occurred via differential visual predation: the unstriped color-pattern confers high survival on *Ceanothus* but low survival on *Adenostoma*, and vice versa for the striped pattern (Sandoval 1994a,b; Nosil 2004). Although studies of phytophagous insects generally consider physiological traits when referring to performance, divergent selection on color also results in performance trade-offs.

The second trait is host preference, which has also diverged between populations. Populations feeding on *Ceanothus* exhibit a stronger preference for that host than do populations feeding on *Adenostoma* (Nosil et al. 2006). Population divergence in host preference has a genetic basis and occurs between adjacent populations that use alternative hosts and between geographically-separated populations that each use single but different hosts. The latter observation indicates that host preference is under selection even in homogeneous sites (those with a single host), perhaps due to search and efficiency costs acting directly on host preference (Bernays and Wcislo 1994). Because these insects mate exclusively on their hosts, divergent host preferences confer partial premating isolation. A detailed study of divergence in mean host preference between populations has shown that mean host preference evolves via a balance between selection and gene flow and will appear elsewhere (Nosil et al. 2006); here we focus on genetic covariance. Less is known about the genetic determination of host preference than that of

color-pattern, but additive gene action is suggested by the fact that crosses between populations yield offspring with intermediate preferences (Nosil et al. 2006).

We compared the genetic covariance between color-pattern and host preference within populations at heterogeneous sites with the covariance at homogeneous sites. Walking-sticks have relatively low motility (Sandoval 1993), allowing selection to cause divergence between populations within a heterogeneous site (Sandoval 1994a; Nosil et al. 2003; Nosil 2004). Morphological and molecular data indicate, however, that some migration between populations on different hosts does occur in heterogeneous sites (Sandoval 1994a, Nosil et al. 2003; Nosil and Crespi 2004). For example, divergence in traits means and in DNA is consistently lower between adjacent populations at the same site than between geographically-separated populations, indicative of migration. The migration rate into populations at heterogeneous sites (defined as the mean proportion of individuals that are immigrants arriving from the other host species in that generation) is estimated as 0.043 (maximum = 0.232). These estimates are derived using the coalescent-based methods of Beerli and Felsenstein (2001), but are rough as they consider only a single locus (mitochondrial DNA; Nosil et al. 2003). In homogeneous sites, by contrast, migration between populations does not occur because only one population on a single host exists. Thus genetic covariance between color-pattern and host preference caused by migration is only expected within populations from heterogeneous sites.

Thus the main prediction we test is that the genetic covariance between color-pattern and host preference will be higher within populations from heterogeneous sites. We then go on to ask how a quantitative measure of habitat heterogeneity (the relative abundance of the two hosts) varies with the genetic covariance. Genetic covariance is expected to increase as the two hosts become more similar in relative abundance because greater population divergence occurs under such scenarios (Sandoval 1994a; Nosil et al. 2003; Nosil and Crespi 2004; Nosil 2004), and covariance generated by migration is proportional to the degree of population divergence. Greater population divergence itself is observed as relative host abundances become similar because migration rates between pairs of populations at the same site become more symmetric, but not necessarily lower overall (Nosil et al. 2003 for details). Increased symmetry in migration promotes divergence because one population does not incur such disproportionately high levels of

homogenizing migration that swamping occurs. This illustrates how migration can have opposing effects: increased migration causes greater covariance for a given level of population divergence, but can decrease the amount of divergence between the populations.

To motivate the predictions more precisely, we will now develop a simple mathematical model, based on the ecology and genetics of the walking sticks, which shows how the genetic covariance depends on migration.

6.4 Mathematical Model

We are interested in predicting the genetic covariance, which we denote G_{CH}, that develops between color-pattern and host preference as the result of migration between two populations. We assume the two traits are controlled by separate sets of autosomal loci (no pleiotropy) that are expressed equally in males and females. In order to quantify the color-pattern phenotypes, we give striped individuals a score of $c = 0$ and unstriped individuals a score of $c = 1$; the frequency of striped individuals in the population is denoted f_s. Consistent with the data from *T. cristinae*, we assume color-pattern is controlled by a single locus C, with allele 1 for unstriped coloration dominant over the allele 0 for striped. Note that the additive genetic variance is well-defined even though color-pattern is controlled by a single locus with a dominant allele (Lynch and Walsh 1998 chap. 21).

We assume genetic variation in host preference is caused by an arbitrary number of loci that show no dominance or epistasis. Each of these loci have two alleles, also denoted 0 and 1, and different loci may make unequal contributions to host preference. An individual's host preference, denoted h, is defined as the probability that an individual will chose *Ceanothus*, and the frequency that host is chosen across all individuals in the population is written $\bar{h}$. For simplicity, we assume that individuals within a population mate randomly. Although there is some evidence for assortative mating by color-pattern within populations, it does not vary according to site heterogeneity such that it could confound our test for an association between genetic covariance and site heterogeneity

(Nosil et al. 2002). Note that Nosil et al. (2003) examined assortative mating between (rather than within) populations, and that their findings do not apply to estimating covariance within populations.

The supplemental equations at the end of this chapter derive the dynamic equation and equilibrium for the genetic covariance between color-pattern and host preference under these fairly general assumptions. The results simplify under several scenarios. If there is only a single host preference locus or if all host preference loci have equal effects and equal allele frequencies, we find that the genetic covariance at equilibrium is

$$G_{CH} \approx m(\sqrt{f_s^M f_s} - f_s)(\bar{h} - \bar{h}^M)/\tilde{r}, \qquad (1)$$

where m is the migration rate between populations, f_s is the frequency of striped individuals and $\bar{h}$ is the frequency of individuals preferring *Ceanothus* within the focal population, M superscripts denote corresponding values among arriving migrants, and $\tilde{r}$ is the harmonic mean recombination rate between the color-pattern and host preference loci. Equation (1) is an approximation that assumes that migration is weak relative to recombination ($m << \tilde{r}$).

As we expect intuitively, the covariance increases with the migration rate and with the genetic differences between the populations in the two traits. The covariance decreases with larger recombination rates between the color-pattern and host preference loci.

An alternative scenario also leads to a simple expression for the covariance. If the color-pattern locus is unlinked to any of the host preference loci, even if preference loci have unequal effects and allele frequencies, then Equation (1) also applies if $\tilde{r}$ is replaced by ½.

With this model as motivation, we now present the methods used in our empirical study.

6.5 Methods

6.5.1 Population divergence

For migration to generate genetic covariance between color-pattern and host preference, populations must differ in both traits. Morph frequencies were obtained by randomly sampling individuals from the nine populations at the six study sites (total n = 2542 individuals; Table 6.1 for population-specific sample sizes). Mean host preference for each population was estimated with randomly sampled individuals from the nine populations, using protocols described below for the quantitative genetic experiment (total n = 636 individuals; Table 6.1). Differences among populations were assessed using χ^2 tests. Seven of our populations were used in previous studies (Nosil et al. 2002, 2003; Nosil and Crespi 2004; Nosil 2004). Sites 1 to 5 of the present study correspond respectively to Sites P, LA, PE, (MBOXC and MBOXA), and (MC and MA) of the earlier work.

6.5.2 Genetic covariance

To estimate genetic covariance, crosses between individuals from within the eight of the nine populations were carried out in 2003. Sexually-immature instars were captured in the field using sweep nets. The sexes were reared separately on *Ceanothus* cuttings in glass jars. Within a few days of sexually-maturity, a single virgin male and a single virgin female were housed together in a petri dish until copulation was observed. In order to maximize the probability that we would detect genetic covariance between color-pattern and host preference, individuals were paired assortatively by color. Thus all matings were between individuals of the same color-pattern morph, with approximately equal numbers of pairs of each morph; this allowed a reasonable number of crosses to be conducted using the rarer morph within a population. Each pair was maintained on *Ceanothus* cuttings until the female died (females lay eggs singly and daily).

Offspring were scored for host preference within a few days of emergence. In total, 428 offspring were scored from 64 families, with 9, 12, 8, 10, 13, 12 families for sites 1 - 6 respectively. The number of crosses from the *Ceanothus* population was 6, 0, 9

in heterogeneous sites 4 - 6, respectively. Between 39 and 108 nymphs per population and 3 to 21 per family were tested.

Individual offspring were placed in the bottom of a 500ml plastic cup (height, 15cm) with one 12cm cutting from each host species in the cup. The bottom end of each cutting was in a water-filled aqua-pick which held the cutting upright and kept it fresh. Containers were covered with wire netting secured by elastic bands. Assays were initiated in the evening and left in darkness overnight. In the morning, the host each individual was resting on was recorded. For assays where the test individual did not choose a host, the container was left overnight until a host was chosen (for up to two nights). Each individual was used only once. All scoring was done blind to parental population and morph by P. Nosil. Color-pattern was not scored because of difficulties rearing the nymphs until it was distinguishable.

The genetic covariance between color-pattern and preference was estimated as twice the phenotypic covariance between the mean parental color-pattern and the mean offspring host preference (Lynch and Walsh 1998). The genetic covariances were estimated using full sib families and thus represent broad sense genetic measures. Additionally, because the parents used in the genetic crosses were initially captured in the field we cannot explicitly rule our maternal effects. However, the fact that all parents were reared to maturity in a common environment, along with several other lines of reasoning, indicates that covariance has a strong genetic basis (see Discussion).

The assortative mating used in the breeding design increases the covariance between parents and offspring, leading to upwardly-biased estimates of the genetic covariance. In principle, it is possible to apply a correction for this bias, but when there is genetic dominance (as with color-pattern in these insects) the correction involves genetic quantities that we cannot estimate directly. Our primary goal is to test whether the covariance is correlated positively with site heterogeneity, rather than to make precise estimates of the covariance. The assortative mating in the breeding design would only confound our results if the bias also varied positively with site heterogeneity. In contrast, if a bias does exist we expect it to work in the opposite direction; homogeneous sites have more extreme color-pattern frequencies and preferences, so the nonrandom mating used

in the breeding design will inflate the parent-offspring covariance more in these crosses than in those from heterogeneous sites. In brief, the quantitative genetic experiment will give upwardly-biased estimates of the covariance for all populations, but it is conservative with respect to the correlation with habitat structure that we seek to test.

Difference in host preference between offspring derived from striped versus unstriped parents is indicative of genetic covariance between host preference and color-pattern. We tested for this difference using ANOVA analyses on family means. The analysis included parental morph (striped or unstriped), parental population and parental habitat-type (heterogeneous or homogeneous) as factors, as well as the interaction between parental morph and habitat type. We are primarily interested in the interaction term, which tests whether genetic covariance differs between habitat types.

We tested for an association between the magnitude of genetic covariance and site heterogeneity using a quantitative index of host-plant diversity. Our measure of site heterogeneity is the Simpson species diversity index, which for our case of two host species is equal to $2p_i(1-p_i)$, where p_i is the proportion of the patch occupied by *Ceanothus* (thus maximal values are achieved when both species are equally abundant). This proportion was estimated using aerial photographs and ground-proofing (as in Sandoval 1994a; Nosil et al. 2003).

6.5.3 Phenotypic covariance

In addition to our study of the genetic covariance, we also looked for a positive relationship between site heterogeneity and the phenotypic covariance of color-pattern and host preference within natural populations. This association was estimated by comparing the difference in host preference between color-pattern morphs that were captured in the field. Laboratory choice tests were conducted on individuals collected in 7 populations from 6 sites in 1992 (2 sites where 'homogeneous'). We offered insects one 30 cm high branch of each host species (branches kept 10 cm apart and out of contact in a Styrofoam sheet floating in a container with water, thereby keeping the plant fresh and preventing insects from escaping). Approximately 10 insects from the same population and of the same morph were placed on the styrofoam, midway between the two branches,

and left overnight (this is considered one replicate). The following morning the number of insects on each branch was counted by C.P. Sandoval. The choice test was replicated for each site and morph type based on insect availability (total number of individuals = 604; total number of replicates = 79, mean number of replicates per morph type per population = 5.6). Each insect was used only once. Mean preference from each replicate (% individuals choosing *Ceanothus*) was used as a single data point when calculating the mean preference of each morph within each population.

6.5.4 Genetic variances

In addition to its effects on genetic covariance, migration may influence the genetic variance of each trait. We therefore estimated the genetic variances of host preference and color-pattern within the study populations. Our main focus was to compare genetic covariance between populations, and logistical constraints limited the experimental designs we were able to use. Our estimates for the genetic variances in host preference should be interpreted with caution (and thus we prefer not to report genetic correlations explicitly).

Because of its simple genetic determinism, we can directly estimate the additive genetic variance for color-pattern in each site from morph frequencies. Color-pattern is controlled by a single locus with the allele for unstriped coloration dominant over the striped allele (Sandoval 1993). The additive genetic variance for color-pattern is therefore $G_C = 2p^3q$, where p is the frequency of the striped allele (Falconer and Mackay 1996, Equation 8.6). We assume the color-pattern locus is in approximate Hardy-Weinberg equilibrium, and estimated the allele frequency as $p = \sqrt{\bar{c}}$, where $\bar{c}$ is the frequency of the striped morph.

We estimated genetic variance in host preference from the genetic experiment described above by comparing variation within families to variation between families (i.e. a full-sib analysis). We used two methods, both based upon one-way ANOVA. First, we estimated broad-sense genetic variance and heritability using all the data with a bootstrapping method developed by Phillips and Arnold (1999). We ran 10,000 bootstrap replicates using their program H2boot to assess whether these parameters were

significantly different than zero. Second, we used a full-sib analysis to estimate the genetic variance in host preference (G_H) for each population separately. Our estimator is $2(MS_T - MS_W)$, where MS_T is the total mean square and MS_W is the mean square within full-sib families (Falconer and Mackay 1996 p. 167). This estimate is biased upwards by genetic dominance and common environmental effects (including maternal effects). Further, the estimates have large errors because of only a small number of families was measured. For these two reasons, we interpret the results with great caution.

6.6 Results

6.6.1 Population divergence

Population divergence in color-pattern and host preference is required for migration between populations to generate genetic covariance between these traits. This precondition is fulfilled. Morph frequencies (% striped individuals) differ among populations using different hosts ($\chi^2 = 489.27, p < 0.001$, data from populations of the same host pooled). They also differ between adjacent pairs of populations on different hosts at the same site ($\chi^2 = 9.48, 13.50, 54.64$ for sites 4-6 respectively, all $p < 0.01$; Table 6.1 for frequencies). Likewise, host preferences (% preferring *Ceanothus*) differ among populations using different hosts ($\chi^2 = 91.45, p < 0.001$), and between adjacent pairs of populations on different hosts at the same site ($\chi^2 = 10.21, 14.62, 77.30, p = 0.07, 0.006, <0.001$ for sites 4-6 respectively; Table 6.1).

6.6.2 Genetic covariances

The genetic covariance between color-pattern and host preference is greater within populations from heterogeneous sites than within populations from homogeneous sites. Three lines of statistical argument support this conclusion.

First, estimates for the genetic covariance in the five populations at heterogeneous sites are all larger than those from the three populations at homogeneous sites (Table 6.1; Fig. 6.1). The probability that this pattern would arise by chance is $(5!)(3!)/(8!) = 1/56 < 0.05$, and so we conclude that the association between site heterogeneity and genetic covariance is statistically significant. We stress that the individual estimates for the

covariance have low precision because of the small number of families tested from each single population. Nevertheless, among populations the predicted pattern of larger covariance at heterogeneous sites is strongly supported.

Second, the difference in host preference between offspring derived from striped versus unstriped parents varied significantly between the two types of sample sites, such that this difference was greater in populations from heterogeneous sites (parental morph x sample-site type interaction, $F_{1,64} = 15.32, p < 0.001$). These data are shown in Figure 6.1.

Third, when all 8 populations are considered, the genetic covariance is positively correlated with our quantitative measure of site heterogeneity (Spearman nonparametric correlation $\rho = 0.952, p < 0.001$, one-tailed probabilities due to the a priori expectation of a positive association). The pattern is shown in Figure 6.2a. This association is suggestive but not significant when only the 5 populations from heterogeneous sites are considered ($\rho = 0.791, p = 0.056$ using only these 5 populations). The same association with the quantitative index of site heterogeneity was observed for the phenotypic covariance between color-pattern and host preference. Consistent with greater phenotypic covariance in more heterogeneous sites, the difference between morphs in host preference was positively correlated our quantitative measure of site heterogeneity ($\rho = 0.67$, $p < 0.05$, for all 7 populations, $\rho = 0.87, p < 0.05$ using only the 5 populations from heterogeneous sites). These associations are depicted in Figure 6.2b.

Although the qualitative agreement between the data and the theory is good (e.g. correlation between estimated and predicted covariance is $\rho = 0.90$, $p < 0.01$, $n = 8$), the quantitative agreement is not. The estimates for the genetic covariance are all much larger than our model predicts (Table 6.1). We return to this discrepancy in the Discussion.

6.6.3 Genetic variances

We detected significant genetic variation in host preference. Combining data across sites, the bootstrap method estimates heritability as $h^2 = 0.20$ (s.e. = 0.07) and the genetic variance as 0.041 (s.e. = 0.013); these estimates are significantly greater than 0 (p

< 0.002). Likewise, color-pattern is genetically variable, although this was already known from data on phenotypic variation and the simple inheritance of this trait.

Estimates of the genetic variances for both traits for each individual population are shown in Table 6.1. In contrast to the results for the genetic covariance, there is no evidence for a systematic association between habitat heterogeneity and either the additive genetic variance for host preference or for color-pattern.

6.7 Discussion

We detected broad sense genetic covariance between color-pattern and host preference within populations of walking-stick insects. This genetic covariance was greater in habitats where migration occurs between genetically-divergent populations. Moreover, this pattern does not appear to solely reflect levels of genetic variation, because genetic variance did not vary systematically with habitat heterogeneity. Even if migration did affect covariance by affecting levels of genetic variance, variability in covariance is nonetheless explained by migration regimes. These findings suggest that genetic covariance can evolve in response to migration, and is therefore not only determined by underlying developmental mechanisms.

Genetic covariance caused by linkage disequilibrium between physically-unlinked loci is often considered to be transitory, as recombination can erode it. Thus genetic covariance between traits affected by unlinked loci might have a weaker effect on future evolution than genetic covariance facilitated by pleiotropy or physical linkage (Hawthorne and Via 2001). However, genetic covariance can persist even between traits affected by physically-unlinked loci, so long as migration between genetically-differentiated populations occurs (Nei and Li 1973, model used in this study). The degree to which migration and pleiotropy are mutually-exclusive mechanisms for the generation of genetic covariance is an interesting remaining question, because it might influence the longer-term evolutionary consequences of genetic covariance generated by migration. Could covariance generated by migration affect whether genetic covariance due to other causes arises? We do not claim that migration is the only factor explaining variable covariance, and our results do not preclude a role for other factors, such as variation in

pleiotropy and physical linkage (Hawthorne and Via 2001). Rather, our results show that migration itself can play an important role in the evolution of genetic covariance.

Population divergence is required for migration to generate genetic covariance. Our results also demonstrate that divergent selection can play an integral role in the generation of genetic covariance by causing the required population divergence. For example, both the traits examined in the current study diverge between populations due to selection (Sandoval 1994a,b; Nosil 2004). Moreover, the greatest covariance was observed when both hosts were equally common. This indicates that greater genetic covariance was generated when selection could best overcome the homogenizing effects of asymmetric gene flow, to cause greater population divergence.

Irrespective of its underlying cause, an important consequence of the increased genetic covariance is that it will cause a greater correlated response to selection in heterogeneous than in homogeneous habitats. This is because the change in the mean of an indirectly selected trait is the product of the selection gradient on the directly selected trait and the genetic covariance between the two (Lande 1979). Thus direct selection on color-pattern, for example, is expected to cause a greater change in the mean habitat preference when genetic covariance is high (as in heterogeneous sites) than when it is low (as in homogeneous sites). Thus our findings have implications for understanding the evolution of host preference in this system: host preferences can evolve as a correlated response to selection acting on color-pattern in heterogeneous habitats, but not in homogeneous sites (see Nosil et al. 2006 for detailed consideration of divergence in mean host preference among populations).

The host preferences detected in this study were atypical in that they were relative, not absolute. When offspring derived from different morphs differed in preference (i.e. in heterogeneous sites), offspring from unstriped individuals strongly prefer to rest on *Ceanothus* whereas offspring from striped individuals tend to exhibit no preference. Consideration of the biology of this system, in particular the relative fitness of the four different combinations of color-pattern and preference, yields a potential explanation. The striped morph has higher survival on *Adenostoma* and the unstriped morph has higher survival on *Ceanothus* (Sandoval 1994a,b; Nosil 2004), but both

morphs have higher fecundity on *Ceanothus* (Sandoval and Nosil 2005). Thus the genotypic combination of unstriped with preference for *Ceanothus* will have particularly high fitness as it will accrue both high survival and high fecundity. Conversely, the combination of unstriped with preference for *Adenostoma* will suffer both low survival and low fecundity. Thus the former genotypic combination should be much more highly represented in nature such that most unstriped individuals prefer *Ceanothus* (as observed). The picture is somewhat different for the host preference of striped individuals where both genotypic combinations might accrue similar fitness; striped with preference for *Ceanothus* will gain high fecundity whereas striped with preference for *Adenostoma* will gain high survival. This similarity in 'total' fitness could result in both genotypic combinations being maintained in populations from heterogeneous sites such that only half of striped individuals prefer *Adenostoma* (as observed).

What maintains genetic variance in homogeneous patches, where ongoing migration cannot help maintain variation? Several hypotheses can be proposed. For color-pattern, visual predation may be frequency-dependent such that it maintains variation (Bond and Kamil 2002). Additionally, color (95% of individuals are green) and stripe are controlled by separate loci such that the stripe allele is hidden from selection when found in the red/grey individuals (which occur at about 5% and do not express the stripe; Sandoval 1993). For host preference, evolution away from an ancestral preference for *Ceanothus* (the likely ancestral host) may be ongoing (Crespi and Sandoval 2000). Thus allopatric populations of *Ceanothus* may have simply retained the ancestral preference whereas allopatric *Adenostoma* populations are in the process of evolving away from it (and thus do not exhibit a strong preference for their native host). Finally, rare or episodic gene flow between currently allopatric populations could introduce variation in both traits.

One additional consideration is maternal effects, because the parents used in the genetic crosses were initially captured in the field. For example, offspring derived from unstriped parents might be more likely to prefer *Ceanothus* than offspring from striped parents because unstriped parents were more likely to have experienced *Ceanothus* (with this environmental effect then influencing offspring preference). Several lines of reasoning indicate that such maternal effects are extremely unlikely to account for our

results: (1) all the parents were reared from first-instar to sexual maturity in a common environment, (2) in heterogeneous sites the differences in preference among offspring from different parental morphs occurred when the different morphs originated from the same host species and (3) a separate reciprocal-rearing experiment has shown that rearing environment (host reared upon from first-instar to sexual maturity) has no effect on host preference whereas genotype (host or population of origin) has a strong effect (Nosil et al. 2006). Thus the covariance detected in the current study most likely has a strong genetic basis.

Our estimates of the genetic covariance between color-pattern and habitat preference are all substantially larger than the values predicted by our model (Table 6.1). There are three obvious explanations for this discrepancy. First, and perhaps most importantly, the breeding experiment used assortative mating based on color-pattern (this method was required to generate enough crosses), which upwardly biases the estimates (Lynch and Walsh 1998). Because of dominance at the color-pattern locus, we cannot correct for this bias. But we expect this bias to work against the association between habitat heterogeneity and the size of genetic covariance (see the Material and Methods). Consequently, this bias is conservative with respect to the hypothesis we are testing. A second factor that would cause the covariance to be larger than the predicted values shown in Table 6.1 is physical linkage between the color-pattern and host preference loci. A third possibility is assortative mating by color-pattern within the natural populations from which the insects used in the genetic experiment were derived (Nosil et al. 2002).

Genetic covariance will not evolve by the selection / migration mechanism described in our study when 1) divergent selection does not cause population divergence and 2) when the geographic arrangement of hosts or substrates results in low opportunity for migration between genetically divergent populations. We note that a Web of Science search using terms 'migration' and 'genetic' and 'covariance' did not recover any studies examining both migration regimes and the genetic covariance between preference and performance. Likewise, a Web of Science search using 'migration' and 'genetic correlation' did not recover any such studies. Our results clearly suggest that future studies should consider the effects of habitat structure and migration on variation in genetic covariance.

Two factors make it difficult to assess systematically whether variation in migration has contributed to the discordant results from previous studies of genetic covariance between preferences and performance. First, most previous studies examined only a single population or pooled estimates among populations or subpopulations (Via 1986; Thompson 1988; Singer et al. 1988); thus variation within studies cannot be evaluated. Second, estimates of covariance in populations which use only a single host are almost completely lacking, precluding even an among-study comparison of covariance in studies where migration between hosts does versus does not occur (i.e. only the former tend to exist). As documented here, habitat heterogeneity and levels of migration can still differ among populations that are in geographic contact, although again this has not been examined previously.

Via (1986) and Singer et al. (1988) provided the first demonstrations of strong genetic covariance between preference and performance. They studied areas where migration between hosts could occur, and so migration may have contributed to the covariance they found. In both studies, there was the potential for variation in migration rates at different sites. Via (1986) examined covariance in aphids sampled from tomato and pea fields that were close together and in isolated field. Singer et al. (1998) noted the presence of both pure and mixed patches of the two host plants for their butterflies. In both studies, results from these different kinds of sites are pooled. It would be of interest to reanalyze the data to look for the association between migration and genetic covariance that we report here.

Two studies of butterflies found variation in genetic covariance that are suggestive of effects of selection and migration on genetic covariance. Bossart (2003) detected greater covariance in some instances in polyphagous populations than in monophagous populations. Forister (2004) reported variation in covariance between preference and performance among multiple populations of butterflies. Although a clear association with the opportunity for migration (i.e. geographical allopatry versus sympatry) was not evident, preference/performance correlations did appear to reflect different levels of local adaptation. For example, groups having undergone recent host shifts exhibited weaker associations.

Variable genetic covariances also have several implications for understanding speciation. This is particularly the case for traits such as performance and habitat preference, which can cause reproductive isolation (Diehl and Bush 1989; Funk 1998; Hawthorne and Via 2001; Funk et al. 2002; Coyne and Orr 2004). Although speciation is generally thought to occur between geographically-separated populations (Coyne and Orr 2004), a general feature of models of speciation without geographic isolation is the evolution of genetic covariance between traits under selection and traits conferring reproductive isolation (Felsenstein 1981; Kirkpatrick et al. 2002; Kawecki 2004). Thus migration can have dual effects. Although its homogenizing effects may generally prevent population differentiation and thus speciation (Slatkin 1987; Coyne and Orr 2004), migration might affect speciation and evolutionary divergence by generating genetic covariance.

In conclusion, predicting evolutionary change requires understanding not only how selection acts on traits but also how other evolutionary forces affect the genetic properties of populations (Arnold 1992; Turelli 1988; Roff 2000). Habitat structure may be integral to how these genetic properties evolve.

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6.9 Supplemental Equations

Our goal is to calculate the genetic covariance G_{CH} that develops between color-pattern and host preference as the result of migration between two populations. We assume these two traits are controlled by separate sets of diploid autosomal loci (there is no pleiotropy) that are expressed equally in males and females. We assign striped

individuals a phenotypic score of $c = 0$ and unstriped individuals a score of $c = 1$. The frequency of unstriped individuals in the population is denoted $\bar{c}$. We assume coloration is controlled by a single locus C, with allele 1 for unstriped coloration dominant over the allele 0 for striped. Genetic variation in host preference is caused by an arbitrary number of loci; we assume they show no dominance or epistasis. Each of these loci have two alleles, also denoted 0 and 1, and different loci may make unequal contributions to host preference. An individual's host preference, denoted h, is defined as the probability that an individual will chose *Ceanothus*, and the frequency that host is chosen across all individuals in the population is written $\bar{h}$.

Assuming that departures from Hardy-Weinberg in zygotes can be neglected (which implies that drift is weak and mating nearly random), the genetic covariance between color and host preference is

$$G_{\mathrm{CH}} = 2b_{\mathrm{c}}\sum_{i} b_i D_{ci}\ , \qquad (1)$$

With allele 1 fully dominant to allele 0 at the color-pattern locus, the additive effect b_{c} is equal to q_{c}, the frequency of allele 0 at that locus (Fisher 1918); thus we have $b_{\mathrm{c}} = q_{\mathrm{c}} = \sqrt{f_{\mathrm{s}}}$, where f_{s} is the frequency of striped individuals. Under our assumption of no dominance at the host preference loci, the additive effect b_i is simply equal to the difference in the average host preference of heterozygotes and homozygotes at locus i.

We next find an expression for the linkage disequilibrium D_{ci}. We assume that this disequilibrium evolves to an equilibrium under migration and recombination (which implies that correlational selection acting within each host is weak or absent). Following migration, which occurs at rate m, the disequilibria between alleles at color locus C and host preference locus i that were inherited from different parents is

$$D_{ci}^{'} = (1-m)D_{ci} + mD_{ci}^{\mathrm{M}} + m(1-m)(p_{\mathrm{c}} - p_{\mathrm{c}}^{\mathrm{M}})(p_i - p_i^{\mathrm{M}}), \qquad (2)$$

where superscripts M denote values among arriving migrants (equation 36 in Kirkpatrick et al. 2002). This expression applies to both the cases where the alleles were

inherited from different parents and where they were inherited from the same parent. When applied to alleles inherited from different parents, however, the two disequilibria in zygotes that appear on the right hand side (D_{ci} and D_{ci}^{M}) are zero. When considering alleles inherited from the same parent, Equation (2) requires a value for D_{ci}^{M}, the disequilibria among immigrants. For simplicity, we will assume that it equals D_{ci}, the association among zygotes in the focal population. This would be the case, for example, at an equilibrium between two symmetric populations (i.e. of similar size and exchanging equal numbers of migrants). The within-gamete disequilibrium among zygotes at the start of the next generation is then

$$D_{ci}'' = (1-r_{ci})[D_{ci} + m(1-m)(p_c - p_c^{M})(p_i - p_i^{M})] + r_{ci}m(1-m)(p_c - p_c^{M})(p_i - p_i^{M}), \quad (3)$$

where r_{ci} is the recombination rate between the two loci.

At an equilibrium, the differences in allele frequencies between the populations is maintained by divergent selection pressures acting on these loci. Equation 3 ignores the effects that selection has on the disequilibrium. These effects, however, will be negligible if migration is weak relative to recombination, $m << r_{ci}$ (the "quasi-linkage disequilibrium" approximation; see Kirkpatrick et al. 2002). Proceeding under this assumption, the equilibrium is found by setting D_{ci}'' equal to D_{ci} and solving, which gives

$$\hat{D}_{ci} \approx m(p_c - p_c^{M})(p_i - p_i^{M})/r_{ci} . \quad (4)$$

Substituting into Equation (1) produces

$$G_{CH} \approx 2m(\sqrt{f_s^{M} f_s} - f_s)\sum_i \frac{1}{r_{ci}} b_i(p_i - p_i^{M}) . \quad (5)$$

As we expect intuitively, the genetic covariance is inversely proportional to the recombination rates between the color-pattern and host preference loci.

This result simplifies under several scenarios. If the color-pattern locus is not linked to any of the host preference loci ($r_{ci} = ½$),

$$G_{CH} \approx 2m(\sqrt{f_s^M f_s} - f_s)(\bar{h} - \bar{h}^M) \,. \qquad (6)$$

If there is only a single host preference locus or if all the host preference loci have equal effects and equal allele frequencies,

$$G_{CH} \approx m(\sqrt{f_s^M f_s} - f_s)(\bar{h} - \bar{h}^M)/\tilde{r}, \qquad (7)$$

where $\tilde{r}$ is the harmonic mean recombination rate between the color-pattern and host preference loci. This is Equation 1 of the text.

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Table 6.1 Estimated genetic covariance (G_{CH}) between color-pattern and host preference in homogeneous versus heterogeneous sites. Sites 1-3 are 'homogeneous sites', whereas sites 4-6 are 'heterogeneous sites'. Also shown are the covariances predicted by Equation (1) assuming free recombination with low ($m = 0.043$) and high ($m = 0.232$) migration rates (these values are the mean and maximum estimates of m from Nosil et al. 2003), the percent of the site occupied by *Ceanothus* (C), the percent of individuals preferring to rest on C, the estimated genetic variance in host preference (G_H), percent unstriped individuals (%U), and the estimated genetic variance in color-pattern (G_C). Numbers in parentheses are sample sizes. No data was available for genetic covariance in population 5C.

Site	Estimated G_{CH}	Predicted G_{CH} (low m)	Predicted G_{CH} (high m)	% C at site	% prefer C	G_H	% U	G_C
1	0.03	0	0	100%	93% (149)	0.60	82% (501)	0.09
2	0.01	0	0	0%	63% (139)	0.27	14% (608)	0.12
3	0.00	0	0	100%	100% (33)	0.08	60% (154)	0.19
4C	0.10	0.002	0.009	5%	87% (52)	0.12	23% (190)	0.17
4A	0.07	0.002	0.010	5%	60% (68)	0.37	8% (99)	0.07
5C	-	0.002	0.011	39%	81% (16)	-	45% (29)	0.21
5A	0.15	0.003	0.014	39%	62% (52)	0.41	16% (174)	0.13
6C	0.26	0.004	0.024	70%	90% (70)	1.11	61% (444)	0.18
6A	0.12	0.006	0.031	70%	44% (57)	0.26	35% (343)	0.20

Figure 6.1 Results of the genetic experiment showing greater genetic covariance between color-pattern and host preference in heterogeneous than in homogeneous sites.

Populations are labelled below the y-axis (where the letter A and light boxes denote *Adenostoma* populations and the letter C and dark boxes denote *Ceanothus* populations). A) Mean % of offspring preferring *Ceanothus* (± 95% CI; the mean of the family means is depicted) for offspring derived from each morph for each population. The difference in preference between offspring derived from striped versus unstriped parents is proportional to the genetic covariance between color-pattern and host preference. This difference varies among habitat types such that it is greater in heterogeneous habitats ($p < 0.001$; ANOVA, see results). B) Estimated genetic covariance between color-pattern and host preference within each population. The probability that covariance would be greater in all five heterogeneous populations than in any of the three homogeneous populations is < 0.05 (results for details).

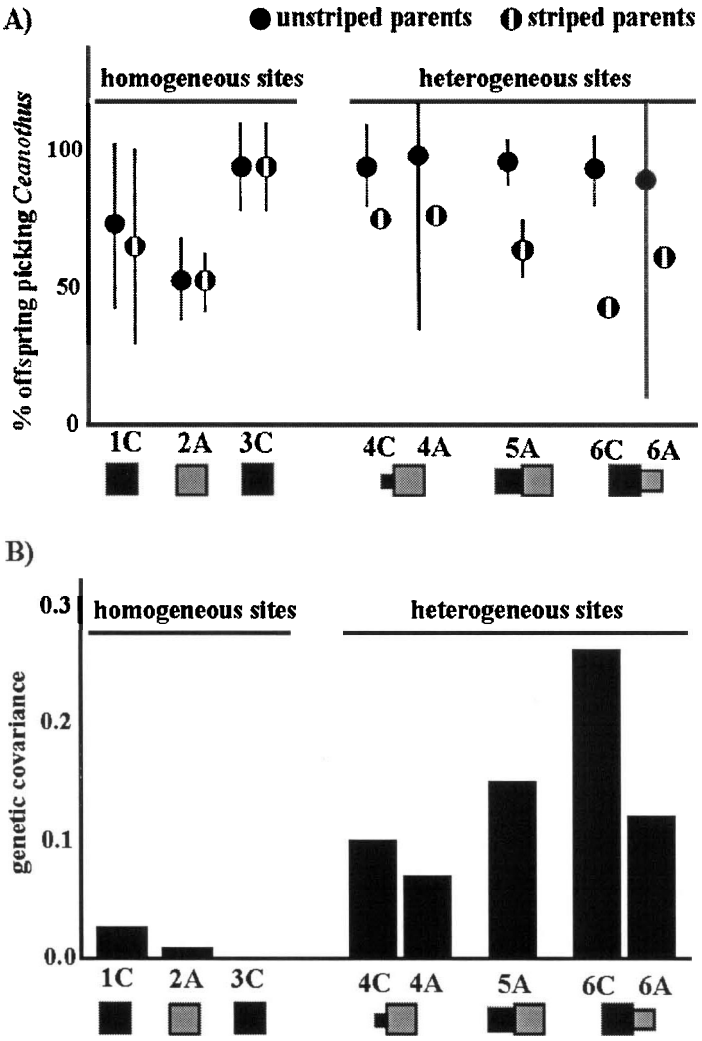
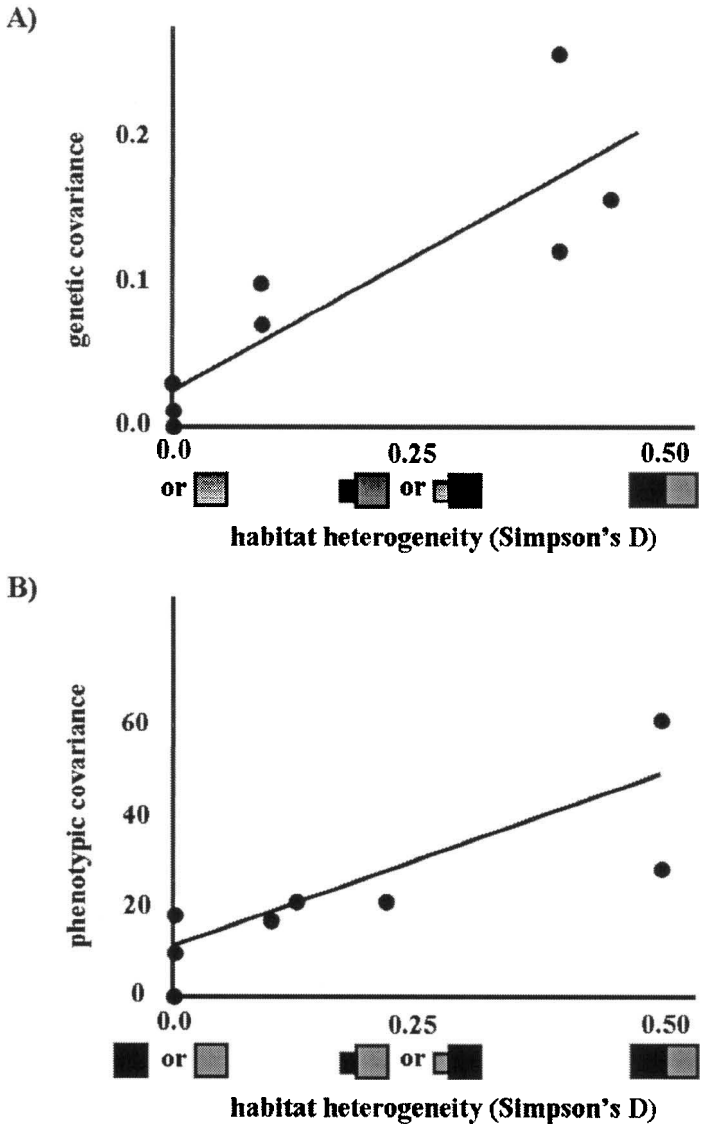


Figure 6.2 A positive association was detected between site heterogeneity and the covariance between color-pattern and host preference.
Site heterogeneity was estimated using Simpson' D, where maximal heterogeneity is achieved when each of the two host species occurs at roughly equal proportion (depicted by boxes below the y-axis). A) Genetic covariance estimated from the quantitative genetic experiment. B) Phenotypic covariance, depicted here as the difference in host preference between morphs collected in the wild (unstriped minus striped preference for *Ceanothus*).



CHAPTER 7.
PERSPECTIVE:
REPRODUCTIVE ISOLATION CAUSED BY NATURAL SELECTION AGAINST IMMIGRANTS FROM DIVERGENT HABITATS*

*A version of this chapter appears as Nosil, P., Vines, T., and Funk, D.J. 2005. Perspective: Reproductive isolation caused by natural selection against immigrants from divergent habitats. Evolution 59: 705-719. Reprinted with permission from the Society for the Study of Evolution.

7.1 Abstract

The classification of reproductive isolating barriers laid out by Dobzhansky and Mayr has motivated and structured decades of research on speciation. We argue, however, that this classification is incomplete and that the unique contributions of a major source of reproductive isolation have often been overlooked. Here, we describe reproductive barriers that derive from the reduced survival of immigrants upon reaching foreign habitats that are ecologically divergent from their native habitat. This selection against immigrants reduces encounters and thus mating opportunities between individuals from divergently adapted populations. It additionally reduces the likelihood that successfully mated immigrant females will survive long enough to produce their hybrid offspring. Thus, natural selection against immigrants results in distinctive elements of premating and postmating reproductive isolation that we hereby dub "immigrant inviability". We quantify the contributions of immigrant inviability to total reproductive isolation by examining study systems where multiple components of reproductive isolation have been measured, and demonstrate that these contributions are frequently greater than those of traditionally recognized reproductive barriers. The relevance of immigrant inviability is further illustrated by a consideration of population-genetic theory, a review of selection against immigrant alleles in hybrid zone studies, and an examination of its participation in feedback loops that influence the evolution of additional reproductive barriers. Because some degree of immigrant inviability will commonly exist between populations that exhibit adaptive ecological divergence, we emphasize that these barriers play critical roles in ecological modes of speciation. We hope that the formal recognition of immigrant inviability and our demonstration of its evolutionary importance will stimulate more explicit empirical studies of its contributions to speciation.

7.2 Introduction

Scientists expect their conceptual terminology to provide precise and complete descriptions of the phenomena to which they are applied. Terminological ambiguity impedes scientific progress because concepts often structure the thought, motivate the research, and influence the interpretations of scientists (Keller and Lloyd 1992). Concepts play a particularly important role in evolutionary biology (Mayr 1997). Consider the classification of reproductive isolating mechanisms laid out by Dobzhansky (1937) and Mayr (1942, 1963) (see Table 7.1), a conceptual structure that has profoundly influenced thinking about the speciation process and the history of speciation studies (Coyne and Orr 2004). This classification aims to present, in chronological order of occurrence, the totality of intrinsic biological tendencies that might impede the mating of individuals from two populations and the meeting and mixing of their alleles across generations. It also illustrates how the naming of concepts affects our thinking.

For example, the term "isolating mechanism" was itself originally coined to reflect the view that reproductive isolation functioned as an adaptation that preserved coadapted gene pools (Fisher 1930, p. 130; Dobzhansky 1937). However, criticism of this adaptive interpretation of reproductive isolation and of the continued use of isolating mechanism terminology (e.g., Paterson 1982) motivated the introduction of "reproductive barrier" as an adaptively neutral alternative. Nonetheless, the original conceptual classification has continued to motivate various research programs to evaluate the roles of traditionally recognized reproductive barriers in speciation (Coyne and Orr 2004). For example, much recent work on the ecology of speciation has specifically focused on demonstrating the consequences of ecological population divergence for mating time (temporal isolation) and place (habitat isolation) and for hybrid inviability (Arnold 1997; Schluter 2000; Berlocher and Feder 2002; Drès and Mallet 2002; Funk et al. 2002; Rundle 2002).

If the suite of currently classified reproductive barriers is truly comprehensive, then empirically evaluating each of them should allow the accurate quantification of total reproductive isolation between populations and of the relative contributions of particular reproductive barriers (e.g. Ramsey et al. 2003). But what if it is incomplete? In this

paper, we describe and evaluate a distinct yet unnamed and understudied category of reproductive barrier.

7.3 The Concept of Immigrant Inviability

In providing the first widely read definitions of what Mayr (1963, p. 92) would later refer to as 'premating' isolating mechanisms, Dobzhansky (1937, p. 231-232) offered the following descriptions: (1) Ecological isolation (= "habitat isolation" as used by Mayr (1963) and throughout this paper) exists when "...the potential parents are confined to different habitats (ecological stations) in the same general region, and therefore seldom or never come together, at least during the reproductive age or season." (2) Temporal isolation exists when "...the representatives of two or more species reach the adult stage at a different season, or the breeding periods fall at different times of the year." (3) Sexual isolation exists when "...copulation does not occur because of the lack of mutual attraction between the individuals of different species".

Thus, following Dobzhansky and Mayr, knowledge of the willingness of two populations to mate in each others' habitats, their overlap in mating time, and their acceptance of each other as mates should be sufficient to predict the overall frequency of matings between them and thus their degree of premating isolation. However, this is not necessarily the case. To understand why, imagine the following scenario: two populations mate over the same time period, migrate to and mate within each others' habitats as readily as their own, and accept mates from each population with equal frequency. Guided by the traditional classification, one would expect random mating between these populations. However, imagine further that due to genetically based adaptive differentiation of these populations, individuals migrating to the foreign habitat survive there at a lower rate than do the native residents of that habitat. Under this scenario, inter-population matings will be reduced relative to intra-population matings because some proportion of immigrants will die in the less suitable foreign habitat prior to mating. In this way, gene flow will be restricted through a degree of positively assortative mating (Table 7.1). Furthermore, those immigrant females that do survive and mate may nonetheless perish before they have produced all or any of their potential offspring, further reducing potential opportunities for genetic exchange through hybrid progeny.

Following Futuyma's (1998) definition of "isolating mechanism" as "A genetically determined difference between populations that restricts or prevents gene flow between them," the lowered survivorship of ill-adapted unmated or mated immigrants constitute legitimate reproductive barriers, which we dub premating and postmating 'immigrant inviability', respectively. In many respects, immigrant inviability barriers represent prezygotic analogs to the postzygotic hybrid inviability that results when ecologically divergent parents produce ecologically ill-adapted hybrid offspring (e.g. Rundle 2002). Thus, we find it striking that the former has received little explicit attention as a reproductive barrier per se whereas the latter has been the focus of appreciable recent study (Arnold and Hodges 1995 for review; Arnold 1997; Emms and Arnold 1997; Wang et al. 1997; Hatfield and Schluter 1999; Campbell and Waser 2001; Rundle and Whitlock 2001; Rundle 2002). These observations notwithstanding, Coyne and Orr (2004) have recently included habitat-associated fitness differences as contributors to prezygotic isolation. These authors classify these contributions under habitat isolation, thus pooling them with the habitat preference traits more traditionally associated with this reproductive barrier.

Although we agree with Coyne and Orr's consideration of these novel elements of reproductive isolation, we further believe that they should be considered and classified separately from habitat isolation because habitat isolation and immigrant inviability are biologically and chronologically distinct. Whereas Dobzhansky and Mayr (1963, p.12) describe habitat isolation in terms of a lack of migration to foreign habitats, immigrant inviability reflects the reduced survival of those individuals that do indeed successfully migrate to the foreign habitat (Table 7.1). This biological difference can be further appreciated by the fact that habitat-associated preference and viability traits may often result from different genes, as reflected in their treatment as independent variables in many speciation models (e.g., Coyne and Orr 2004, p. 130). This biological independence means that these two types of barriers can act in isolation from each other, such that studying one may possibly provide no insight on the other and the contributions of each must be independently assessed. Such independent assessments of habitat isolation and immigrant inviability will furthermore facilitate study of the important evolutionary interactions between them that are discussed later in this paper. None of

these insights are possible if these distinctive phenomena are not appropriately distinguished. This is not merely a semantic distinction, but an important ontological one.

At this point, we should be clear about the relationship between the concerns of this paper and the contributions of previous students and models of speciation, many of which have invoked habitat-associated fitness differences between populations. For example, as pointed out by Coyne and Orr (2004, p. 131), various models of sympatric speciation, from the classic work of Maynard Smith (1966) and Felsenstein (1981) onwards treat speciation as the evolution of particular combinations of assortative mating, niche preference, and niche adaptation (i.e., habitat-associated fitness). In these models, genes affecting habitat-specific fitness are described as contributing to speciation primarily through their pleiotropic association with, or by being in linkage disequilibrium with, genes affecting assortative mating (i.e., forms of reproductive isolation other than immigrant inviability). However, habitat-associated fitness is not itself described as a source of reproductive isolation per se in these models. Hybrid zone and parapatric speciation models come closer to treating habitat-associated fitness differences as an isolating barrier in their incorporation of selection against ill-adapted alleles and their recognition of the reduction in gene flow that results (e.g. Slatkin 1973; Endler 1977; Barton 1983; Gavrilets 2000, 2004). Here, we call for the more widespread and explicit recognition that habitat associated fitness differences, in and of themselves, are a source of reproductive isolation that act through the above-described mechanisms of immigrant inviability.

We should also be clear that some other past authors have implicitly or explicitly recognized selection against immigrants as a cause of reproductive isolation (see Hendry 2004 for theoretical treatment). For example, elevated predation on *Heliconius* butterfly mimics that immigrate to regions occupied by different wing pattern races has long been identified as a cause of reproductive isolation (e.g., Mallet 1989, Mallet and Barton 1989; Mallet et al. 1998). Via has distinguished between host plant choice (i.e., habitat isolation) and selection against immigrants as complementary causes of host-associated reproductive isolation between clover- and alfalfa-associated pea aphids (Via 1999, Via et al 2000). Funk (1998) coined the term "physiological isolation" to describe the

premating and postmating reproductive isolation resulting from the death of ill-adapted insect immigrants on unsuitable host plants prior to mating or ovipositing, respectively. Other workers have undoubtedly made related observations (e.g. Young 1996; Nagy and Rice 1997; Hendry et al. 2000; Riechert and Hall 2000; Riechert et al. 2001; Nosil 2004).

Why then has immigrant inviability not been more generally recognized as a reproductive barrier? Perhaps this is because most students of divergent adaptation are understandably less interested in reproductive isolation, while students of prezygotic barriers focus on the reproductive behavior of living individuals rather than on the selective processes determining who survives to mate. Perhaps this is because of the foreignness of the concept of 'selection *as* reproductive isolation' – which describes immigrant inviability – as opposed to the more familiar idea of selection *promoting* the evolution of reproductive barriers. And perhaps this simply reflects the fact that these barriers have not previously been named (but see Funk 1998) and so have not been highlighted as a defined subject of study. Here, we recommend formally inserting the premating and postmating elements of immigrant inviability within the accepted classification of reproductive barriers (Table 7.1).

7.4 The Prevalence and Importance of Immigrant Inviability

For the reasons described above, there exists little in the way of literature that explicitly quantifies the contributions of immigrant inviability to reproductive isolation. However, the role of immigrant inviability in a given study system can nonetheless be quantified given published data on relative viability in native versus foreign habitats that are occupied by potential mates, plus data on additional reproductive barriers.

7.4.1 Literature Survey I: Identifying Studies on Adaptive Population Divergence

We identified study systems with data appropriate for these calculations by surveying the literature on reciprocal transplant experiments and local adaptation. Such investigations often provide estimates of immigrant and resident viability for each study population with regard to their respective habitats and are thus designed to detect the divergent adaptation that results in immigrant inviability. To identify pertinent literature, we conducted two searches using the Web of Science (Thomson ISI). For the first, we

searched on the Topic of "reciprocal transplant*" with no restrictions on journal of publication. For the second, we searched on the Topic of "local* adapt*" while restricting the journals searched to: *The American Naturalist*, *Ecology*, *Evolution*, *Evolutionary Ecology*, *Evolutionary Ecology Research*, *Journal of Evolutionary Biology*, *Oecologia*, and *Oikos*. This restriction was necessary because over 16,000 papers were recovered otherwise, almost entirely from irrelevant sources. Using the abstracts of papers from both searches, we eliminated non-pertinent papers, i.e., those that did not present studies of adaptive divergence in congeneric populations. For each of the remaining papers, we conducted a further literature search to identify any studies of reproductive isolation conducted on the focal species. Thus, we searched on the Topic of "[species name] AND (isolation or barrier* or gene flow or speciati*)" and then scanned titles and abstracts to identify appropriate literature. In this way, we identified taxa for which data had been collected that were relevant to immigrant inviability plus at least one other reproductive barrier.

7.4.2 Quantifying Contributions to Reproductive Isolation

In general, we quantified each reproductive barrier as described below. Sexual isolation was estimated as described because data on all four possible Sex x Population mating combinations were not available for many studies, precluding the use of more powerful estimators (Rolan-Alvarez and Cabarello 2000). When a reproductive barrier was studied more than once in a system, we averaged results across studies. These cases did not appreciably affect our conclusions, however, because separate studies tended to yield similar results. See online Supplementary Materials for details concerning each study system.

Habitat isolation (choice experiments) = 1 – (% of trials where foreign habitat was chosen).

Habitat isolation (no-choice experiments) = 1 – (% of trials where foreign habitat was accepted)

Immigrant inviability = 1 – (immigrant viability / resident viability).

Sexual isolation = 1 – (heterotypic mating frequency / homotypic mating frequency).

Floral (pollinator) isolation = 1 – (no. cross-species foraging bouts / total no. foraging bouts)

Pollen competition = 1 – (no. hybrids in mixed pollination crosses / no. parentals in homotypic crosses).

Hybrid inviability (genetic) = 1 – (hybrid viability / parental viability).

Hybrid inviability (ecological) = 1 – (hybrid ecological fitness / parental ecological fitness)

Sexual selection against hybrids = 1 – (hybrid mating success / parental mating success)

For each of our study systems, we calculated the "individual", "absolute", and "relative" contributions of each studied barrier to reproductive isolation, as well as the "total" reproductive isolation between study populations (Tables 7.2, 7.3). These values may range from negative infinity through zero (no reproductive isolation) to one (complete reproductive isolation). Negative values indicate increased gene flow relative to the random expectation, as, for example, under negatively assortative mating. Individual contributions indicate the magnitude of reproductive isolation caused by a particular reproductive barrier if it were to act in isolation. Absolute contributions indicate the magnitude of reproductive isolation caused by a particular reproductive barrier considering the restrictions on gene flow already contributed by previously acting barriers. Thus, if the individual contribution of two reproductive barriers are equal, the barrier that acts later in life-history will make a smaller absolute contribution because the earlier barrier will have already reduced the number of individuals that might potentially be removed by the later barrier. As suggested by Ramsey et al. (2003), the absolute contribution (AC) of a component of reproductive isolation (that is, of the individual contribution = RI) at stage *n* in the life history was calculated as:

$$AC_1 = RI_1, \qquad (1)$$

$$AC_2 = RI_2 (1 - AC_1), \text{ and} \qquad (2)$$

$$AC_3 = RI_3[(1 - (AC_1 + AC_2)]. \qquad (3)$$

and more generally: $$ACn = RIn(1 - \sum_{i=1}^{n-1} ACi). \qquad (4)$$

Total reproductive isolation can be calculated either by multiplying across the individual contributions of all barriers or by summing across the absolute contributions of all barriers, an approach proposed by Coyne and Orr (1989) for two components of reproductive isolation and extended to *n* components by Ramsey et al. (2003). Thus, for *m* components of isolation, total reproductive isolation (T) may be calculated as:

$$T = \sum_{i=1}^{m} ACi. \qquad (5)$$

Finally, the relative contribution of a particular barrier to total reproductive isolation indicates the proportion of the total reproductive isolation that owes to the absolute contributions of that barrier. The relative contribution (RC) of an individual component at stage *n* in life history is thus:

$$RCn = \frac{ACn}{T}. \qquad (6)$$

We calculated these measures of reproductive isolation using the Excel (Microsoft, Redmond, WA) spreadsheet employed by Ramsey et al. 2003, available at http://www.plantbiology.msu.edu/schemske.shtml.

7.4.3 Results – Literature Survey I

The existence of ecologically divergent and locally adapted populations is now well documented and widely recognized (e.g. Mopper and Strauss 1998). Furthermore, the great biological generality of this phenomenon is suggested by a prior literature review (Schluter 2000) that identified evidence for local adaptation in 36 of 42 pertinent studies. Our own literature review further suggests that the direct relevance of such divergent adaptation for reproductive isolation is rarely evaluated. To wit, out of 145

plant and metazoan genera recovered by our literature search on reciprocal transplant experiments testing for local adaptation, only 13 included species that have also been studied with respect to specific reproductive barriers. By including some additional systems not recovered by our searches, we assembled a total of 20 pertinent population comparisons for our calculations, representing arthropod, mollusc, vertebrate, and plant taxa (Table 7.2).

These calculations reveal that immigrant inviability commonly represents a strong reproductive barrier, often stronger than more conventionally studied barriers such as sexual isolation (Tables 7.3, 7.4, Fig. 7.1). Indeed, among reproductive barriers evaluated in more than two study systems, only the contributions of habitat isolation were found to be comparably high. Our analyses thus suggest that immigrant inviability commonly represents a biologically important reproductive barrier. This conclusion does not depend on the high absolute contribution of immigrant inviability, which partly reflects the early life history stage at which these reproductive barriers act. Although absolute and relative contributions are indeed high, so too are individual contributions, which are the data we present in Figure 7.1. These individual contributions show that immigrant inviability, even when acting alone, is capable of considerably restricting gene flow between divergently adapted populations.

7.5 Hybrid Zones and Immigrant Inviability

Hybrid zones can be profitably exploited for studies of speciation and the contributions of various reproductive barriers to reproductive isolation (Harrison 1990). The inherently spatial nature of hybrid zones provides opportunities for addressing the role of immigrant inviability because the structure of hybrid zones depends on the strength of reproductive isolation and rate of dispersal. Selection against immigrants, i.e., immigrant inviability, may therefore play a major role in the structure and maintenance of hybrid zones. 'Mosaic hybrid zones' (Harrison and Rand 1989) -- those with genetic structures that are spatially patchy rather than clinal -- are of particular relevance to immigrant inviability. Mosaic hybrid zones can be formed by long distance dispersal into the previously unoccupied region between two advancing populations (Nichols and Hewitt 1994, Ibrahim et al 1996). However, when a close correspondence between

particular genotypes and discernible environmental patches is observed, this explanation is implausible (Barton and Hewitt 1985). In such situations, the patchiness of these zones is more readily attributed to some combination of active habitat preference on the one hand and selection against immigrant alleles on the other.

Reciprocal transplant experiments that demonstrate the divergent ecological adaptation of hybridizing taxa to their respective habitat patches provide direct evidence for the role of immigrant inviability in maintaining mosaic hybrid zones. Phenotypic differentiation of these populations that is apparently associated with divergent habitats provides more indirect evidence of this phenomenon. Additional indirect evidence is had when genetic structure is associated with habitat patches, yet experiments reveal no evidence for divergent habitat preferences in the hybridizing populations. In such instances, immigrant inviability seems the most plausible alternative explanation even in the absence of explicit tests of habitat-associated fitness. Furthermore, since habitat preferences are unlikely to evolve in the absence of local adaptation (Rice and Hostert 1993), it could be argued that the demonstration of divergent habitat preferences itself constitutes indirect evidence for immigrant inviability.

7.5.1 Literature Survey II: Identifying Studies on Mosaic Hybrid Zones

We identified mosaic hybrid zone study systems through a Web of Science search on the topic of "mosaic* AND hybrid* AND zone*" and through following references listed in these papers. Among the recovered studies, we selected for our survey those study systems for which hybrid zones were explicitly described as mosaics by authors or that presented a map showing one or more spatial patches within the hybrid zone. For each such study system, we investigated associated literature to determine whether authors observed evidence for the following with respect to parental genotypes: (a) a spatial correspondence between an environmental variable and phenotypes within the zone, (b) divergent habitat preferences (e.g. MacCallum et al. 1998; Vines et al. 2003), and (c) habitat-associated performance/viability differences or phenotypic divergence suggestive of same.

7.5.2 Results – Literature Survey II

Our second literature survey identified 27 mosaic hybrid zones (Table 7.5). Twenty of these (74%) are habitat-associated, a pattern that itself is highly suggestive of a common role for ecological adaptation in hybrid zone maintenance. This proportion may overestimate or underestimate the fraction of mosaic zones that are truly habitat-associated, depending on which is more likely to be overlooked and understudied: habitat-independent zones or cryptic mosaic-associated environmental variables, respectively. Our primary interest, however, was in how commonly immigrant inviability appears to act in habitat-structured zones.

On this point, our survey revealed that only two of these 20 hybrid zones exhibited clear evidence of divergent adaptation and thus direct evidence for immigrant inviability. However, eight more zones showed putatively adaptive phenotypic divergence, providing indirect evidence for this phenomenon. Studies on an additional six zones provided evidence against divergent habitat preferences, leaving anti-immigrant selection as the most plausible cause for the habitat association. Of the remaining four habitat-associated zones, three have not been evaluated for either habitat preference or divergent adaptation. Thus, out of 17 habitat-associated mosaic hybrid zones in which the necessary data have been collected, direct or (mostly) indirect evidence for immigrant inviability has been found in 16. Although this survey indicates the need for additional and direct experimental investigations on divergent adaptation in mosaic hybrid zones, it also is highly consistent with a general role for immigrant inviability in their maintenance.

7.6 Theoretical and Population Genetic Aspects

From a population genetic perspective, selection against immigrants has generally been regarded as roughly equivalent to selection against heterozygotes with respect to maintaining reproductive isolation in the face of migration (Barton and Gale 1993, Kruuk et al 1999b). This position stems from the observation that clines maintained by a balance between migration and either form of selection are the same width under a wide range of conditions (Kruuk et al 1999b). However, there are several theoretical reasons why

selection against immigrants may be able to maintain population differentiation under higher levels of migration than selection against heterozygotes.

Consider a simple mainland-island model, where the island population receives immigrants at rate m from the mainland population. These two populations differ at a single biallelic locus. The frequency of allele A is denoted by p, and its frequency is initially assumed to be close to 1.0 on the island. The frequency of the alternative allele a is denoted by q and is assumed to be fixed on the mainland. Under this scenario, when selection only acts against heterozygotes it must be relatively strong to maintain the local allele. For example, under random mating, the fitness of heterozygote hybrids must be about 92% that of homozygotes (i.e. $s \sim 0.08$) in order to preserve the local allele when m is only 0.01. The minimum value of s increases to ~ 0.62 when $m = 0.1$, and when the migration rate exceeds 0.2, even a hybrid fitness of 0 cannot preserve the local allele. This is because some AA individuals are inevitably involved in AA x aa matings each generation and produce offspring that die, resulting in the loss of A alleles. This problem does not affect aa genotypes because their numbers are more than replenished every generation by migration. Furthermore, given $p < 0.5$ prior to migration, any amount of selection against heterozygotes will actually decrease the frequency of the island allele. In contrast, if selection favors genotypes that are adapted to the island environment then, providing there is no dominance, selection will be capable of countering higher immigration rates because both Aa and aa will have low fitness. Indeed, Haldane (1932, p.122) showed that as long as $s > m$, the island allele will be maintained, and thus any amount of immigration can potentially be countered by selection (see also Slatkin 1981; Vines et al. 2003).

These observations indicate that selection against immigrants may play a critical role in facilitating speciation in the face of gene flow. They show that populations can more readily diverge adaptively and maintain critical levels of reproductive isolation when immigrant inviability is acting to counter the potential effects of migration on gene flow.

7.7 Further Implications of Immigrant Inviability

So far, we have pointed out that divergent ecological adaptation commonly results in strong reproductive barriers that we refer to as 'immigrant inviability'. Like other reproductive barriers, immigrant inviability restricts gene flow between populations and in this way may obviously and directly contribute to speciation. In this section, however, we discuss some additional evolutionary implications of immigrant inviability for population differentiation and speciation (Fig. 7.2).

For example, immigrant inviability can select for population divergence in mating preferences through forms of selection that are analogous to reinforcement (e.g., Servedio 2001). In this case, it is not the threat of producing unfit hybrid offspring that selects for discrimination against foreign (here, immigrant) mates (Dobzhansky 1937; Servedio and Noor 2003 for review); instead, it is the increased likelihood that one's immigrant mate will die before producing offspring. The same logic applies if residents themselves exhibit reduced survival as a function of mating with immigrants. Studies of *Timema* stick insect populations associated with two host plants provide examples of both these phenomena (Nosil et al. 2003). Two points are relevant: each host-associated population of these insects has a pattern that is more cryptic on its native host, and these animals mate for extended periods of time. The result is that matings between residents and immigrants yield elevated rates of predation on the ill-camouflaged immigrants as well as on residents while they are in copula with their conspicuous mates. The reduced viability of immigrants in foreign habitats should furthermore favor alleles that increase the degree of preference for the native habitat and thus reduce immigration to foreign habitats, yielding increased habitat isolation (Balkau and Feldman 1973 for theory; Rice and Hostert 1993; Kruuk and Gilchrist 1997; Via et al. 2000). Since local adaptation appears to be common, this strengthening of habitat isolation should be a general phenomenon, though empirical tests are lacking.

Immigrant inviability also participates in various feedback loops that may influence the evolutionary dynamics of the speciation process. Consider, for example, the selection for increased habitat preference discussed above. Such increased restriction to a particular habitat should increasingly result in specialized adaptations to that habitat

as countervailing selection pressures associated with other, increasingly ignored, habitats correspondingly decrease. This process will thus promote increased adaptive divergence and immigrant inviability between populations in a positive feedback loop. However, the strength of this feedback should ultimately fade due to decreasing numbers of immigrants, and thus diminishing selection for habitat preference, each generation (Hendry 2004).

Like any reproductive barrier, immigrant inviability restricts the homogenizing force of gene flow (Slatkin 1987; Lenormand 2002), facilitating reinforcement (Servedio and Noor 2003 for review), divergent adaptation, and increased immigrant inviability. Notably, this increased adaptive divergence may itself indirectly (pleiotropically) contribute to the evolution of any reproductive barrier (Mayr 1942, 1963; Funk 1998; Schluter 2000; Hendry et al. 2000, 2001; Jiggins et al. 2001; Nosil et al. 2002, 2003; Presgraves et al. 2003). These relationships describe further feedback loops (Hendry 2004) that, together with those described above, may have complex dynamics and interactions, with important consequences for speciation. Additional theoretical treatment of all these issues is needed.

Lastly, it is perhaps in the context of the recent renaissance in the study of ecological speciation (Schluter 2000; Funk et al. 2002) that immigrant inviability will be of the broadest interest to evolutionary biologists. Many reproductive barriers may evolve in the absence of ecological divergence, such as through environment-independent sexual selection, sexual conflict between the sexes (Arnqvist et al. 2000; Martin and Hosken 2003) or genetic drift (Templeton 1980; Gavrilets 2004). In contrast, premating and postmating immigrant inviability (along with ecological hybrid inviability: Schluter 2000; Rundle and Whitlock 2001; Rundle 2002) should usually evolve only when populations have ecologically diverged, and thus are inherently ecological reproductive barriers. Intriguingly, immigrant inviability also softens theoretical objections to sympatric speciation, in two respects. First, because selection itself is the cause of reproductive isolation (i.e. reduced gene flow), the same genes affecting fitness also affect reproductive isolation. Thus, a pleiotropic relationship exists between reduced (immigrant) fitness and assortative mating (due to immigrant mortality) that obviates the oft-cited need for a genetic association between separate loci controlling habitat-

associated fitness and mating preferences (Felsenstein 1981; see; Kirkpatrick and Ravigne 2002; Gavrilets 2004 for reviews of speciation models). Second, it represents direct selection on genes promoting reproductive isolation and so should be more effective than indirect selection acting through imperfect genetic associations (Kirkpatrick and Barton 1997).

7.8 Caveats and Qualifiers

Before concluding this paper, a few issues must be raised that may have occurred to the reader and that bear on the importance of immigrant inviability as compared to other reproductive barriers. Most basically, it should be reiterated that, like other reproductive barriers, immigrant inviability is not an all or nothing phenomenon at the population level, even though it may be thought of in these terms (life versus death) at the individual level. Rather, partial immigrant inviability may be said to act even when some immigrants survive to mate and reproduce in the foreign habitat, so long as the overall rate of immigrant survivorship and reproduction is lower than that of the resident population.

It is, however, quite important to recognize that immigrant inviability will come into play only to the extent that migration between habitats occurs, providing the immigrants on which selection might act. Thus, one might predict immigrant inviability to restrict gene flow the most in taxa with particular tendencies, such as: frequent dispersal of both sexes to foreign habitats and a willingness to settle in them; occupation of regions that are ecologically heterogeneous; and a tendency to disperse early in the life-history, such that immigrants must long endure habitats they find stressful. Thus, the potential role of immigrant inviability will be intimately linked to the biology of the taxon. For example, it may play a relatively small role in angiosperms that disperse through pollen (e.g., Arnold et al. 1991) rather than the seeds that produce immigrant plant individuals. Nonetheless, this issue is not special to immigrant inviability. Rather, there are particular circumstances and taxa for which each reproductive barrier is relatively more or less likely to play an active role in restricting gene flow. Furthermore, barriers that do not presently restrict gene flow despite their potential to do so should not

be overlooked given the possibility of future changes, such as secondary contact between currently separated populations, that might bring them to bear.

On this point, one might argue that the geographic scale of successful migration is relevant for determining whether foreign habitats are reached in the first place. Issues of spatial scale are certainly critical to an understanding of how all premating barriers restrict gene flow. However, an adequate treatment of this issue is beyond the scope of the present paper. Furthermore, our interest here is on how intrinsic factors (i.e. those reflecting genetic variation) rather than geographic factors influence reproductive isolation between coexisting populations. The spatial scale that implicitly underlies our arguments is that of divergent habitats existing within the ordinary movements or "cruising ranges" of populations. Thus, we are primarily concerned with what happens to immigrants once they have arrived at the foreign habitat, rather than with the (e.g., spatial) factors influencing whether they get there (see Coyne and Orr 2004 for further discussion of geographic versus reproductive barriers to gene flow).

Finally, a critical but often overlooked issue for speciation studies is the fact that the current contributions of particular reproductive barriers are not necessarily indicative of the historical role they have played in speciation. Consider that while reproductive barriers evolving early in the speciation process may play a particularly large role in promoting speciation, barriers that evolve after speciation has reached completion may presently obscure the contributions of those earlier barriers. For example, if immigrant inviability drove speciation between two populations, after which strong habitat isolation evolved, a modern investigator might mistakenly conclude that habitat isolation was largely responsible. Evaluating the likely time course for the evolution of reproductive barriers through comparative studies (Etges 2002; Coyne and Orr 2004) will thus be necessary to accurately evaluate the role of immigrant inviability or any reproductive barrier as an actual contributor to speciation.

7.9 Conclusions

Collectively, the lines of evidence presented here suggest that the reduced survival of immigrants to foreign environments represents an important form of

reproductive isolation that likely plays important and diverse roles in the ecology of speciation. We suggest the formal recognition of the twin premating and postmating elements of immigrant inviability in an extended classification of reproductive barriers. We also hope for increased empirical study of these barriers for their own sake, but also in conjunction with other reproductive barriers in order to further tease apart the relative contributions of each (e.g. Ramsey et al. 2003). The many consequences of immigrant inviability for adaptive differentiation, the evolution of other reproductive barriers, and gene flow further provide opportunities for theoretical investigations of the little-studied feedback loops among these phenomena and how these play into speciation dynamics. Increased attention to all these issues will help further our understanding of the complex processes of speciation.

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Young, N.D. 1996. An analysis of the causes of genetic isolation in two Pacific Coast Iris hybrid zones. Can. J. Bot. 74 : 2006-2013.

Figure 7.1 Mean individual contributions (± s.e.) to reproductive isolation across reproductive barriers.

Each bar represents mean values across those study systems for which data for that barrier were available (see Table 7.3 for raw data). Numbers above bars indicate number of systems contributing data. One extreme data point was excluded in the calculation for the "ecological hybrid inviability" bar. Including this data point (from a study where hybrid fitness was much higher than parental fitness) yields a mean value slightly less than zero. Note the high value for immigrant inviability relative to traditionally recognized reproductive barriers.

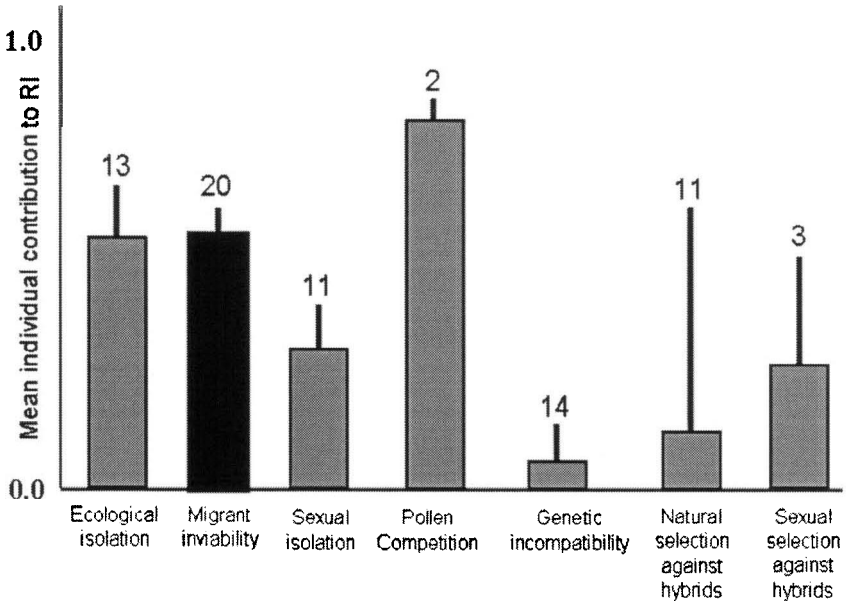

Figure 7.2 A diagrammatic summary of the various direct and indirect effects of immigrant inviability on other aspects of adaptive divergence and reproductive isolation and of the resulting evolutionary feedback loops in which it participates.
See text for details.

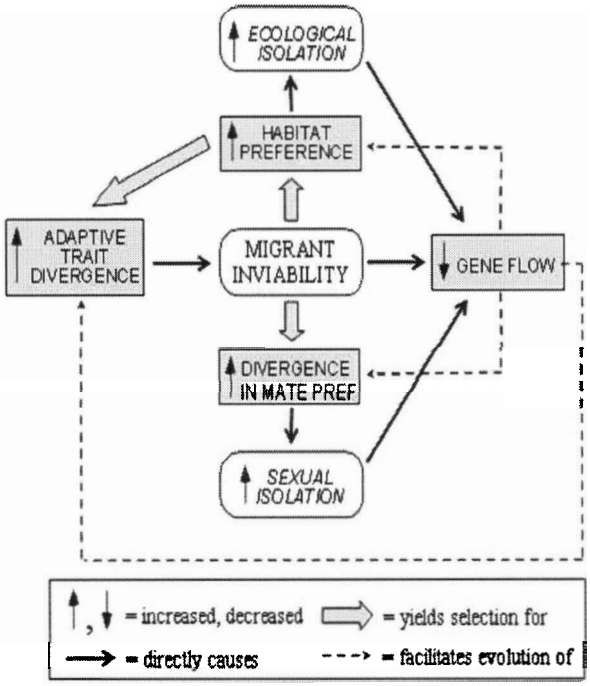

Table 7.1 An extended classification of intrinsic reproductive barriers = "isolating mechanisms". Modified from Mayr (1963), Futuyma (1998).
Novel additions to the traditional classification are presented in boldface. This paper treats immigrant inviability; papers discussing other novel barriers are cited below.

Form of reproductive isolation	Description
Premating, prezygotic barriers	
Temporal = allochronic isolation	reduced encounters of potential mates due to different mating times
Habitat = ecological isolation	reduced encounters of potential mates due to different mating sites
Immigrant inviability, premating	reduced encounters of potential mates due to mortality of maladapted immigrants
Sexual = behavioural = ethological isolation	reduced mating due to divergent courtship signals, mate preferences
Postmating, prezygotic barriers	
Mechanical isolation	reduced transfer of sperm during mating due to poor genitalic compatibility
Gametic incompatibility	reduced fertilization of eggs by ill-suited sperm
Postmating, postzygotic barriers	
Immigrant inviability, postmating	reduced production of offspring due to mortality of mated, maladapted immigrants
Zygotic mortality	reduced survival of zygotes soon after fertilization
Hybrid inviability (genetic)	reduced survival of hybrid offspring independent of the environment
Hybrid inviability (ecological)[a]	reduced survival of hybrid offspring where viability is environment-dependent
Sexual selection against hybrids[b]	reduced mating success of hybrid offspring
Hybrid sterility	reduced fertility of hybrid offspring
F2 breakdown	reduced survival or fertility of subsequent hybrid generations

[a] Schluter 2000, Rundle and Whitlock 2001; [b] Schluter 2000, Naisbit et al. 2001

Table 7.2 Study systems for which contributions of multiple reproductive barriers to reproductive isolation were quantified. These represent taxa for which data from both reciprocal transplant experiments and additional assays of reproductive isolation were available. This table presents information on the populations, species, or "forms" compared, the aspect of fitness assayed, the site of the transplant experiments, and the putative source of selection yielding the observed divergent adaptation.

Study system	Common name	Form 1	Form 2	Fitness	Lab or field?	Putative agent(s) of selection	Pertinent references
Acyrthosiphon pisum	pea aphids	clover host race	alfalfa host race	fecundity	field	physiological adaptation	Via 1999; Via et al. 2000
Agelenopsis aperta	desert spider	arid land	riparian	survival	field	food availability, predation risk	Riechert and Hall 2000; Riechert et al. 2001
Artemesia tridentata	big sagebrush	basin subspecies	mountain subspecies	survival, growth	field	-	Wang et al. 1997
Bombina spp.	toads	pond-adapted *bombina*	puddle-adapted *variegata*	survival, growth	both	biophysical conditions, predation	MacCallum et al. 1995, 1998; Kruuk and Gilchrist 1997; Kruuk et al. 1999a
Eurosta solidaginis	galling fly	*altissima* race	*gigantean* race	survival	lab	physiological adaptation	Craig et al. 1993. 1997, 2001
Galerucella nymphaeae	water lily leaf beetle	Nymphaeaceae race	Polygonaceae race	survival	lab	physiological adaptation	Pappers et al 2002a,b

Study system	Common name	Form 1	Form 2	Fitness	Lab or field?	Putative agent(s) of selection	Pertinent references
Galerucella spp.	water lily leaf beetle	*nymphaeae*	*sagittariae*	survival	lab	physiological adaptation	Nokkala and Nokkala 1998
Gasterosteus aculeatus	freshwater stickleback	limnetic	benthic	growth, survival	field	foraging efficiency, competition, predation	Schluter 1993, 1994, 1995; Hatfield and Schluter 1999; Rundle 2002; Vamosi 2002
Gilia capitata	herbaceous plant	inland subspecies	coastal subspecies	survival	field	biophysical conditions	Nagy 1997a,b; Nagy and Rice 1997
Heliconius erato	mimetic butterflies	postman	rayed	longevity	field	visual predation	Mallet et al. 1998; Mallet 1989; Mallet and Barton 1989
Ipomopsis spp.	phlox flowers	*aggregata*	*tenuituba*	survival	field	biophysical factors	Campbell and Waser 2001; Campbell et al. 2003; Campbell 2003
Iris spp. 1	perennial flowers	*douglasiana*	*innominata*	survival	field	biophysical factors	Young 1996
Iris spp. 2	perennial	*fulva*	*hexagona*	survival	field	-	Arnold 1997

Study system	Common name	Form 1	Form 2	Fitness	Lab or field?	Putative agent(s) of selection	Pertinent references
Littorina saxatilis	intertidal snails	upper shore	lower shore	survival	field	physical factors, predation	Johannesson et al. 1993; Rolan-Alvarez et al. 1997
Mimulus spp.	monkey-flower	*lewissii*	*cardinalis*	survival, ecogeographic isolation	field	climatic conditions	Hiesey et al. 1971; Ramsey et al. 2003
Mitoura spp.	butterfly	host races	host races	survival	lab	physiological adaptation	Forister 2004
Neochlamisus bebbianae	leaf beetles	*Acer* host form	*Betula, Salix* host forms	survival	lab	physiological adaptation	Funk 1998
Polemonium viscosum	alpine flower	Krummholz low elevation	Summit high elevation	survival	field	climatic conditions	Galen and Kevan 1980; Galen 1985; Galen et al. 1991
Rhagoletis spp.	fruit flies	*mendax*	*pomonella*	survival	lab	physiological adaptation	Bierbaum and Bush 1990
Timema cristinae	cryptic walking-sticks	*Ceanothus* host form	*Adenostoma* host form	survival	field	visual predation	Sandoval 1994a,b; Nosil et al. 2002; 2003; Nosil 2004

Table 7.3 Individual components of reproductive isolation for focal study systems.

Note that environment-independent ("genetic") and environment-dependent ("ecological") aspects of hybrid inviability are treated separately. Values may range from negative infinity through zero (no reproductive isolation) to 1.0 (complete reproductive isolation). The negative values in this table indicate cases where hybrid viability exceeded that of parentals, a phenomenon that would actually increase gene flow. See text for details of calculations.

Study system	Habitat isolation	Immigrant inviability	Sexual / Floral isolation	Pollen competition	Hybrid inviability (genetic)	Hybrid inviability (ecological)	Sexual selection on hybrids
A. pisum	0.927	0.970	-	-	0.000	0.470	-
A. aperta	-	0.632	0.06	-	-0.15	-	-
A. tridentata	-	0.900	-	-	-	-5.250	-
Bombina spp.	0.660	0.437	-	-	0.190	-	-
E. solidaginis	0.962	0.877	0.365	-	0.000	0.344	-
G. nymphaeae	0.855	0.772	0.000	-	0.000	0.322	-
Galerucella spp.	0.904	0.938	-	-	0.000	-0.710	-
G. aculeatus	-	0.363	0.633	-	0.000	0.255	0.777
G. capitata	-	0.704	-	0.800	0.813	-	-
H. erato	0.000	0.520	0.000	-	0.000	0.450	-
Ipomopsis spp.	-	0.429	-	-	0.000	-0.125	0.155
Iris spp. 1	0.000	0.955	0.000	-	-0.620	-	-
Iris spp. 2	-	0.087	-	-	-	-0.008	-
L. saxatilis	0.742	0.691	0.518	-	0.000	-0.259	0.000

Study system	Habitat isolation	Immigrant inviability	Sexual / Floral isolation	Pollen competition	Hybrid inviability (genetic)	Hybrid inviability (ecological)	Sexual selection on hybrids
Mimulus spp.	-	0.587	0.976	0.833	0.415	-	-
Mitoura spp.	0.696	0.208	-	-	0.000	-	-
N. bebbianae	0.947	0.990	0.720	-	-	-	-
P. viscosum	-	0.795	0.140	-	-	-	-
Rhagoletis spp.	0.555	0.368	-	-	-	0.606	-
T. cristinae	0.252	0.340	0.222	-	-	-	-

Table 7.4 Absolute and relative contributions to total reproductive isolation of investigated barriers from focal study systems. Also presented are estimates of total reproductive isolation that were calculated both with and without the contribution of immigrant inviability. Note that environment-independent ("genetic") and environment-dependent ("ecological") aspects of hybrid inviability are treated separately. These calculations assume that immigrant inviability occurs prior to sexual/floral isolation during life-history. However, for systems where sexual/floral isolation has been evaluated we also provide estimates (in brackets) of the lower bound on the contribution of immigrant inviability assuming that sexual/floral isolation acts prior to immigrant inviability. Values ordinarily range from negative infinity through zero (no reproductive isolation) to 1.0 (complete reproductive isolation). The negative values in this table indicate cases where hybrid viability exceeded that of parentals, a phenomenon that would actually increase gene flow. The values that are >1 represent unusual artefacts of the method of calculating relative/absolute contributions to reproductive isolation when certain individual components are negative. See text for details of calculations.

Study system	Habitat isolation	Immigrant inviability	Sexual / Floral isolation	Pollen Comp-etition	Hybrid inviability (genetic)	Hybrid inviability (ecological)	Sexual selection on hybrids	Total isolation	Total isolation (no immigrant inviability)
A. pisum									
absolute	0.927	0.071	-	-	0.000	0.001	-	0.999	0.961
relative	0.928	0.071	-	-	0.000	0.001	-		
A. aperta									
absolute	-	0.632 (0.594)	0.022 (0.060)	-	-0.052	-	-	0.602	-0.080
relative	-	1.05 (0.986)	0.04 (0.10)		-0.086	-	-		

Study system	Habitat isolation	Immigrant inviability	Sexual / Floral isolation	Pollen Comp-etition	Hybrid inviability (genetic)	Hybrid inviability (ecological)	Sexual selection on hybrids	Total isolation	Total isolation (no immigrant inviability)
A. tridentata									
absolute	-	0.900	-	-	-	-0.525	-	0.375	-5.25
relative	-	2.400	-	-	-	-1.400	-		
Bombina spp.									
absolute	0.660	0.148	-	-	0.036	-	-	0.844	0.725
relative	0.781	0.176	-	-	0.040	-	-		
E. solidaginis									
absolute	0.962	0.034 (0.021)	0.002 (0.014)	-	0.000	0.001	-	0.998	0.984
relative	0.964	0.034 (0.021)	0.002 (0.014)	-	0.000	0.001	-		
G. nymphaeae									
absolute	0.855	0.112	0.000	-	-	0.011	-	0.978	0.902
relative	0.875	0.115	0.000	-	-	0.011	-		
Galerucella spp.									
absolute	0.904	0.090	-	-	0.000	-0.004	-	0.990	0.836
relative	0.914	0.091	-	-	0.000	-0.004	-		

Study system	Habitat isolation	Immigrant inviability	Sexual / Floral isolation	Pollen Comp-etition	Hybrid inviability (genetic)	Hybrid inviability (ecological)	Sexual selection on hybrids	Total isolation	Total isolation (no immigrant inviability)
G. aculeatus									
absolute	-	0.363 (0.133)	0.403 (0.663)	-	0.000	0.060	0.135	0.961	0.939
relative	-	0.378(0.139)	0.419 (0.658	-	0.000	0.062	0.141		
G. capitata									
absolute	-	0.703	-	0.238	0.048	-	-	0.989	0.963
relative	-	0.711	-	0.240	0.049	-	-		
H. erato									
absolute	0.000	0.520	0.000	-	0.000	0.216	-	0.736	0.450
relative	0.000	0.707	0.000	-	0.000	0.293	-		
Ipomopsis spp.									
absolute	-	0.429	-	-	0.000	-0.071	0.100	0.457	0.049
relative	-	0.938	-	-	0.000	-0.156	0.218		
Iris spp. 1									
absolute	0.000	0.945	0.000	-	-0.028	-	-	0.926	-0.619
relative	0.000	1.030	0.000	-	-0.030	-	-		
Iris spp. 2									
absolute	-	0.0868	-	-	-	-0.008	-	0.079	-0.008
relative	-	1.092	-	-	-	-0.092	-		

Study system	Habitat isolation	Immigrant inviability	Sexual / Floral isolation	Pollen Comp-etition	Hybrid inviability (genetic)	Hybrid inviability (ecological)	Sexual selection on hybrids	Total isolation	Total isolation (no immigrant inviability)
L. saxatilis									
absolute	0.742	0.179 (0.086)	0.041 (0.134)	-	0.000	-0.010	0.000	0.953	0.843
relative	0.779	0.188(0.090)	0.043(0.141)	-	0.000	-0.011	0.000		
Mimulus spp.									
absolute	-	0.587 (0.014)	0.403 (0.976)	0.008	0.001	-	-	0.999	0.998
relative	-	0.588 (0.014)	0.403 (0.977)	0.008	0.001	-	-		
Mitoura spp.									
absolute	0.696	0.063	-	-	0.000	-	-	0.759	0.696
relative	0.917	0.083	-	-	0.000	-	-		
N. bebbianae									
absolute	0.947	0.053 (0.015)	0.000 (0.038)	-	-	-	-	1.000	0.985
relative	0.947	0.053 (0.015)	0.000 (0.038)	-	-	-	-		

Study system	Habitat isolation	Immigrant inviability	Sexual / Floral isolation	Pollen Comp-etition	Hybrid inviability (genetic)	Hybrid inviability (ecological)	Sexual selection on hybrids	Total isolation	Total isolation (no immigrant inviability)
P. viscosum									
absolute	-	0.795 (0.684)	0.029 (0.140)	-	-	-	-	0.824	0.140
relative	-	0.965 (0.830)	0.035 (0.170)	-	-	-	-		
Rhagoletis sp									
absolute	0.556	0.163	-	-	-	0.170	-	0.889	0.825
relative	0.625	0.184	-	-	-	0.191	-		
T. cristinae									
absolute	0.252	0.254 (0.198)	0.110 (0.166)	-	-	-	-	0.616	0.418
relative	0.409	0.413 (0.321)	0.178 (0.269)	-	-	-	-		

Table 7.5 Evidence on ecological divergence in mosaic hybrid zones. HA = habitat-associated genetic structure, HP = evidence for divergent habitat preferences, DA = evidence for divergent ecological adaptation.
The term 'indirect' in the DA column denotes evidence for local adaptation stemming from phenotypic divergence (see text for details). A question mark indicates an absence of pertinent data. Patch type refers to the environmental feature putatively responsible for the mosaic.

Hybridising taxa	Type	HA?	Patch type	HP?	DA?	Source
Allonemobius fasciatus x A. socius	Cricket	Yes	Temperature	?	?	Howard 1986; Britch et al. 2001
Ambystoma tigrinum subspecies	Tiger salamander	Yes	Elevation & vegetation	?	?	Jones and Collins 1992
Baptisia sphaerocarpa x B. leucophaea	False indigo	Yes	Soil type	No	?	Alston and Turner 1963; Leebens-Mack and Milligan 1998
Bombina bombina x B. variegata	Fire-bellied toad	Yes	Aquatic habitat	Yes	Indirect	MacCallum et al. 1995,1998; Vines et al. 2003; Kruuk et al. 1997
Bufo americanus x B. fowlerii	Toad	Yes	Vegetation type	?	?	Green and Parent 2003
Chamerion angustifolium diploid x tetraploid	Fireweed	?	n/a	No	?	Husband and Schemske 1997
Chorthippus brunneus x C. jacobsi	Cricket	Yes	Vegetation	No	?	Bridle et al. 2001; Bailey et al. 2004
Gryllus pennsylvanicus x G. firmus	Cricket	Yes	Soil type	?	Indirect	Rand and Harrison 1989
Iris fulva x I. brevicaulis	Iris	Yes	Light & soil water	No	Indirect	Cruzan and Arnold 1993; Johnston et al. 2001

Hybridising taxa	Type	HA?	Patch type	HP?	DA?	Source
Iris fulva x I. hexagona	Iris	Yes	Light & soil water	No	Yes	Arnold and Bennett 1993; Emms and Arnold 1997
Limnoporus dissortis x L. notabilis	Water strider	No	---	No	?	Sperling and Spence 1991
Littorina saxatilis morphs	Snail	Yes	Humidity & exposure	Yes	Yes	Otero-Schmitt et al. 1997; Rolán-Alvarez et al. 1997
Monobella grassei subspecies	Springtail	No	---	No	?	Deharveng et al. 1998
Mus musculus domesticus chromosomal races	Mouse	No	---	No	?	Hauffe and Searle 1993
Mytilus edulis x M. galloprovincialis	Mussel	Yes	Exposure & salinity	Yes	Indirect	Bierne et al. 2003
Nucella lapillus races	Dog whelk	Yes	Humidity & exposure	?	Indirect	Kirkby et al. 1997
Palaemonetes kadiakensis type A x type B	Grass shrimp	?	---	?	?	Garcia and Davis 1994
Papilio canadensis x P. glaucus	Butterfly	Yes	Host plants & temperature	?	Indirect	Hagen 1990; Scriber 2002
Piriqueta caroliniana x P.viridis	Piriqueta	Yes	Soil type	No	?	Martin and Cruzan 1999
Polystichum imbricans x P. munitum	Fern	Yes	Light & soil water	No	?	Kentner and Mesler 2000
Quercus affinis x Q. laurina	Oak	Yes	Elevation	No	Indirect	González-Rodríguez et al. 2004
Quercus grisea x Q. gambelii	Oak	Yes	Elevation & Soil type	No	?	Howard et al. 1997
Ranunculus lappaceus group (7 species)	Buttercup	Yes	Soil water	No	?	Briggs 1962

Hybridising taxa	Type	HA?	Patch type	HP?	DA?	Source
Sceloporus grammicus races	Lizard	Yes	Oak/pine	Yes	?	Sites et al. 1995
Solenopsis invicta x S. richteri	Fire ant	No	---	No	?	Shoemaker et al. 1996
Triturus cristatus x T. marmoratus	Newt	Yes	Aquatic habitat	Yes	Indirect	Arntzen and Wallis 1991
Triturus vulgaris x T. montandoni	Newt	No	n/a	No	?	Babik and Rafinski 2001

7.12 Supplemental Materials

Here, we provide details on the calculations of individual components of reproductive isolation within each study system, as well as pertinent references. In reciprocal transplant experiments where multiple measures of fitness (e.g. size, mass, growth, survival, etc.) were assayed in the same experiment but a composite measure of fitness was not presented, we used survival as the most direct means of calculating immigrant inviability. For studies providing composite measures of fitness, we used total fitness for our calculations.

For *Acyrthosiphon pisum*, host preference was estimated as the mean from two experiments: one examining the distribution of diagnostic allozyme loci between fields of alfalfa and clover and one examining the tendency for aphids from each host race to settle on alfalfa versus clover in laboratory choice experiments (Via 1999). These two experiments gave comparable estimates of host preference (0.889 and 0.965 respectively). Their mean was used in all calculations in the current study. The fitness (fecundity) of immigrants versus residents was estimated in a reciprocal transplant experiment where winged forms (i.e. the natural dispersers) of a set of aphid genotypes from each host were experimentally 'migrated' to one of the two crops. Hybrid ecological fitness was estimated as the fecundity of F1 hybrids relative to each parental form in its native habitat (Via et al. 2000).

For *Agelenopsis aperta*, immigrant inviability was estimated using survival data from Riechert and Hall 2000. Sexual isolation was estimated using the laboratory mating trials reported in Riechert et al. 2001. Genetic incompatibility was estimated using the data on spiderling mass and survival to sexual maturity for parental and F1 hybrid crosses reported in Riechert et al. 2001 (averaged across these two measures of fitness). Notably, differences among genetic classes in these latter two forms of reproductive isolation were not statistically significant.

For *Artemisia tridentata*, (Wang et al. 1997), immigrant inviability was estimated using the composite fitness of native versus foreign plants in each parental environment (basin and mountain). Natural selection against hybrids was estimated by comparing the

composite fitness of hybrids versus each parental form (averaged over both parents) within the hybrid environment, where hybrids were much more fit, resulting in negative isolation values.

For *Bombina* spp., clear evidence for divergent habitat preferences has been obtained using allele-frequency data, rather than by habitat-preference experiments. We used the estimated difference in allele frequencies at habitat preference loci between puddles and ponds as an indirect estimate of the degree of habitat isolation between puddle-adapted *B. variegata* and pond-adapted *B. bombina* (see MacCallum et al. 1998, p. 237 for details). Immigrant inviability was also difficult to estimate because a reciprocal transplant experiment suggested that immigrants were selected against in one environment (puddles) but favored in another (ponds) when predators were excluded (MacCallum et al. 1995). However, predation trials indicated that immigrants would be selected against in ponds when predators were present (Kruuk and Gilchrist 1997). Furthermore, the reciprocal transplant experiment was flawed due to mixed species egg batches. We adopted the conservative strategy of assigning immigrants and residents equal fitness in ponds and dividing the estimate of immigrant inviability from puddles in half to yield a rough estimate averaged across the two environments. Intrinsic hybrid inviability was assessed by comparing the viability of egg batches from the center of a hybrid zone to that of egg batches from parental types, with viability estimated using total mortality: the product of embryonic and larval mortality for each batch (as in Kruuk et al. 1999a).

For *Eurosta solidaginis*, host preference was estimated as the mean from three experiments (0.974, 0.969 and 0.942 for no-choice host preference, choice host preference, and mating site host preference experiments respectively (Craig et al. 1993). The survival of immigrants versus residents on each host was estimated from the proportion of eggs oviposited on each host that yielded adults. Sexual isolation was taken as the mean from two experiments where mating preferences were measured in the absence of hosts, including one no-choice scenario and one multiple-choice scenario (Craig et al. 1993). A lack of intrinsic inviability was inferred from consistently high hybrid survival on some plant genotypes of both host-plant species (Craig et al. 1997; Itami et al. 1997; Craig et al. 2001). Ecological hybrid inviability was inferred by

comparing emergence success (proportion of eggs oviposited on each host that yielded adults) of hybrids to that of each parental form on its native host (with hybrid fitness averaged across the two hosts, Craig et al. 1997).

For *Galerucella nymphaeae*, all data were taken from Pappers et al. 2002a. (Although Pappers et al. 2002b report similar data and nearly identical results, the former study was much larger and controlled in some cases for environmental and maternal effects.) In all instances, results were averaged across the four treatments (two Nymphaeaceae hosts and two Polygonaceae hosts). Habitat preference was calculated from the results of an oviposition preference experiment, and immigrant inviability was calculated from the survival of insects on hosts from their native versus foreign host family. Sexual isolation and genetic hybrid inviability were considered to be absent because all types of crosses (within and between host family) interbred equally and hybrid offspring outperformed parental forms when fitnesses were averaged across all treatments.

For *Galerucella* spp., we used data from Nokkala and Nokkala (1998) to estimate components of reproductive isolation between *G. nymphaeae* and *G. sagittariae*, in all cases averaging the results from the two forms of *G. sagittariae* that were examined. Habitat isolation was estimated from habitat choice tests where insects were scored for the host on which they were sitting and on which they oviposited, with results averaged across sitting and oviposition assays. Percent larval survival of each parental form and of hybrids on each host provided estimates of immigrant inviability and ecological hybrid inviability. All hybrids were fertile, indicative of a lack of genetic hybrid inviability.

For *Gasterosteus aculeatus*, estimates of immigrant versus resident fitness were averaged across three studies. Schluter (1995) and Rundle (2002) provide estimates of growth rates of benthic and limnetic forms in each habitat, whereas Vamosi (2002) examined survival under predation. These studies gave comparable estimates (0.418, 0.393, and 0.280 respectively) and were chosen because the fitness of both limnetic and benthic fish was measured in both habitats in natural populations. Studies using laboratory feeding trials, semi-natural pond experiments, or that performed experiments in only one habitat were excluded but yielded similar conclusions (Schluter 1993, 1994;

Hatfield and Schluter 1999; Vamosi et al. 2000; Vamosi and Schluter 2002). Sexual isolation was estimated by comparing the probability of spawning between males and females from the same lake and same ecotype to the probability of spawning between different ecotypes from the same lake (data from Rundle et al. 2000, which include data from Nagel and Schluter 1998). Hatfield and Schluter (1999) found no evidence for genetic hybrid inviability, and assessed growth rate of F1 hybrids versus each parental form in its native habitat Analogous data from Schluter 1995 is for F10 hybrids and thus was excluded, but trends were similar. With respect to sexual selection against hybrids, a recent field experiment found reduced mating success of F1 hybrid males relative to limnetic males (Vamosi and Schluter 1999), indicating that the lack of a hybrid mating disadvantage found in laboratory mating trials by Hatfield and Schluter (1996) does not apply in the wild; the latter study was thus excluded.

For *Gilia capitata*, selection against immigrants was estimated using data on the proportion of immigrant versus resident plants to flower, averaged across the multiple years of the experiment (Nagy and Rice 1997). Whether pollen competition occurs between subspecies is unclear, but hybridization rates are only 20% of that expected based on the pollen mixture applied in a greenhouse experiment, indicative of a fertility barrier between inland subspecies male function and coastal subspecies female function (see Nagy 1997a, p. 711, for discussion). Genetic hybrid inviability was estimated from mean number of seeds per fruit for parental versus F1 hybrid crosses (Nagy 1997a). Ecological hybrid inviability has been documented (Nagy 1997b), but not in a manner that allows a standardized index of hybrid versus parental fitness to be calculated.

In *Heliconius erato* postman and rayed mimetic races, Mallet (1989) noted a lack of host preferences. Ecological selection against immigrants was quantified using estimates of the longevity of immigrants and residents. A lack of sexual isolation was inferred from no evidence for assortative mating in crosses (Mallet 1989), while population genetic analyses indicated random mating and a lack of hybrid inviability in the field (Mallet et al. 1990). Breeding experiments indicated full fertility and viability of hybrids, backcrosses, F2, and future hybrid generations (Mallet 1989). Ecological hybrid inviability was estimated using the probability of establishment of hybrids versus residents (i.e. the local race) at a single site (Mallet and Barton 1989).

For *Ipomopsis spp.*, immigrant inviability was estimated from the percentage of plants surviving to flower or to five years of age for immigrant and resident species at four parental sites (Campbell and Waser 2001). A lack of genetic hybrid inviability was evidenced by the equal means of seeds per flower produced by hybrid versus pure species crosses (Campbell and Waser 2001; Campbell et al. 2003). Floral isolation is also likely in this system (see Campbell 2003 for review), but published data did not allow us to easily construct an appropriate index. Ecological hybrid inviability was estimated by comparing the percentage of plants surviving to flower or to five years of age for hybrid versus parental species in a hybrid environment in a reciprocal transplant experiment (Campbell and Waser 2001). Sexual selection against hybrids was estimated by comparing the number of seeds per fruit produced by crosses where the pollen was purely from hybrids versus mixed pollen of hybrids and *I. aggregata* (Campbell et al. 2003).

For *Iris spp. 1,* overlap in flowering times indicated a lack of temporal isolation (Young 1996); immigrant inviability was estimated from survivorship of reciprocally-transplanted individuals, a lack of floral isolation was inferred from pollinator observations, and genetic hybrid inviability was estimated from the seed set of parental and hybrid crosses from controlled pollination experiments (in which hybrids outperformed parentals but not significantly so, Young 1996).

For *Iris spp. 2,* immigrant inviability was estimated using the survival of immigrant versus resident species in each parental habitat (averaged across the two parental habitats) and ecological hybrid inviability was estimated by comparing the survival of F1 hybrids to each parental form in each of the two hybrid sites (averaged across sites and parental forms, Emms and Arnold 1997).

In *Littorina saxatilis* ecotypes, direct tests of habitat preference were unavailable, but migratory differences were quantified between ecotypes. The tendency for smooth unbanded morphs from the lower shore to move towards the sea when transplanted to the upper shore and the tendency for ridged and banded morphs from the upper shore to move towards land when transplanted to the lower shore were thus used as a measures of habitat isolation (Erlandsson et al. 1998). This tendency was estimated using the mean angular dispersion for each morph, averaged across treatments, when transplanted to the

foreign shore (Erlandsson et al. 1998 for details). Immigrant inviability was estimated using cross-product estimates of the viability of each pure morph at upper and lower shore levels, using only morphs originating from these two shore levels. Viability estimates pooled among sites were used and were averaged across four comparisons (small individuals on upper and lower shores, large individuals on upper and lower shores). Estimates of sexual isolation came from two sources (both contained in Rolan-Alvarez et al. 1999, which includes data from Johannesson et al. 1995). First, total assortative mating in natural populations was found to be 0.71 in summer and 0.75 in the fall, but arises from a combination of microspatial differences and divergent mating preferences among morphs. Computer simulations showed that about 40% and 31% of the total isolation comes from microhabitat differences for summer and fall samples, respectively, requiring individual components of 0.52 and 0.64 for sexual isolation to yield the observed levels of total assortative mating (mean individual component = 0.58). A laboratory estimate of sexual isolation was based on small sample sizes, but was comparable (0.46). We used the mean from the field and lab estimates of sexual isolation. There was no evidence for genetic hybrid inviability (Rolan-Alvarez et al. 1997; Cruz et al. 1998; Johannesson et al. 2000). We include estimates of ecological hybrid inviability, but note that results were not statistically significant because hybrids exhibited high survival in the midshore region where they are found (midshore results were only used for this component of isolation; Rolan-Alvarez et al. 1997). Finally, although sexual selection against hybrids was not detected in a recent study (Cruz et al. 2001), there was some evidence that extreme forms within hybrids (i.e. those resembling each parental morph) did have higher mating success.

For *Mimulus* spp., all data are from Ramsey et al. (2003), who estimated multiple components of reproductive isolation between species of monkeyflowers. We used their measure of ecogeographic isolation as an estimate of immigrant inviability (see Ramsey et al. 2003 for details) but congruent results were obtained by Hiesey et al. 1971. We also used their estimates of reproductive isolation caused by pollinator fidelity (i.e. floral isolation), pollen competition, and total postzygotic isolation. We provide this estimate of total postzygotic isolation in the genetic hybrid inviability category but note that it is a composite measure of postmating isolation that includes measures of fitness that might be

considered natural or sexual selection against hybrids, e.g., pollen viability of hybrids (see Ramsey et al. 2003 for details). In all cases, we used the average for the two species.

For *Mitoura* spp., data are from Forister 2004, who examined oviposition preference and larval performance of three host races (Cedar, Juniper and Cypress races) on four different hosts (one Cedar species, one Juniper species and two Cypress species). Oviposition preference provided a measure of habitat isolation and was estimated as percent ovipositing on their native host, averaged across the different populations within a host race category and then averaged across host races. Larval survival provided a measure of immigrant inviability and for simplicity we have averaged the results such that immigrant inviability is estimated as the mean relative survival of all the other host races on the native host of a race, averaged across the three host races. The different host races can freely interbreed in the lab and produce fertile offspring, indicative of a lack of genetic incompatibility (Forister unpublished).

In *Neochlamisus bebbiannae*, habitat isolation was estimated as the average population level host preference from six experiments, all of which gave comparable results (see Funk 1998 for details; means across populations from each experiment: percent eggs laid on non-native host = 7.08; percent of time spent on non-native host = 12.86; larval feeding on non-native host under no-choice conditions = 8.54; larval feeding on non-native host under choice conditions = 1.20; adult feeding on non-native host under no-choice conditions = 1.88; adult feeding on non-native host under choice conditions = 0.50). Immigrant inviability was estimated by comparing the survival of larvae transplanted to their non-native host to the survival of larvae reared on their native host. Sexual isolation was inferred from the isolation indices reported in Funk 1998 (Levene's isolation index, transformed to a scale of zero to one).

For *Polemonium viscosum*, immigrant inviability was estimated using the survival of immigrant versus resident plant ecotypes in each of two environments in a reciprocal transplant experiment (Galen et al. 1991). Floral isolation was estimated indirectly from information on (1) the relative frequency of different floral scent morphs at low versus high elevation sites, and (2) the seed set of naturally pollinated plants of each morph at each elevation (i.e. 'floral sexual fitness'). One-third of the individuals at low sites were

the sweet morph and two-thirds were the skunk morph, whereas at the high elevation site two-thirds of the individuals were the sweet morph (Galen and Kevan 1980). At the low site, the sweet and skunk morphs produced 1.37 versus 2.67 seeds per flower, respectively, and at the high site 2.73 versus 1.84 seeds per flower, respectively. Thus, immigrants to the low site were assigned a floral sexual fitness of: one-third x 2.67 + two-thirds x 1.37, whereas residents of that site were assigned a floral sexual fitness of: one third x 1.27 + two-thirds x 2.67. Migrants to the high site were assigned a floral sexual fitness of: one-third x 2.73 + two-thirds x 1.84, whereas residents of that site were assigned a floral sexual fitness of: one third x 1.84 + two-thirds x 2.73. Although reduced seed set of the less-frequent morph at each site might reflect pollen competition or postzygotic isolation, floral isolation seems more likely based upon pollination and weeding experiments (see Galen 1985 for details).

For *Rhagoletis spp.,* habitat isolation was estimated from percent oviposition on apple versus blueberry in no-choice trials, immigrant inviability from percent larval survival to adulthood of each species on each host, and ecological hybrid inviability from percent larval survival to adulthood for hybrids versus parental forms in their native environment (all data from Bierbaum and Bush 1990).

Methodology for *Timema cristinae* walking-stick insects is given in detail in Nosil et al. 2002, 2003 (sexual isolation) and Nosil 2004 (immigrant inviability). For sexual isolation, mating frequencies for parapatric and allopatric populations were pooled to estimate homo- and heterotypic mating frequencies. The strength of immigrant inviability was estimated using the mean survival of between-host immigrants versus residents averaged between allopatric and parapatric pairs of populations. Host preference data are from Nosil (unpublished), who has conducted choice experiments in the lab. In all cases, only populations using alternate host-plant species were used in the calculations.

CHAPTER 8.
REPRODUCTIVE ISOLATION CAUSED BY VISUAL PREDATION ON MIGRANTS BETWEEN DIVERGENT ENVIRONMENTS*

*A version of this chapter appears as Nosil, P. 2004. Reproductive isolation caused by visual predation on migrants between divergent environments. Proceedings of the Royal Society of London B 271: 1521-1528. Reprinted with permission from the Royal Society of London.

8.1 Abstract

In theory, natural selection can drive adaptation within species while simultaneously promoting the formation of new species by causing the evolution of reproductive isolation. Cryptic colouration is widespread in nature and is generally considered a clear, visual example of adaptation. Here I provide evidence that population divergence in cryptic colouration can also cause reproductive isolation. First, a manipulative field experiment using walking-stick insects demonstrates that the relative survival of different colour-pattern morphs depends on which host-plant species they are resting, but only in the presence of avian predation. Second, natural populations adapted to different host plants have diverged in colour-pattern morph frequencies such that between-host migrants are more likely to be the locally less-cryptic morph than are residents. Collectively, these data indicate that high rates of visual predation on less-cryptic migrants are likely to reduce encounters, and thus interbreeding, between host-associated populations. Comparison with previous estimates of sexual isolation revealed that the contribution of selection against between-host migrants to total premating isolation is as strong or stronger than that of sexual isolation. These findings highlight the potential role of natural selection against migrants between divergent environments in the formation of new species.

8.2 Introduction

Classical work on speciation focused on the circumstances under which new species are formed, with a particular emphasis on the likelihood and frequency of speciation when populations are geographically-separated (allopatry) versus in geographic contact (parapatry or sympatry) (Mayr 1942, 1963). Recent years have seen a resurgence of interest in the exact mechanisms of speciation. In particular, much work has concerned the potential role of adaptive divergence in the evolution of reproductive isolation (Hendry et al. 2000; Jiggins et al 2001; Rundle et al. 2000; Schluter 2000 for review; Funk et al. 2002; Nosil et al. 2002). Most such studies of 'ecological speciation' have quantified reproductive isolation caused by divergent mate preferences (sexual isolation), divergent habitat preferences, or ecological selection against hybrids.

Here I consider a less-recognized, yet general, form of reproductive isolation: reduced survival of between-population migrants (see also Mallet & Barton 1989; Funk 1998; Hendry et al. 2000; Via et al. 2000; Hendry in review). This process is often not considered a form of reproductive isolation but does result in partial reproductive isolation (i.e. reduced gene flow due to a non-geographic barrier) when it reduces encounters, and thus interbreeding, between individuals from different populations. Reduced migrant fitness may commonly facilitate speciation because it 1) directly reduces gene flow between populations whenever local adaptation occurs, 2) can result in partial reproductive isolation without a genetic correlation between separate traits affecting local adaptation and mate preference, overcoming the main theoretical objection to the sympatric evolution of reproductive isolation (Felsenstein 1981), 3) imposes selection for further forms of reproductive isolation, such as increased preference for local habitats and mates (Via et al. 2000), and 4) causes reductions in gene flow which will increase the efficacy of selection (Hendry et al. 2001). Existing examples of natural selection against migrants are convincing and come from diverse taxa (Mallet & Barton 1989; Via et al. 2000; Schluter 2000 for review; Vamosi & Schluter 2002), but are usually not framed in terms of the contribution of this process to reductions in gene flow between diverging taxa (i.e. reproductive isolation, but see Mallet & Barton 1989; Funk 1998; Hendry et al. 2000; Via et al. 2000).

Cryptic colouration is extremely common in nature and is usually considered a clear, visual example of adaptation within species (Cott 1940; Kettlewell 1973). In fact, industrial melanism in the peppered moth is perhaps the most commonly used example of adaptation via natural selection (Kettlewell 1973). However, population divergence in cryptic colouration can also promote speciation whenever high rates of predation on less-cryptic migrants reduce encounters, and thus interbreeding, between individuals from different populations. Despite this intuitive link between crypsis and reproductive isolation, and despite the popularity of crypsis as an example of adaptation within species, its potential role in speciation has received little attention (but see Tauber & Tauber 1977, Endler & Houde 1995).

Timema are wingless, phytophagous insects distributed throughout western North America (Vickery 1993). Selection against less-cryptic migrants is likely to have played a role in the diversification of the genus *Timema* because host-specific divergence in colour-pattern between closely-related sympatric and parapatric species is common (Crespi & Sandoval 2000). *Timema* cristinae exhibits genetically-determined colour-pattern morphs (Sandoval 1993), with an unstriped morph more common on the host plant *Ceanothus spinosus* (mean frequency = 81%) and a striped morph more common on *Adenostoma fasciculatum* (mean frequency = 72%) (Sandoval 1994a; Nosil et al. 2002). Controlled predation trials using wild scrub-jays and captive lizards have demonstrated that each of these morphs is most cryptic on the plant on which it is more common (Sandoval 1994b). Dominance of the unstriped morph is incomplete (Sandoval 1993), and thus a third, intermediate morph (i.e. bearing a faint stripe) is also found at low (<5%) frequency in some populations. Local morph-frequencies are temporally stable and geographic variation indicates that they are determined by a gene flow – selection balance between host-plant patches exhibiting the different selective regimes (Sandoval 1994a). However, previous work on selection for crypsis in *T. cristinae* has not estimated the relative survival of each morph on each host plant in nature and fitness in the absence of predation is unknown, precluding estimates of the magnitude of reproductive isolation caused by visual predation on between-host migrants.

Two criteria are required for crypsis to contribute to reproductive isolation between populations of *T. cristinae* adapted to the use of different host-plant species.

First, the survival of different colour-pattern morphs in natural populations must depend on which host-plant species they are resting, but only in the presence of visual predation. Second, for immigrants to exhibit reduced fitness relative to residents, populations using different hosts must have diverged in colour-pattern morph frequencies such that immigrants are more likely to be the locally non-matching morph than are residents. Here I use a manipulative field experiment and data on morph-frequencies in natural populations of walking-stick insects to test whether these conditions are fulfilled. Second, I consider the effects of relative population sizes on the strength of selection against migrants. Finally, previous studies of reproductive isolation in this system have examined only sexual isolation, and thus I compare the relative contributions of selection against between-host migrants versus sexual isolation to total premating isolation (data on sexual isolation from Nosil et al. 2002, 2003).

8.3 Materials and Methods

8.3.1 Mark-recapture experiment

Individual *T. cristinae* were collected using sweep nets in the Santa Ynez Mountains, California in February 2003 (latitude 34 30.55, longitude120 4.17). Animals were captured in the first instar on both host plants and reared to maturity in the lab on *C. spinosus*. Prior to release, each walking-stick was scored for colour-pattern (unstriped, striped, intermediate) on two separate days, photographed with a digital camera, and individually marked with a fine-tipped permanent marker on the abdomen, such that the mark would not be visible when the insects were resting in their natural position on the host-plant. Colour-pattern was scored by one individual (P. Nosil) and was highly repeatable (380 of 384 individuals given identical scores on both occasions). All individuals were marked and released within 5 days of attaining maturity.

I used a replicated, random blocks design with four treatment levels (*Ceanothus* versus *Adenostoma* with avian predators present versus absent, one bush per treatment). Avian predators were excluded using chicken-wire enclosures (3cm mesh). Each of the four treatments was represented twice within each of two study sites with 24 individuals released onto each bush (n = 96 individuals for each of 4 treatments; each bush

previously cleared of all *Timema*; area near latitude 34 30.90, longitude 119 48.01). Upon release, sex ratios were equal and morph frequencies were similar among bushes. Sample bushes were separated from all other suitable host plants by a minimum distance of 5m (12m is a typical maximum per-generation dispersal distance; Sandoval 1993). Availability of mature test animals forced me to start the experiments at each of the two sites on slightly different dates (March 26th, 2003 for site 1 and April 14th, 2003 for site 2).

Recapture surveys were conducted 3, 10, 17, and 24 days following release (no individuals were recaptured on the final recapture session). For each sample bush, I placed a white, cotton sheet underneath the bush, visually inspected the bush for walking-sticks insects and then shook each branch such that any undetected insects would fall onto the sheet. I recorded which insects were recaptured and then each specimen was released back where they were captured. A recapture session was considered complete when no walking-stick insects were found after fifteen-minutes of shaking the branches of a particular bush. On the initial release date, and on each subsequent recapture date, I identified which birds were present at the study sites. At both sites, the following insectivorous bird species were observed foraging on or near the experimental bushes; *Aphelocoma californica, Sayornis saya, Pipilo maculates, Pipilo crissalis, Sturnella neglecta, Psaltriparus minimus, Turdus migratorius*. Identifying these birds was the most difficult part of my thesis.

The probability of post-release recapture (recaptured versus not recaptured) was analyzed in a four-factor logistic regression model that included sex, treatment (C-pred: *Ceanothus* with predators, A-pred: *Adenostoma* with predators, C-nopred: *Ceanothus* without predators, A-nopred: *Adenostoma* without predators), morph (unstriped, intermediate, striped) and bush number (1 to 4 – a 'block' effect), as well as all possible interactions among these factors (adding 'site' as a factor does not influence the results). Morph-specific recapture probability dependent on treatment, independent of bush and sex, is indicated by a significant interaction between morph and treatment in a reduced regression model derived using backward elimination (initial model included all factors and interactions but then removed all terms for which the significance of –2 log LR was > 0.10).

8.3.2 Estimating morph-specific survival

Morph-specific survival probabilities were then estimated using MARK (White and Burnham 1995). For each treatment separately, I started with a fully time-dependent Cormack-Jolly-Seber model (CJS) (Lebreton et al. 1992) that included morph, time (one time period for each recapture session) and the interaction between morph and time for both survival and recapture probabilities. In all cases, this full model provided a good fit to the data (goodness of fit tests using chi-square values or a nonparametric bootstrap approach, 1000 replicates, all $p > 0.10$), and thus represents a reasonable starting point for the analyses (Lebreton et al. 1992). Because the full models fit the data well, I then used Akaike information criteria (AIC) (Akaike 1973) and likelihood-ratio tests (LR) (Edwards 1992) to find models that best fit the data. The best-fit model was compared to other models using AIC and LRT criteria and was used to estimate survival differences among morphs, where appropriate (best fit models for each treatment were, C-pred: CJS fully time-independent survival differences among morphs; A-pred : CJS survival differences among morphs dependent on two time periods (1 : release to first recapture, 2: all other recapture sessions); C-nopred: CJS time-dependent survival, time-independent recapture; A-nopred: CJS time-dependent survival, morph-dependent recapture).

In all cases, I corrected for over- or under-dispersion in the data using estimates of the variance inflation factor (c-hat)(Lebreton et al. 1992), derived by comparing the deviance of the full model to the mean deviance of 1000 simulated datasets (using nonparametric bootstrap of the original dataset). Analyses conducted using a constant c-hat of 1.0 gave results congruent with analyses adjusting for lack-of-fit (results not shown).

8.3.3 Inferring migrant and resident survival

The survival of between-population migrants versus residents was inferred by extrapolating from the field experiment to 15 different pairs of natural populations. A 'population' of walking-sticks is defined as the insects collected within a homogenous patch of a single host plant (validated by previous mark-recapture and molecular data

indicating that most individuals in a patch are residents)(Sandoval 1993; Nosil et al. 2003; Nosil & Crespi 2004). 'Residents' are defined as the walking-stick insects captured within a focal population and 'Migrant' refers to potential migrants, which are walking-stick insects captured outside of the focal population and within one or more of the other study populations (usually a neighbouring population using the alternate host). 'Parapatric' insect populations are in contact with a population of insects adapted to the alternative host, while 'allopatric' populations are separated from all other populations adapted to the alternative host by distances > 50 times the 12m per-generation gene flow distance (Sandoval 1993).

Colour-pattern morph frequencies were surveyed in 15 pairs of populations by catching walking-stick insects with sweep nets during February-June in 2000-2003 (n = 5233; 12 parapatric pairs comprised of one population using each host plant, 2 allopatric pairs using the same host plant, 1 allopatric pair using different host plants). Chi-square tests were used to test for differences among paired populations in morph-frequencies.

Selection against between-population migrants was estimated using the following data; 1) morph-frequencies of the residents within a focal population ($\%U_R$, $\%I_R$, and $\%S_R$ = %unstriped, intermediate and striped morph within a focal population), 2) morph-frequencies in another population from which potential migrants originated ($\%U_M$, $\%I_M$, and $\%S_M$ = %unstriped, intermediate and striped morph within the population that migrants originate from) and 3) the mean relative survival of each colour-pattern morph in the mark recapture experiment (using the treatments where predators were present; S_{UC}, S_{IC}, S_{SC} = relative survival of unstriped, intermediate and striped morph respectively on *Ceanothus* and S_{UA}, S_{IA}, S_{SA} = relative survival of unstriped, intermediate and striped morph respectively on *Adenostoma*).

This calculation entailed four steps. First, the relative survival of each morph on each host plant was calculated by assigning a value of one to the morph with the highest survival on each host plant and then scaling the survival of the other two morphs appropriately (e.g. 1 / absolute survival of the morph with highest survival provides the factor by which the survival of the other two morphs is multiplied). Second, the frequency of each morph within a population was then multiplied by that morphs relative

survival on a particular host plant, and these values from each of the three morphs summed, to yield the mean relative survival of individuals from a given population on a given host plant. For example, the mean survival of residents of a *Ceanothus* population would be calculated as [(%U_R x S_{UC}) + (%I_R x S_{IC}) + (%S_R x S_{SC})] and the mean survival of migrants from another population would be calculated as [(%U_M x S_{UC}) + (%I_M x S_{IC}) + (%S_M x S_{SC})]. The same calculations were then carried out for the other population in a pairwise comparison, this time reversing the resident and migrant designations (notably when *Adenostoma* populations were considered S_{UA}, S_{IA}, S_{SA} values would be used). Third, the strength of selection against migrants within each population was estimated as (1 - mean survival of migrants / mean survival of residents). Fourth, the strength of selection against migrants for a population pair was estimated by averaging the mean values that were calculated for each single population.

For parapatric populations, I examined the relationship between the magnitude of selection against migrants (averaged across a population pair) and asymmetry in population size between a pair of adjacent populations. Population size was inferred using the relative area of the host-plant patch used by each population (patch size has been shown to be strongly and positively correlated with population size, $r^2 = 0.63$ and 0.53 for *Ceanothus* and *Adenostoma* patches respectively, n = 13 patches of each host) (Sandoval 1994b).

8.3.4 Relative contributions of reduced migrant survival and sexual isolation

I estimated total premating isolation caused by the combined effects of selection against migrants and sexual isolation, and the relative contribution of each of these two individual components to total isolation (Ramsey et al. 2003 for details of estimation procedure). In brief, individual components of reproductive isolation (RI) specify the magnitude of reproductive isolation caused by a given barrier to gene flow and generally vary from zero to one. The individual contribution of selection against migrants (RIm) was estimated as [1 – (migrant survival / resident survival)] and the individual contribution of sexual isolation (RIs) as [1 – (heterotypic mating frequency / homotypic mating frequency)] (migrant survival estimated as above, mating data from Nosil et al. 2002, 2003). Total reproductive isolation is computed as multiplicative function of the

individual components at sequential stages in the life history, but a given component of reproductive isolation can only eliminate gene flow that has not been eliminated by a previous component. Selection against migrants act before sexual isolation in the life history and thus the absolute contribution of selection against migrants is (ACm = RIm), the absolute contribution of sexual isolation is (ACs = RIs (1 – ACm)) and total isolation is (ACm + ACs). The relative contribution of any component is simply the absolute contribution divided by total isolation.

First, using the populations examined in the current and a previous study I estimated components of reproductive isolation between pairs of populations under three major eco-geographical scenarios : allopatric pairs of populations using the same host-plant species (two pairs from the current study to estimate migrant survival; all mating trials from Nosil et al. 2003 where the sexes were from the same population (homotypic matings) versus from different allopatric populations (heterotypic matings) using the same host to estimate sexual isolation), allopatric pairs of populations using different host-plant species (single pair from the current study to estimate migrant survival, all mating trials from Nosil et al. 2003 where the sexes were from the same population versus from different allopatric populations using the alternate host to estimate sexual isolation), and parapatric pairs of populations using alternate host-plant species (morph-frequencies from the twelve pairs in the current study pooled to estimate mean migrant survival, all mating trials from Nosil et al. 2003 where the sexes were from the same population versus from a population that has an adjacent, neighbouring population on the alternate host to estimate sexual isolation).

Second, I estimated levels of reproductive isolation under a range of relative sizes of the neighbouring population using each of the twelve study populations in Nosil et al. 2003 in a single comparison (all the populations examined in the current study were examined in Nosil et al. 2003 but not vice-versa). I calculated the level of premating isolation due to natural selection against migrants and the level of sexual isolation that each of the twelve focal populations examined in Nosil et al. (2003) exhibits against all other study populations from Nosil et al. 2003 that use the alternate host plant (individuals within a population considered resident and individuals from all other populations considered migrants; within-population mating trials considered homotypic

and all between-population mating trials considered heterotypic). This averaging among populations should not bias the results, although it will decrease the precision of the estimates. I then examined the association between geographic scenario (i.e. the relative size of the neighbouring population, allopatric populations assigned a value of zero) and reproductive isolation using regression analyses. Departures from linearity were detected in some cases and thus partial F-tests were used to test whether a model including both a linear and a quadratic term provided a better fit than a model with only a linear term.

8.4 Results

8.4.1 Mark-recapture experiment

Mark-recapture experiments show that the recapture probability (% recaptured) of different colour-pattern morphs is dependent on host-plant and on the presence of avian predators (mean recapture rates for each morph in each treatment are shown in Fig. 8.1; morph x treatment interaction, $LR = 14.16$, $df = 6$, $p < 0.05$). Consequently, I used maximum-likelihood techniques that are designed specifically for the analysis of mark-recapture data (i.e. that independently estimate survival and recapture probabilities) to estimate survival differences among the colour-pattern morphs within each treatment.

8.4.2 Host-specific survival of colour-pattern morphs

When avian predators were present, the colour-pattern morphs differed in survival (*Ceanothus*: best model includes a morph survival term, AIC=108.3, 2.26 times better than the next best model; $LR = 5.95$, $df = 2$, $p < 0.05$; *Adenostoma*: best model includes a morph survival term, AIC=164.93, 5.88 times better than next best model; LR=7.88, $df = 2$, $p < 0.05$). The unstriped morph exhibited the highest survival on *Ceanothus* and the striped morph exhibited the highest survival on *Adenostoma* (Fig. 8.2). The intermediate morph exhibited survival similar to that of the striped morph (low of *Ceanothus* and high on *Adenostoma*), suggesting that a faint stripe functions similarly to a pronounced stripe. The best fit-model included a time component on *Adenostoma*, but not on *Ceanothus*, and the results are presented as such in Fig. 8.2 (see methods for details of model-testing procedures).

Survival differences among colour-pattern morphs disappear when predators are excluded (for both *Ceanothus* and *Adenostoma*, the best model does not include a morph survival term, AIC=177.95, 196.03 respectively, 436 and 123 times better than the best models that include a morph survival term respectively). Thus survival of different colour-pattern morphs is dependent on host-plant species only in the presence of avian predation. Notably, morph-specific dispersal cannot account for these results as walking-sticks could disperse from both predator and predator-free treatments, yet morph-specific survival occurred only when predation was present. Collectively, these data demonstrate divergent, host-specific selection on colour-pattern and indicate that differential visual predation is the agent of selection.

8.4.3 Estimates of reproductive isolation caused by reduced migrant survival

Populations of *T. cristinae* using different hosts have diverged in colour-pattern morph frequencies such that immigrants are more likely to be the locally non-matching morph than are residents (Table 8.1). Thus between-host migrants are likely to exhibit reduced survival relative to residents (s, strength of selection against between-host migrants averaged across the thirteen pairs using alternate hosts = 0.18). This reduced survival of migrants will cause reproductive isolation if it lowers encounter rates, and thus interbreeding, between host-associated populations.

The degree of fitness reduction exhibited by migrants was variable and dependent on the ecological and geographical scenario examined (Fig. 8.3). Under a scenario of secondary contact between allopatric populations, different populations using the same host-plant species would exhibit no difference in the fitness of migrants versus residents (due to similarity in morph frequencies and in the selective environment; $s = 0.00$). Conversely, the geographically-separated populations using different host-plants are strongly differentiated in morph frequencies and thus would exhibit high levels of reduced migrant fitness ($s = 0.53$). Among adjacent, parapatric populations using different host-plants, selection against migrants, taken as the mean in the two populations, weakens as asymmetry in population size between paired-populations increases (range of $s = 0.02$-0.30, mean $s = 0.15$, s.d. $= 0.08$; $r = 0.51$, $p < 0.05$; $n = 12$ paired populations, Spearman Rank Correlation).

8.4.4 Relative contributions of reduced migrant survival and sexual isolation

In *T. cristinae*, total premating isolation is nonexistent for allopatric pairs using the same host, strongest for allopatric pairs using alternate hosts, and intermediate for parapatric pairs using alternate hosts (Fig. 8.4). Selection against between-host migrants can contribute to total reproductive isolation between populations using different hosts as strongly as does sexual isolation (relative contributions to total reproductive isolation of 0.68 versus 0.32, 0.90 versus 0.10, and 0.38 versus 0.62 for all, allopatric only, and parapatric only populations respectively).

Consideration of the potential for gene flow into each of the 12 study populations examined in Nosil et al. 2003 revealed a more refined picture of the evolution of reproductive isolation (Fig. 8.4). The individual component of reproductive isolation caused by selection against migrants was highest between allopatric populations and declined with increasing migration into a focal study population (linear regression, r^2 = 0.81, B = -0.42, s.e. = 0.07, $p < 0.001$; F-change between linear and quadratic model = 1.91, $p = 0.20$). Conversely, the individual component of reproductive isolation caused by sexual isolation was weak between allopatric populations, increased until the size of a study population was similar to that of its neighbouring population using the alternate host plant, and decreased as the neighbouring population became relatively larger (linear regression, $r^2 = 0.05$, B = -0.05, s.e. = 0.07, $p = 0.48$; quadratic regression, $r^2 = 0.44$, B = -0.53, s.e. = 0.21, $p < 0.05$; F-change between linear and quadratic model = 6.30, $p < 0.05$; as reported in Nosil et al. 2003). Thus total premating isolation was high across a wide range of geographical scenarios, with selection against migrants contributing strongly to total isolation when the size of the population adjacent to the study population was small (or when the study population is allopatric) and sexual isolation contributing most strongly to total isolation when the sizes of the study and the neighbouring population were similar (Fig. 8.4).

8.5 Discussion

Natural selection against migrants between divergent environments can cause reproductive isolation by reducing encounters, and thus interbreeding, between

individuals from populations exhibiting divergent local adaptations (Mallet & Barton 1989; Funk 1998; Via et al. 2000). In this study, a manipulative field experiment and data on morph-frequencies in natural populations were used to infer the survival of migrant versus resident walking-stick insects under various geographic and ecological scenarios. The results show that migrants between populations adapted to the use of different host plant species (i.e. divergent environments) are likely to exhibit increased predation rates and thus low survival relative to residents. This process will cause reproductive isolation between populations using different host plants when it reduces encounters, and thus interbreeding, between host-associated populations. These findings are of particular interest because the agent of selection (differential visual predation) is well understood and differences in survival among morphs disappeared it was excluded.

The survival of between-population migrants versus residents was inferred by extrapolating from the field experiment to 15 different pairs of natural populations. Thus the results may slightly overestimate selection against migrants as they do not account for the reduced predation pressure on less-cryptic prey that can arise from density- or frequency-dependent predation (Bond & Kamil 2002). Nonetheless, geographic variation in morph-frequencies is also consistent with increased survival of the more common morph on each host-plant species in nature (Sandoval 1994b). Furthermore, lower relative survival of the less-cryptic morph in the mark-recapture experiment was independent of time on *Ceanothus* and was strongest in the latter stages of the experiment on *Adenostoma*, providing evidence that the less-cryptic morph will indeed be subject to increased predation even as it becomes increasingly rare.

My results are consistent with previous studies of host-associated pea aphids (Via et al. 2000) and leaf beetles (Funk 1998), mimetic *Heliconius* butterflies (Mallet & Barton 1989) and sympatric sticklebacks (Schluter 1995; Vamosi & Schluter 2002) which have also explicitly noted that natural selection against migrants between divergent environments is likely to reduce gene flow between diverging taxa. In particular, the results compliment the work on *Heliconius*, where migrants suffer increased rates of visual predation due to a loss of mimicry rather than crypsis. However, reduced migrant fitness (i.e. local adaptation) is widespread in nature and thus many other potential examples exist (Schluter 2000 for review). Notably, reproductive isolation caused by

selection against migrants is independent of actual mating preferences, which may increase or decrease total levels of reproductive isolation (if foreign males are discriminated against or preferred as mates respectively).

The current study expands on previous work on reproductive isolation caused by selection against migrants by explicitly examining the role of relative population sizes (i.e. population demography). Colour-pattern morph frequencies among populations of *T. cristinae* are determined by a balance between gene flow and selection such that morph frequencies are strongly differentiated between allopatric populations using different host-plant species, moderately differentiated between adjacent populations of similar size that use different hosts, weakly differentiated between adjacent populations of unequal size that use different hosts, and almost identical between populations using the same host plant (Sandoval 1994a). Thus selection against migrants, taken as the mean in the two populations, weakens as asymmetry in population size between paired-populations increases. These data indicate that selection against migrants is most likely to promote speciation when secondary contact between allopatric populations is accompanied by 1) ecological divergence and 2) equality in the sizes of diverging populations (i.e. when asymmetric gene flow is least likely to erode population differentiation).

Although natural selection against migrants may commonly reduce gene flow between populations, such reproductive isolation is environment-dependent and incomplete. Nonetheless, in *T. cristinae* selection against between-host migrants can contribute to total reproductive isolation as strongly as sexual isolation. Second, there are both theoretical and empirical data indicating that selection against migrants can facilitate the evolution of further forms of reproductive isolation. For example, the initial reduction in gene flow caused by selection against migrants may facilitate the evolution of reproductive isolation as a by-product of local adaptation (Hendry et al. 2001) or the evolution of reinforcement (Servedio & Kirkpatrick 1997). The exact importance of selection against migrants in speciation might depend on what stage of the process is examined. For example, anti-migrant selection might be particularly important in the early stages of speciation as it can evolve rapidly whenever new environments are colonized, but may play a more minor role in the latter stages of speciation where sexual isolation or postmating incompatibilities act as strong barriers to gene flow.

Natural selection against migrants can also promote speciation by imposing selection for increased efficiency of habitat choice and increased mating discrimination against between-population migrants (Via et al. 2000), a process analogous to reinforcement (Dobzhansky 1951). In *T. cristinae*, the costs of mating with locally less-cryptic migrants are twofold; 1) males ride on the back of the female during the mating period and thus females that mate with less-cryptic males are likely to suffer reduced individual survival during mating, and 2) females that mate with less-cryptic males will produce a higher frequency of less-cryptic offspring than females that mate with resident males (Sandoval 1993). Sexual isolation between populations of *T. cristinae* has apparently evolved in parallel with divergence in host-plant use and is greater in geographic regions where between-host migration occurs than in regions where geographically-separated populations do not exchange migrants (Nosil et al. 2002, 2003). These data suggest that the costs of mating with migrants have played a role in driving the evolution of sexual isolation. However, between-population mate choice is not based on colour-pattern (Nosil et al. 2002) and thus reproductive isolation caused by selection against migrants versus divergent mate preferences is not based on the same trait(s).

The results of this study have broad implications for understanding both adaptation and speciation as they illustrate how divergent selection for local adaptation in general, and cryptic colouration in particular, can play a role in the speciation process. Moreover, they highlight the potential role of selection for crypsis and visual predation in the origin of species and thus provide some of the only evidence for the general role of predation in adaptive radiation (Schluter 2000).

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Figure 8.1 **Percent recapture (± 1 S.E.) of different colour-pattern morphs of *Timema cristinae* under four selection regimes (*Ceanothus* (C) versus *Adenostoma* (A) as host plants in the presence versus absence of avian predation).**

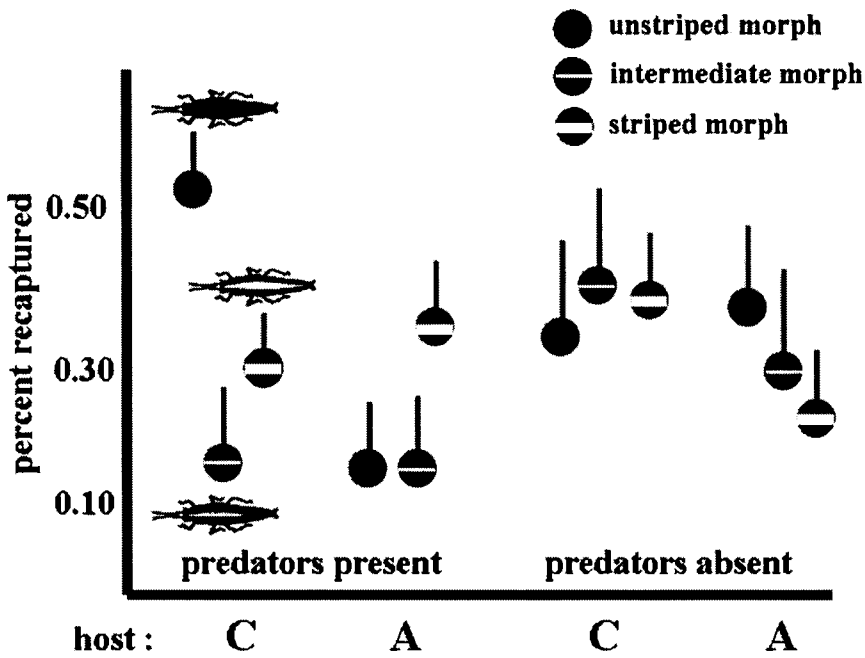

Figure 8.2 When exposed to visual predation, survival probabilities varied among colour-pattern morphs of *T. cristinae* on two different host-plant species ($p < 0.05$ on both hosts).
On *Ceanothus*, survival was independent of time with the unstriped morph exhibiting the highest survival during (see methods for model-testing procedures). Conversely, in the best-fit model for *Adenostoma* survival was dependent on an interaction between time and morph, with the striped morph exhibiting somewhat higher survival early in the experiment (between initial release and the first recapture session) and much higher survival for the remainder of the experiment ('late survival'). When visual predation was excluded, survival did not vary among morphs (results not shown, both $p > 0.25$). Populations using different host-plants have diverged in morph frequencies such that each morph is most common on the host-plant on which it has the highest survival. Thus, on average, individuals that migrate to the alternate host-plant exhibit reduced fitness relative to residents (mean $s = 0.18$). A) *Ceanothus* – lines show 95% C.I. B) *Adenostoma* – ellipses show 95% C.I.

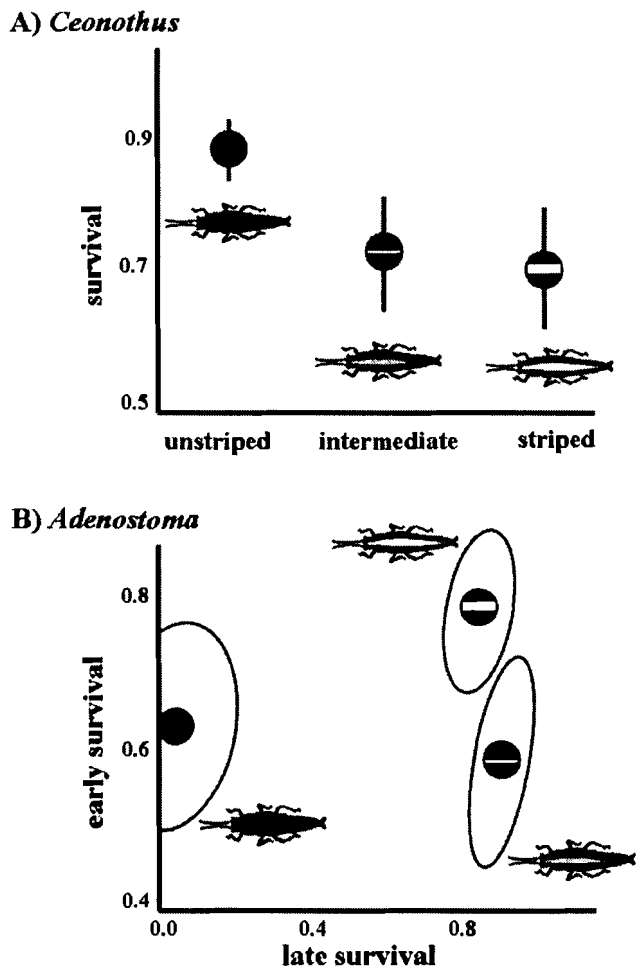

Figure 8.3 The strength of selection against between-host migrants for 12 different pairs of adjacent populations, under various geographical scenarios (x-axis).

Each population pair consists of one population that uses *Ceanothus* as host plant and one that uses *Adenostoma* (total n = 5233, Table 8.1 for population-specific sample sizes). The values on the y-axis represent the strength of selection against migrants (1 – migrant survival / resident survival), averaged across the two populations in a pairwise comparison. Reduced fitness of migrants is greatest when population sizes are similar (i.e. when asymmetric gene flow is least likely to homogenize morph-frequencies). The curve was estimated using the non-parametric cubic spline (dashed lines show standard errors from 1000 bootstrap replicates) (Schluter 1988). The geographical scenario for each parapatric pair of populations is denoted by black circles on the x-axis. Also shown for comparative purposes is the strength of selection against migrants under a scenario of secondary contact between two allopatric populations using different host-plants (top-right).

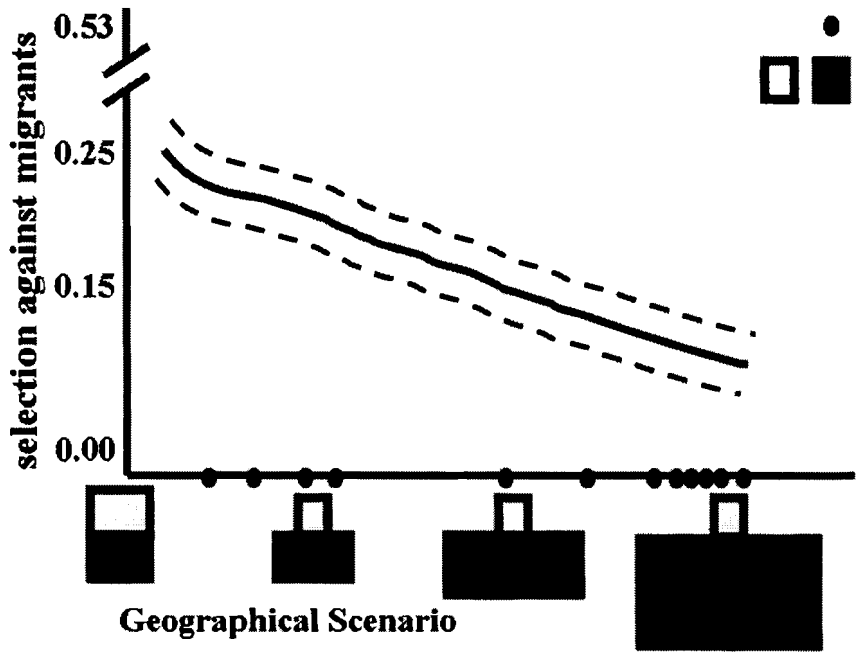

Figure 8.4 Components of reproductive isolation under different ecological and geographic scenarios.

A) Absolute contributions of natural selection against migrants and sexual isolation to total premating isolation for walking-stick insect populations under three different ecological and geographic scenarios. The relative contribution of each component is simply its absolute contribution divided by total isolation. B) For the 12 populations also examined in Nosil et al. 2003, the average magnitude of reproductive isolation that a study population exhibits against all other populations using the alternate host (total isolation and individual components are shown). The x-axis represents the relative size of the population on the alternate host that neighbours a study population (study population, grey box; neighbouring population, black box). Similar levels of total premating isolation are observed under a range of geographical scenarios (i.e. roughly until the size of the neighbouring population exceeds that of the study population), but arise via different individual components of reproductive isolation.

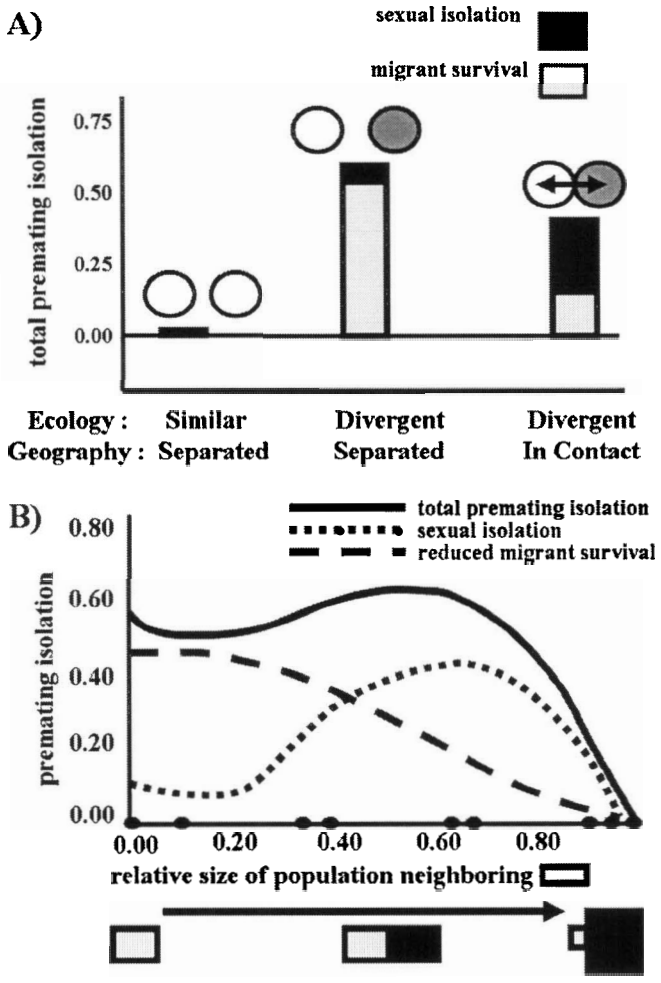

Table 8.1 Morph frequencies in the sample populations of *T. cristinae*.
Numbers of each morph found within 30 populations (U - unstriped, S – striped, I – intermediate). Populations were paired such that 12 pairs of populations were adjacent, parapatric pairs using alternate hosts (pairs 1-12) and 3 pairs were allopatric (pair 13 consists of one population using each host and pairs 14, 15 consist of two allopatric populations each using *Ceanothus*). For parapatric pairs, %C refers to the total area of the two populations that is occupied by *Ceanothus*. Also shown are chi-square values testing whether morph-frequencies differ between paired populations and overall.

	***Ceanothus*+ Population**				***Adenostoma*+ Population**			**%C**	**Chi-square**
	U	S	I		U	S	I		
Parapatric									
1	28	43	6		101	485	14	34	26.01***
2	5	6	2		21	133	8	39	9.52**
3	447	44	0		213	79	9	94	59.78***
4	10	10	0		70	322	7	8	13.12**
5	52	12	3		201	166	13	67	15.83***
6	158	97	80		86	175	81	20	43.55***
7	7	3	3		3	4	2	5	1.26
8	12	7	4		10	47	9	65	14.30***
9	30	21	0		11	5	1	12	3.54
10	27	68	0		3	27	0	5	4.24*
11	30	82	3		6	51	1	1	7.09*
12	2	12	4		5	56	10	2	1.19
pooled	808	405	105		730	1550	155		387.51***
Allopatric									
13	423	5	1		69	337	4	N/A	576.87***
14	382	90	14		45	21	3	N/A	6.12*
15	35	0	0		43	2	2	N/A	3.13

+this host designation applies only to parapatric pairs in the table

*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

CHAPTER 9.
HOST-PLANT ADAPTATION DRIVES THE EVOLUTION OF SEXUAL ISOLATION*

*A version of this chapter appears as Nosil, P., Crespi, B.J., and Sandoval, C.P. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature 417: 440-443. Reprinted with permission from Nature.

9.1 Abstract

Here we show that ecological divergence can play a crucial role in the early stages of speciation. Populations of the walking-stick insect *Timema cristinae* that use different host-plant species have diverged in body size and shape, host preference, behaviour and the relative frequency of two highly cryptic colour-pattern morphs[1,2]. Mating trials revealed that levels of sexual isolation are higher between pairs of populations using different host-plant species than between pairs of populations using the same host plant. Conversely, mtDNA and nDNA genetic distances did not different between pairs using different versus the same host. Thus the magnitude of sexual isolation detected was positively correlated with inter-population differences in colour-pattern morph frequencies but was uncorrelated with mtDNA or nDNA genetic distances. Collectively, these data demonstrate that divergent selection for host adaptation, and not genetic drift, has promoted the evolution of sexual isolation. Although mate choice itself is not directly based upon cryptic color-pattern, visual predation plays a role in the evolution of reproductive isolation by reducing gene flow between populations using different hosts[1,2], thus facilitating adaptive differentiation.

9.2 Introduction, Results, Discussion

Parallel evolution of similar traits in independent populations that inhabit ecologically-similar environments strongly implicates natural selection as the cause of evolution, as random genetic drift is unlikely to produce such a pattern[3]. Parallel speciation is a special form of parallel evolution where traits that determine reproductive isolation evolve repeatedly, in closely-related populations, as by-products of adaptation to ecological conditions[3,4]. The outcome of such parallel evolution is that ecologically-divergent pairs of populations exhibit greater levels of reproductive isolation than ecologically-similar pairs of populations of similar or younger age[4-6]. The parallel evolution of reproductive isolation provides some of the strongest evidence for natural selection in the process of speciation[3], yet only one conclusive example from nature is known[4]. In this study, we provide evidence that host-plant adaptation can promote the parallel evolution of reproductive isolation.

Timema walking-sticks are wingless, phytophagous insects that inhabit the chaparral of California, other areas of the western United States, and northern Mexico. Species are mono- or polymorphic for colour, presence of a dorsal stripe, or both, and these patterns match those of their host plants[1,2,7]. Phylogenetic studies suggest that speciation in this genus has involved specialization on different host plants[7]. *Timema* cristinae exhibits two genetically-determined[8] colour-pattern morphs, with an unstriped morph more common on *Ceanothus* spinosus and a striped morph more common on *Adenostoma fasciculatum* (mean frequency of unstriped morph on *Ceanothus* is 81%, range 33-100%; mean frequency of unstriped morph on *Adenostoma* is 28%, range 0-100%). Predation on *T. cristinae* by birds and lizards is strong and each morph is most cryptic on the host plant on which it is more common[1,2]. Thus populations of *T. cristinae* using different host-plant species are exposed to intense divergent selection for crypsis. Patches of these two host plant species are distributed in parapatric mosaics and local morph frequencies are determined by a gene flow – selection balance between host-plant patches exhibiting the different selective regimes[2].

Local adaptation to host-plant species in *T. cristinae* is also indicated by a number of other phenotypic traits that are more divergent between pairs of populations using

different host plants than between pairs using the same host; these include body size (PC1: males, Mantel test $t = -1.86$, $p < 0.05$; females, Mantel test $t = -1.49$, $p < 0.05$), body shape (CVA1 : males, Mantel test $t = -1.51$, $p < 0.05$; CVA2 : females, Mantel test $t = -1.69$, $p < 0.05$; first and second canonical variate axes from population-level discriminant analyses), host preference[8] and cryptic resting behavior (% resting where visible from above, the side and below respectively; from *Ceanothus* : 36%, 33%, 31%; from *Adenostoma* : 16%, 70%, 15%; chi-square = 62.34, $p < 0.001$, $n = 537$). Such host-specific differentiation suggests that host-plant adaptation involves divergence in a suite of complex morphological and behavioural traits. However, physiological trade-offs in host-plant use have not been detected, with females of both morphs exhibiting higher fecundity on *Ceanothus*[8].

Phylogenetic analyses indicate that different populations using the same host plant do not form monophyletic groups (Shimodaira-Hasegawa tests[9]: on *Adenostoma*: difference in ln likelihood = 77.37 for mtDNA (COI), 22.57 for nDNA (ITS-2), both $p < 0.001$; on *Ceanothus*: difference in ln likelihood = 75.49, $p < 0.001$ for mtDNA, difference in ln likelihood = 6.45, $p = 0.08$ for nDNA; Templeton tests[10]: $p < 0.05$ for all four analyses). This pattern is consistent with the hypothesis that the use of different host-plant species in *T. cristinae* has evolved multiple times, allowing a replicated test of whether sexual isolation has evolved in parallel with ecological divergence in host-plant use (see below for alternative explanations).

No-choice mating trials were conducted between all possible combinations of eight allopatric populations, yielding a total of twenty-eight pairwise comparisons of sexual isolation. The results revealed strong evidence of host-associated sexual isolation: across all populations, walking-sticks were more likely to copulate if paired with an opposite-sex member from the same host-plant species than if paired with an opposite-sex member from the different host-plant species (Fig. 9.1; male host by female host interaction, LR = 23.98, df =1, $p < 0.001$, logistic regression, $n = 1024$ mating trials). Consistent with the main prediction of the parallel speciation hypothesis, the magnitude of sexual isolation detected between pairs of populations using different host plants (I_{PSI} isolation index = 0.24, SD = 0.14, $n = 15$) was significantly greater than the magnitude of

isolation detected between pairs of populations using the same host plant (I_{PSI} = 0.08, SD = 0.10, n = 13)(Mantel test t = 2.24; $p < 0.05$; Table 9.1).

The degree of sexual isolation observed depended strongly on which pairs of populations were compared (LR = 13.61, df = 4, $p < 0.01$, logistic regression). We tested three hypotheses concerning the causes of this variation. First, interpopulation differences in colour-pattern morph frequency (% difference in frequency of unstriped morph) were positively correlated with the magnitudes of sexual isolation observed between pairs of populations (r = 0.38, $p < 0.05$, Mantel test). This result strongly suggests that the net strength of divergent selection for host adaptation to which populations are exposed directly influences the actual magnitude of sexual isolation that evolves. Stronger divergent selection will translate into local adaptation and the evolution of higher levels of sexual isolation. Conversely, gene flow between patches will tend to erode local adaptation[2] and progress towards speciation.

Second, the geographic distance between populations was positively correlated with the degree of sexual isolation detected (r = 0.37, $p < 0.05$, Mantel test), which suggests that geographic isolation facilitates the evolution of sexual isolation. Third, the degree of sexual isolation observed between populations was not correlated with genetic distances between pairs of populations (mtDNA: r = 0.13, p = 0.29; nDNA: r = 0.29, p = 0.10; Mantel tests) and pairs of populations using different host plants were not more genetically divergent from one another (mean genetic distance, mtDNA : 2.80% , SD = 0.81; nDNA : 0.88%, SD = 0.83) than pairs of populations using the same host plant (mean genetic distance, mtDNA : 3.07%, SD = 0.79; nDNA : 1.10%, SD = 0.83; Mantel test t = 0.47, 0.76, respectively, both $p > 0.15$). Given that a molecular clock could not be rejected for the best ML tree inferred from the mtDNA data set, variability in the length of time available for genetic drift to differentiate same-host versus different-host populations of *T. cristinae* is therefore unlikely to account for the increased sexual isolation detected between ecologically-divergent populations.

Our work indicates that divergent natural selection and subsequent host-adaptation, rather than genetic drift, has promoted the parallel evolution of sexual isolation. The non-monphyletic patterns observed in mtDNA and nDNA could also result

from a single origin and incomplete lineage sorting (retention of ancestral polymorphism) or a single origin followed by gene flow and introgression between host forms in multiple contact zones. Such processes are likely to contribute (given the polyphyletic relationships evident in the gene trees), but are unlikely to completely contribute to our results. Strong levels of sequence divergence were observed between populations (1-4% in mtDNA and 1-2% in nDNA), indicating there has been time for lineage sorting. Some gene flow could occur between adjacent populations, but is also unlikely to completely account for our results because all the populations studied are geographically separated (i.e. allopatric) from one another. Independent of these arguments, we stress that percent sequence divergence between populations in the neutral markers represents a measure of the opportunity for divergence via genetic drift (i.e. gene flow reduces sequence divergence, but also counters the effects of drift). Levels of sexual isolation are uncorrelated with sequence divergence, and strongly related to divergence in host-plant use. The most likely scenario is one where divergent host-plant adaptation has promoted the evolution of sexual isolation (above and beyond levels expected by genetic drift alone).

Although mate choice was not based directly upon cryptic colour-pattern (see legend for Fig. 9.1), visual predation plays a role in promoting sexual isolation among populations of *T. cristinae* by limiting gene flow between populations using different hosts. Two processes contribute to this reduction of gene flow. Firstly, between-host migrants will be locally less cryptic and will exhibit reduced survivorship due to predation[1,2]. Secondly, even if locally less cryptic migrants secure mates, they will produce a higher proportion of locally less cryptic offspring because color morph is genetically determined[8]. Given that host adaptation promotes sexual isolation and that gene flow erodes local adaptation[2], differential predation accelerates the evolution of reproductive isolation by reducing the homogenizing effects of gene flow and facilitating adaptive differentiation via natural selection.

9.3 Methods

T. cristinae were collected from eight study sites in the Santa Ynez Mountains of California between February and April 2001. Individuals from different populations and

the sexes were kept separate. Animals were fed foliage of the host on which they were collected and all the test animals used in mating experiments were juveniles that were reared to maturity in the lab.

9.3.1 Population Differentiation in Morphology and Cryptic Resting Behavior

We conducted seven linear measurements on 550 walking sticks (n = 259 males, 291 females). Each trait was measured twice and measurement error was low for all traits (all repeatabilities > 0.90, $p < 0.001$, ANOVA). Mantel tests[11] were used to analyze associations between interpopulation distance matrices. The first principal component (PC1) from a PC analysis exhibited high and positive loadings for all traits in both sexes.

Individual walking-sticks collected from both hosts (n = 643) were placed in the bottom of 500ml plastic cups with one 12cm host cutting from each host-plant species. These assays were initiated in the evening and in the morning we recorded the position from which individuals were visible (for individuals choosing *Ceanothus* only as the thin leaves of *Adenostoma* precluded measurement of this variable).

9.3.2 No-choice Mating Experiment

One male and one female were placed in a 10cm petri dish. At the end of one hour we scored whether the male and female were separate or copulating. Walking-sticks from eight populations were used, yielding 28 pairwise comparisons (13 same-host comparisons, 15 different-host comparisons). Sixteen sets of 16 individuals (one member of each sex from each population) were subjected to mating trials (n = 64 trials per pair-wise comparison). Each individual was tested only once with an opposite-sex member from each of the eight populations, alternating the test order.

9.3.3 Statistical Analysis of Mating Data

Copulation frequencies were analyzed using logistic regression in a model that examined the dependence of copulation on male host, female host and an interaction term, with sexual isolation indicated by a significant interaction and significance assessed using a likelihood ratio test (LR). To test whether colour-pattern contributed to sexual isolation, we used logistic regression to assess whether morph pair-type (male and female

the same or different colour-pattern morphs) in between-population crosses influenced the probability of copulation (using LR tests). To determine whether the degree of sexual isolation observed was dependent on which specific pair of populations was tested, we added male population and female population as terms in the previous logistic regression model and assessed the significance of the four-way interaction between all factors. We also tested for sexual isolation between populations for each pairwise comparison separately.

We calculated an index that summarizes the effects of sexual isolation (I_{PSI})[12], and used 5000 resamplings to calculate the standard deviation and significance of this index.

9.3.4 DNA Sequencing

Individuals were collected from each of the populations used in the mating experiment (mtDNA, COI, n= 40, 5 individuals per population; nDNA, ITS2, n = 23, 2-4 individuals per population). For mtDNA, double-stranded PCR amplifications were performed with the primers S2183-A3014, S2195-A3014, S2183-A2887, and S2195-A2887[13], which amplify most of the 3' half of the cytochrome oxidase I gene. For nDNA, PCR amplifications were performed with the primers AED5.8F : TGTGAACTGCAGGACACATGAAC and 28SBLD : TTCTTTTCCTCC (C/G)CTTA(C/T)T(A/G)ATATGCTTAA. Sequences are in Genbank under accession numbers AF439805-AF439820 (mtDNA) and AF459648-AF459653 (nDNA).

9.3.5 Phylogenetic Analyses

Maximum likelihood trees were estimated using the HKY + G model of DNA substitution for mtDNA (COI) analyses and the F81+G model for nDNA (ITS-2) analyses, as these models gave the optimal fit to the data[14]. *T. cristinae* from a different mountain (Ojala) was used as an outgroup for mtDNA analyses while a closely related species (*T. monikensis*) was used as an outgroup for nDNA analyses.

For mtDNA, analyses performed on unique haplotypes in PAUP 4.0b4b[15] revealed that a clock-constrained ML tree (-ln likelihood = 915.07) did not differ significantly from a clock-unconstrained tree with the same topology (-ln likelihood =

908.44; chi-squared =13.26, d. f. = 14, $p > 0.50$)[16]. For nDNA, a molecular clock was rejected (unconstrained tree, -ln likelihood = 688.21; clock-constrained tree, -ln likelihood = 698.51; chi-squared = 20.60, df = 6, $p < 0.05$).

The ML mtDNA tree, showing bootstrap values from 200 replicates, was: (outgroup, (((P, *L*, *H-HV*-P) : 82 , *L*) : 80 , (P-VPC-*VPA-OUTA*-PR, (((*H* , *H*), (PR, PR-*VPA-OUTA* , VPC , VPC) : 88) : 64 ,(*HV* , *H*) : 66) : 90) , (*L* , P) : 94) ; The ML nDNA tree, showing bootstrap values from 200 replicates, was: (outgroup, P, (*HV-L*, (P-*HV-L*, *L*, (PR-VPC-*OUTA-VPA-H*, VPC) : 54) : 95) : 73); populations using *Adenostoma* as a host are italicized.

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Figure 9.1 Copulation frequencies were higher for ecologically-similar pairs of *T. cristinae* walking-sticks than for ecologically-divergent pairs, where divergence refers to differences in host-plant use ($p < 0.001$, logistic regression).

Numbers of mating trials for each pair type are shown above each bar. In between-population mating trials, morph pair-type did not influence the probability of copulation (copulation frequencies when males and females were the same morph and different morphs : different-host crosses, 35%, n = 219 and 34%, n = 261 respectively, –2log likelihood = 0.18, df = 1, p = 0.67; same-host crosses, 44%, n = 218 and 51%, n = 198 respectively, –2log likelihood = 2.01, df = 1, p = 0.17). This result suggests that colour-pattern is not used during mate choice in between-population tests and that host-plant adaptation, rather than selection for crypsis, has directly promoted the evolution of sexual isolation between populations.

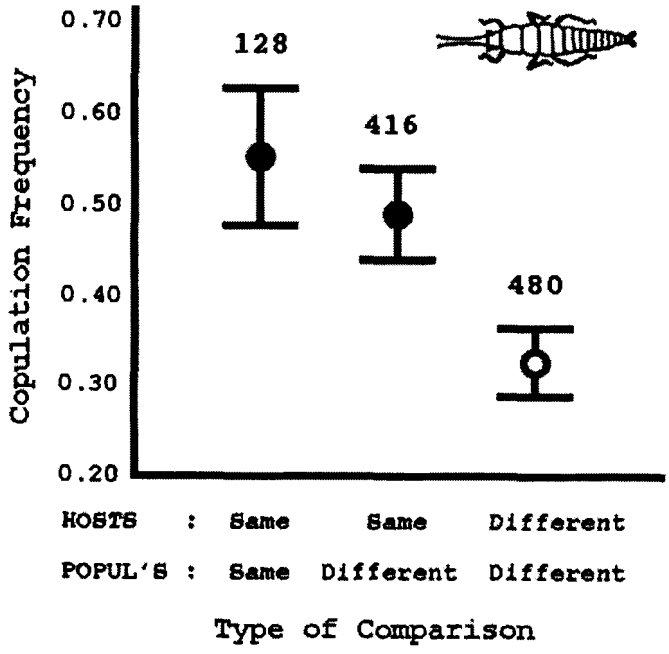

Table 9.1 Sexual isolation indices (I_{PSI} (s.d.)) from no-choice mating trials between host-associated populations of *T. cristinae* (n = 64 mating trials for each pairwise comparison).
Populations using *Adenostoma* are italicized.

Different-host comparisons	IPSI (s.d.)	LR	Same-host comparisons	I_{PSI} (s.d.)	LR
VPC x *HV*	0.52 (0.19)*	9.99**	PR x P	0.08 (0.18)	0.60
VPC x *OUTA*	0.01 (0.21)	0.01	PR x VPC	0.00 (0.20)	0.06
VPC x *L*	0.20 (0.21)	2.27	P x VPC	0.01 (0.20)	0.07
VPC x *H*	0.11 (0.24)	0.17	*VPA* x *OUTA*	0.07 (0.19)	0.25
VPC x *VPA*	0.20 (0.21)	1.60	*H* x *VPA*	-0.02 (0.20)	0.00
PR x *VPA*	0.12 (0.20)	1.01	*H* x *HV*	0.06 (0.19)	0.64
PR x *L*	0.39 (0.18)*	7.85**	*H* x *OUTA*	-0.11 (0.20)	0.57
PR x *H*	0.11 (0.21)	0.31	*HV* x *VPA*	0.28 (0.19)	7.00**
PR x *OUTA*	0.36 (0.20)	4.43*	*HV* x *OUTA*	0.11 (0.18)	0.48
PR x *HV*	0.20 (0.17)	3.23	*L* x *VPA*	0.13 (0.17)	1.01
P x *H*	0.19 (0.20)	2.27	*L* x *H*	0.08 (0.19)	0.58
P x *OUTA*	0.16 (0.19)	1.61	*L* x *OUTA*	0.10 (0.20)	1.05
P x *L*	0.39 (0.17)*	9.22**	*L* x *HV*	0.20 (0.17)	2.01
P x *HV*	0.33 (0.16)*	7.85**			
P x *VPA*	0.41 (0.18)*	8.09**			

*$p < 0.05$, **$p < 0.01$

I_{PSI} is an overall sexual isolation index for each pairwise comparison (range –1 to +1, null = 0, +1 = complete sexual isolation). LR values are from likelihood-ratio tests of the male population x female population interaction term in a logistic regression model, where a significant interaction term indicates sexual isolation. Using both analyses, stronger sexual isolation was detected for different-host comparisons than for same host-comparisons (see also Fig. 9.1).

CHAPTER 10.
REPRODUCTIVE ISOLATION DRIVEN BY THE COMBINED EFFECTS OF ECOLOGICAL ADAPTATION AND REINFORCEMENT*

* A version on this chapter appears as Nosil, P., Crespi, B.J., and Sandoval, C.P. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proceedings of the Royal Society of London B 270: 1911-1918. Reprinted with permission from the Royal Society of London.

10.1 Abstract

Recent years have seen a resurgence of interest in the process of speciation but few studies have elucidated the mechanisms either driving or constraining the evolution of reproductive isolation. In theory, the effects of reinforcing selection for increased mating discrimination where interbreeding produces hybrid offspring with low fitness, and the effects of adaptation to different environments, can both promote speciation. Conversely, high levels of homogenizing gene flow can counteract the forces of selection. We demonstrate the opposing effects of reinforcing selection and gene flow in *Timema cristinae* walking-sticks. The magnitude of female mating discrimination against males from other populations is greatest when migration rates between populations adapted to alternate host plants are high enough to allow the evolution of reinforcement, but low enough to prevent gene flow from eroding adaptive divergence in mate choice. Moreover, reproductive isolation is strongest under the combined, additive effects of reinforcement and adaptation to alternate host plants. Our findings demonstrate the joint effects of reinforcement, ecological adaptation, and gene flow on progress towards speciation in the wild.

10.2 Introduction

Speciation via natural selection can occur as an indirect by-product of adaptive divergence (Mayr 1963; Jiggins et al. 2001) and can also involve selection for reproductive isolation in geographical regions where hybridization is maladaptive (i.e. reinforcement) (Dobzhansky 1957; Butlin 1995; Howard 1993; Noor 1999). When speciation occurs as a by-product of adaptive divergence, ecologically-divergent populations exhibit greater reproductive isolation than ecologically-similar populations of similar age (Funk 1998; Rundle et al. 2000; Schluter 2000; Funk et al. 2002 for review). The key prediction of the reinforcement hypothesis is that non-allopatric (geographically contiguous or overlapping) populations will exhibit greater mating discrimination than allopatric (geographically separated) populations. Previous empirical studies of reinforcement have provided evidence for such a pattern (Noor 1995; Saetre *et al.* 1997; Rundle & Schluter 1998; Higgie *et al.* 2000) but there are few data on how ecological adaptation and reinforcement interact during the speciation process (Schluter 2000). Furthermore, although theoretical models predict that the outcome of reinforcement reflects a balance between the strength of reinforcing selection and the ability for homogenizing gene flow between populations to counteract selection (Sanderson 1989; Servedio & Kirkpatrick 1997; Cain *et al.* 1999; Servedio 2000), this prediction has never been tested using natural populations.

In this paper, we use *Timema* walking-sticks to analyze the joint roles of ecological adaptation, reinforcement, and gene flow in the evolution of reproductive isolation. *Timema* are wingless, phytophagous insects distributed throughout western North America (Crespi & Sandoval 2000). *Timema cristinae* exhibits two genetically-determined colour-pattern morphs (Sandoval 1993), with an unstriped morph more common on the host plant *Ceanothus spinosus* and a striped morph more common on *Adenostoma fasciculatum* (Sandoval 1994a). Predation on *T. cristinae* by birds and lizards is strong and each morph is most cryptic on the plant on which it is more common (Sandoval 1994a,b). Populations using different host plants have also diverged in a suite of other morphological and behavioral traits, including body size and shape, host preference, and cryptic resting behavior (Nosil *et al.* 2002; Nosil & Crespi 2004).

Levels of sexual isolation are higher between pairs of *T. cristinae* populations using different host plants (n = 15 pairs) than between similar-aged pairs using the same host (n = 13 pairs; Nosil *et al.* 2002). Because insect colour-morph (striped versus unstriped) does not influence between-population mate choice, the sexual isolation that has evolved between populations adapted to alternate hosts is independent of colour-pattern (although colour-pattern might influence within-population mate choice; Nosil *et al.* 2002). In this study, we expand previous work by considering the effects of reinforcing selection and gene flow on between-population mating preferences.

T. cristinae exhibits all of the preconditions required for reinforcement to contribute to the reproductive isolation observed between populations. First, interbreeding and gene flow between adjacent populations using different host plants (i.e. 'hybridization' between the host-adapted forms) is strongly implicated by the observations that individuals from adjacent populations on different hosts are well within one per-generation dispersal distance of each other (Sandoval 1993, 1994a). Moreover, geographic variation in morphology is indicative of a balance between host-specific selection and gene flow between adjacent patches (Sandoval 1994a; Nosil & Crespi 2004) and the populations are conspecific. In this study, we use mtDNA sequence variation to provide further evidence of interbreeding and gene flow between adjacent populations of *T. cristinae.*

Second, the evolution of reinforcement traditionally requires reduced hybrid fitness. In *T. cristinae,* between-host migrants are more likely to be the locally non-matching colour-morph than are residents (Sandoval 1994a) and the non-matching morph is at a large selective disadvantage due to differential predation (relative fitness of the less-cryptic morph is 0.30; Sandoval 1994a,b). Thus offspring derived from between-host mating (i.e. 'hybridization') will tend to exhibit reduced fitness relative to offspring derived from within-population mating because females who mate with locally less-cryptic males produce a higher frequency of offspring that are the locally non-matching colour-morph or intermediate in colour pattern (i.e. bear a faint stripe) than do females that mate with cryptic males (e.g. experimental genetic crosses show that only 5% of within-morph crosses produced F1 offspring of the alternate morph or intermediates whereas 80% of between-morph crosses produced both morphs or intermediates;

Sandoval 1993). Although previous work demonstrates that females producing offspring of the non-matching colour-pattern morph will be at a selective disadvantage (Sandoval 1994a,b), the cost of producing offspring that are intermediate in colour-pattern is less well known. In the current study, we test whether selection against the intermediate colour-pattern morph also imposes reinforcing selection, predicting that if the intermediate morph exhibits low fitness then its frequency will decline through time (i.e. between sequential time periods and age classes; Endler 1986). Because populations using different hosts have also diverged in a number of traits other than colour-pattern (Nosil *et al.* 2002; Nosil & Crespi 2004), selection against 'hybrids' may extend beyond that based upon colour-pattern alone.

Finally, we note that recent theoretical work indicates that direct selection against hybrid mating can promote reinforcement in the 'broad sense', even without reduced hybrid fitness (Servedio 2001). In *T. cristinae*, males ride on the back of the female during the mating period and thus females that mate with locally less-cryptic males are likely to suffer reduced individual survival during mating, favoring mating discrimination against migrants from the alternate host.

Given that the preconditions for reinforcement are met, we tested for its presence by assessing levels of female mating discrimination against foreign males (males that were collected from a different population from the females) for walking-stick insects collected from eight parapatric populations (reinforcement possible) and from four allopatric populations (no opportunity for reinforcement). Mating trials were conducted for all 66 possible pairwise combinations between the 12 study populations) (Fig. 10.1). Most patches of these two host plant species are distributed in parapatric patches of varying size, forming a mosaic at the scale of a mountain slope. However, some host patches are geographically separated from all other host patches by regions lacking suitable hosts. We define a 'population' of walking-sticks as all the insects collected within a homogenous patch of a single host plant. 'Parapatric' insect populations are in contact with a population of insects adapted to the alternative host, while 'allopatric' populations are separated from all other populations adapted to the alternative host by distances > 50 times the 12m per-generation gene flow distance (Sandoval 1993).

Reinforcement would be supported by higher between-population copulation frequencies in mating trials involving allopatric females than in trials involving parapatric females.

Reinforcing selection is predicted to be frequency-dependent, with increasing rarity of a population (relative to the population with which it co-occurs) raising the probability of mating with the wrong population and thus the opportunity for reinforcing selection (Howard 1993; Noor 1995). However, increased opportunity for between-population matings also increases the potential for high levels of gene flow between populations, which can retard or prevent reinforcement. Thus, the actual magnitude of female mating discrimination that evolves is expected to reflect a balance between the opposing forces of reinforcing selection and gene flow, with the effects of reinforcement being greatest when population sizes are similar and migration rates intermediate (Sanderson 1989; Servedio & Kirkpatrick 1997; Cain *et al.* 1999; Servedio 2000; Kirkpatrick 2000; Servedio and Noor 2003 for review). Because we quantified female mating discrimination in multiple populations of differing size, we were able to test these key predictions of the reinforcement hypothesis.

Collectively, we provide an assessment of the joints effects of host adaptation, reinforcing selection and gene flow on the evolution of reproductive isolation, predicting that 1) reinforcing selection strengthens premating isolation, 2) that high levels of gene flow counteract the effects of reinforcing selection, and 3) that the strongest reproductive isolation is found under the combined influence of divergent host plant adaptation and reinforcement.

10.3 Materials And Methods

10.3.1 No-choice mating experiment

T. cristinae were collected from multiple study sites in the Santa Ynez Mountains, California in February 2001 and 2002 using sweep nets. Study sites were chosen such that a wide range of geographic arrangements of populations was represented. Other species of *Timema* do not occur in sympatry with *T. cristinae*. Animals were captured in the first instar and reared to maturity in the lab on the foliage of either their native or the alternative host.

Protocols for the no-choice mating trials used in this study have been previously published (Nosil *et al.* 2002) (n = 3320 trials; 1024 of these from Nosil *et al.* 2002; median number of mating trials per pairwise comparison = 275, range = 75 – 497). One male and one female were placed in a 10cm petri dish and at the end of one hour we scored whether the male and female were paired (male on female without genital contact) or not, and copulating or not. Individuals were selected randomly from each population, such that mating trials were conducted using natural colour-pattern morph frequencies.

The probability of copulation at the end of an hour was analyzed using logistic regression, assessing significance using likelihood ratio tests (LR). We tested for reinforcement in a model that examined the dependence of copulation with males from the twelve study populations on male host, female host, male population, female population, allopatry (female from an allopatric or parapatric population), and all possible interaction terms. Host specific sexual isolation is indicated by a significant interaction between male host and female host. Reinforcement is indicated by a significant allopatry term. We do not report the significance of the other terms in the model, as they are peripheral to the topic of the study. All results are from a reduced regression model derived using backward elimination (initial model included all factors and interactions but then removed all terms for which the significance of –2 log LR was > 0.10). Including rearing environment (insect raised on its native or the alternative host) in the logistic regression models yielded no significant interactions (all main effects and interactions, $p > 0.25$), indicating that differences in mate preference likely have a genetic basis. Analogous analyses conducted using males (male from parapatric or allopatric populations) did not yield evidence of reinforcement (all main effects and interactions with allopatry, $p > 0.25$).

The analyses described above are powerful as they analyze large samples (i.e. individuals pooled among populations of the same host and geographic type), but they treat individuals, rather than populations, as the unit of replication. It could be argued that populations are a more relevant unit of replication in tests of reinforcement (note though, that most studies consider only one or a few parapatric populations, Servedio & Noor 2003). Thus we tested whether between-population copulation frequencies were consistently lower for females from parapatric populations than the average from the four

allopatric populations using a Wilcoxon's signed ranks test, treating parapatric population as the unit of replication (i.e. n = 8).

10.3.2 Population rarity and the opportunity for reinforcement

In *T. cristinae*, the area of the host plant patch of the study population relative to its parapatric neighbour serves as a measure of its rarity : the larger the neighbouring population and the smaller the study population, the 'rarer' the individuals of the study population become relative to individuals of the neighbouring population.

The rarity of each study population was calculated as [size of neighbouring patch / (size of study patch + size of neighbouring patch)]. The area of each population and its neighbor (in the case of parapatric patches) was calculated using aerial photographs. Patch size has been shown to be strongly and positively correlated with population size (r^2 = 0.63 and 0.53 for *Ceanothus* and *Adenostoma* patches respectively, n = 13 patches of each host; Sandoval 1994a). The strength of female mating discrimination against foreign males was calculated for each of the twelve study populations as the absolute value of mean copulation frequency of females with foreign males subtracted from mean copulation frequency with males from their own population.

10.3.3 DNA sequencing and estimates of gene flow

A total of 107 mtDNA (COI) sequences, 467 base-pairs in length, were collected from the twelve study populations as well as from each of the populations that neighbor the parapatric study populations (mean number of individuals per population = 6.3, range = 3-11; protocols and 40 sequences from Nosil *et al.* 2002). Haplotypes from the 67 sequences acquired in this study are in Genbank.

We then estimated levels of gene flow between adjacent patches in order to test whether gene flow into a parapatric population, from its neighbouring population of the alternate host, increases with increasing relative size of the neighbouring population. Gene flow was estimated using the coalescent-based methods of Beerli & Felsenstein (2001). First, we used default settings in the program MIGRATE to obtain estimates of the number of migrants per generation (N*m*) into each of the parapatric study populations,

from their neighbouring population of the alternate host. We then estimated migration rates using two, independent approaches. First, we estimated *m* (the proportion of the population represented by migrants, migration rate) from N*m* by calculating total population size (using previously published regression equations for patch size versus population size; Sandoval 1994a), dividing this number by 0.5 to obtain female population size (mtDNA is maternally inherited) and then multiplying by 0.10 to obtain effective populations sizes (Frankham 1995; changing this final scaling value alters only the absolute estimates of *m*, whereas results of our analyses, and their interpretation, depend only on variation in relative migration rates). Second, we also report the migration parameter M (M = *m* / mutation rate), obtained from MIGRATE. We note that our analyses of the relationship between gene flow and neighbouring population size depend on relative migration rates and thus estimating *m* from M using different mutation rates will not affect our results.

10.3.4 Selection against the intermediate pattern morph

The intermediate morph is rare in most populations of *T. cristinae* (<2%). However, one site (Refugio) has relatively high frequencies of the intermediate morph, providing an opportunity to test for selection against the intermediate morph within a natural population. This site contained both host plants, each of which was sampled during March and April in 1996 and 1997. Captured individuals were scored for colour-pattern (unstriped, striped, intermediate) and age class (juvenile, adult – we found juveniles only during March 1996) and released back where captured. All specimens were scored by one individual (C.P. Sandoval).

We first assessed whether the frequency of the intermediate morph was dependent on age class by testing for an association between morph (intermediate or other) and age class (using the March 1996 sample) in a three-way loglinear contingency analysis that included morph, age class and host as factors. Second, we tested whether the frequency of the intermediate morph within adults was dependent upon sample month using a four-way loglinear analysis that also included sample year as a factor. For both analyses, we examined the effects of the interactions in question independent from the effects of other

factors by using partial chi-square values and by assessing the significance of higher-order interactions (Norusis 1993).

10.4 Results

10.4.1 Gene flow and hybridization in the wild

Adjacent pairs of populations using different host plants are weakly or not differentiated at mtDNA (mean F_{st} = 0.07, range = 0.00 to 0.25, n = 7 pairs) while geographically-separated populations are strongly differentiated (mean F_{st} = 0.31, range = 0.00 to 0.79, n = 129 pairs; Mantel's t = 2.33, $p < 0.01$). These data suggest that substantial gene flow between neighboring populations occurs in the wild (see also coalescent-based estimates of gene flow below). Thus, although incomplete lineage sorting (between neighboring populations only) could produce similar patterns, the requirement for reinforcement of recent hybridization appears to be fulfilled.

10.4.2 Selection against intermediate colour-pattern morphs

The frequency of the intermediate morph was higher in juveniles than in adults sampled during the same time period (partial chi = 53.40, d.f. = 1, $p < 0.001$; higher-order interaction, $p > 0.10$; Fig. 10.2). In addition, the frequency of the intermediate morph within the adult age class decreased between March and April. This reduction occurred in both sample years, but overall results were marginally non-significant (partial chi = 2.96, d.f. = 1, p = 0.08; all higher-order interactions, $p > 0.10$). These results demonstrate selection against the intermediate morph during the juvenile stages, and possible continued selection during adult life. Ongoing interbreeding between the parental forms (i.e. striped and unstriped morphs) could potentially account for the persistence of the intermediate morph at the study site, despite selection against it.

10.4.3 Reinforcement of premating isolation

Female mating discrimination against foreign males is significantly stronger when females are from populations where the two host-adapted forms hybridize than when females are from geographically isolated populations (Fig. 10.3; mean between-

population copulation frequencies in mating trials involving females from parapatric populations = 28%, s.d. = 0.45; in trials using females from allopatric populations = 35%, s.d. = 0.47; allopatry term, LR = 11.93, d.f. = 1, $p < 0.001$). Notably, copulation frequencies of females with males from their own population were similar for females from parapatric and allopatric populations (allopatry term in a model including within-population mating trials only; LR = 1.40, d.f. = 1, $p = 0.24$), indicating that reinforcement has strengthened female mating discrimination against foreign males without reducing mating frequencies with local males. Moreover, between-population copulation frequencies were lower for females from parapatric populations than the average from the four allopatric populations in seven out of eight population comparisons (Wilcoxon's signed ranks test (WSR), $Z = 2.24$, $p < 0.05$). These data provide strong evidence for reinforcement of pre-mating isolation.

10.4.4 Population size, gene flow and reinforcement

Coalescent-based analyses indicate that the opportunity for gene flow to erode the effects of reinforcing selection increases with increasing population rarity: levels of gene flow into the parapatric study populations, from their neighboring populations, were highly variable (range of number of migrants (Nm) = 0.68 – 14.7, mean = 3.13; range of migration rates estimated using population sizes (m) = 0.001 – 0.232, mean = 0.043; range of migration parameter (M) = 22.70 – 300.43, mean = 177.83) and increased with increasing population rarity ($r = 0.86, 0.62, 0.92$, $p < 0.01, 0.05, 0.01$ respectively; Spearman rank correlations). Consistent with the balancing effects of frequency-dependent reinforcing selection and gene flow, mating discrimination is low when the study population is allopatric or large relative to its neighbor, it increases rapidly until the sizes of the study and neighboring population are similar, and it then decreases when the study population is relatively rare (Fig. 10.4).

10.4.5 Combined effects of host-adaptation and reinforcement

Host plant adaptation and reinforcement contributed independently and additively to the evolution of reproductive isolation (Fig. 10.3). Thus, copulation frequency was reduced when the sexes were from populations using alternate hosts (for both allopatric

and parapatric females) and mating discrimination is highest when both adaptation and reinforcement occur.

10.4.6 Alternative hypotheses

Numerous processes have been presented which could account for increased mating discrimination in parapatric or sympatric populations relative to allopatric populations (Howard 1993; Butlin 1995; Noor 1999 for review). Each of these hypotheses can be viewed as an alternative to reinforcement. As described below, each of these alternatives hypotheses was unsupported.

First, when similar phenotypes from different populations compete most strongly for resources, frequency-dependent disruptive selection drives population divergence (Slatkin 1980). This phenomenon, called ecological character displacement, results in populations that are in geographic contact with one another to exhibit greater adaptive divergence than allopatric pairs of populations. As a consequence of this greater trait divergence, mating discrimination can be stronger between parapatric than allopatric pairs of populations. Previous work has shown that ecological character displacement in morphology does not occur in *T. cristinae*: divergence in morph frequencies, body size and body shape is greater between allopatric pairs of populations using different hosts than between parapatric pairs (Sandoval 1994a; Nosil & Crespi 2004). In this study, we tested for character displacement in resting behaviour and a correlate of physiology (survival to maturity in the lab) by assessing the effects of host (*Ceanothus* vs. *Adenostoma*), allopatry (allopatry vs. parapatry) and an interaction term on variability among individuals from the twelve study populations. Resting behaviour refers to whether the insects were found resting where visible from the side (versus above or below) in 1073 host preference trials (Nosil *et al.* 2002 for details). For these binary variables, we assessed the effects of host, allopatry and the interaction term using logistic regression.

The character displacement hypothesis is untenable because individuals from parapatric populations using different hosts are less behaviorally and physiologically divergent from one another than are individuals from allopatric populations using

different hosts (difference in mean trait values for individuals from parapatric versus allopatric populations; resting behavior, 8 and 36% respectively, likelihood ratio from interactions term LR = 28.03, $p < 0.001$; survival in lab, 4 and 5% respectively, LR = 0.25, $p > 0.10$).

Second, we evaluated whether population ancestry (i.e. time since divergence) might contribute to levels of reproductive isolation by testing whether values of an index of reproductive isolation (I_{PSI}, Rolan-Alvarez and Caballero 2000) were correlated with neutral mtDNA differentiation (% nucleotide divergence, range 0-5%) between the 66 pairs of study populations, or with differentiation at a nuclear locus among 8 of the 12 populations used in this study (n = 28 pairs of populations; data from Nosil *et al.* 2002).

The population ancestry hypothesis is unsupported because levels of reproductive isolation are uncorrelated with neutral differentiation (mtDNA, $r = -0.11$, $p > 0.50$, Mantel test; nuclear DNA, $r = 0.29$, $p = 0.10$, data from Nosil *et al.* 2002). Moreover, levels of gene diversity, defined as the probability that two randomly-chosen haplotypes are different in the sample, tend to be correlated with population age (Nei 1987) but do not differ between parapatric and allopatric populations (mean = 0.75, s.d. 0.14 versus 0.82, s.d. 0.05 respectively, $p > 0.25$, t-test).

Third, the biased extinction hypothesis predicts that non-allopatric pairs of populations tend to exhibit greater mating discrimination because non-allopatric populations without strong reproductive isolation fuse upon secondary contact, or one population goes extinct (Noor 1999 for review). We tested the key prediction of this hypothesis, that some allopatric populations will exhibit levels of mating discrimination similar to those observed in parapatric populations.

The biased extinction hypothesis is unsupported because all four allopatric populations used in this study exhibited lower discrimination than the average parapatric population ($Z = 1.83$, $p < 0.05$, one-tailed WSR). However, this hypothesis cannot be unequivocally rejected using this approach because only four allopatric populations were sampled (Noor 1999 for discussion). We do note that the biased extinction does not apply as readily to cases with gene flow among conspecific populations, where populations are

defined by the local geography of their host plant, and where populations are parapatric rather than sympatric (i.e. *T. cristinae*) (Noor 1995, 1999).

Fourth, we tested for male preference of allopatric females because such male preferences could result in higher copulation frequencies when males are paired with allopatric versus parapatric females. For a subset of the mating trials (n = 160), we recorded the position of the male every ten minutes, over a four-hour interval. For trials where pairing occurred, we assessed whether males paired more rapidly with females from three allopatric populations (n = 74) than with females from a parapatric population (n = 22). Male post-copulatory guarding behaviour towards allopatric versus parapatric females was examined by observing single male/female pairs for two weeks after the first copulation event and noting when the male first stopped guarding the female (pairs observed twice a day, n = 30, half of the trials conducted with females from allopatric populations, all pairs were from populations using different hosts).

The male preference hypothesis is unsupported because males do not pair more rapidly with allopatric versus parapatric females (mean time until first pairing, 55 and 35 minutes respectively, $Z = 0.67$, $p > 0.50$, Mann-Whitney U-test) and males do not preferentially guard allopatric versus parapatric females (mean time until males dismount = 3.38 and 3.88 days respectively, $Z = 0.35$, $p > 0.50$, Mann-Whitney U-test).

10.5 Discussion

We detected strong evidence for reinforcement in *T. cristinae*: the assumptions of the reinforcement hypothesis were met, and as predicted females from populations where the two host-adapted forms interbreed exhibit greater mating discrimination against foreign males than females from geographically isolated populations. Moreover, each of the alternative explanations for the increased mating discrimination of parapatric females was unsupported (Noor 1999). In contrast, two additional, key predictions of the reinforcement hypothesis were supported (Sanderson 1989; Servedio & Kirkpatrick 1997; Cain *et al.* 1999; Servedio 2000). Specifically, migration between divergent populations acted as both a homogenizing and a diversifying force, such that reinforcement was most likely when migration was high enough to facilitate

reinforcement but low enough to prevent gene flow from eroding adaptive divergence in mate choice. These are the first empirical data to demonstrate the role of relative population sizes and levels of gene flow in the evolution of reinforcement. We note that although maternal effects on mating tendencies have not been explicitly ruled out, such maternal effects are unlikely to produce patterns of mating discrimination that are consistent with a balance between selection and gene flow.

Reinforcement requires a cost to hybridization. In *T. cristinae*, the costs of between-host mating are twofold. First, colour-pattern in *T. cristinae* is genetically-determined (Sandoval 1993) and immigrant males from the alternate host-plant tend to exhibit the locally-less cryptic colour-pattern (Sandoval 1994a,b). Thus females that mate with males from the alternate host-plant tend to produce a higher frequency of less-cryptic offspring (i.e. the locally non-matching morph or offspring that are intermediate in colour-pattern) than do females that mate with resident males. Second, females pairing with males from the alternative host might themselves suffer increased predation rates, favouring increased female mating discrimination. Under both these scenarios, natural selection favours mating discrimination against foreign males (see Servedio 2001 for discussion). Although we are not able to completely disentangle these two costs of hybrid mating we note that direct benefits would likely be obtained at the level of male/female pairing, rather than willingness to copulate in a confined area. Mating trials were conducted in small petri dishes, allowing us to assess whether females discriminate against males once the opportunity for direct benefits is reduced (i.e. males can easily pair with the female but cannot force copulations; the frequency of pairing is 65%), rather than testing whether females discriminate against males prior to pairing (i.e. by actively fleeing).

Previous work has demonstrated that indirect effects of adaptation to alternative host plants also increase reproductive isolation between *T. cristinae* populations (Nosil *et al.* 2002). Our augmented data set affirms these findings, and demonstrates that host plant adaptation and reinforcement contribute independently and additively to the evolution of reproductive isolation (i.e. mating discrimination is highest when both processes occur). The indirect effects of such host plant adaptation may provide the initial degree of

divergence in mate preference that has been predicted to make reinforcement evolve more readily (Liou & Price 1994; Kelly & Noor 1996).

Consistent with the independence of the effects of reinforcement and host adaptation, reinforcing selection exerted a 'universal' effect on mating preferences. Thus, although females are selected to be more discriminating against males from an adjacent population, this selection has indirectly resulted in increased mating discrimination against foreign males from multiple other populations that use either host (trials involving females from parapatric populations exhibited lower copulation frequencies than those involving allopatric females for males from ten of the twelve populations, $Z = 1.83$, $p < 0.05$, one-tailed WSR; significant differences detected only in this direction and in six comparisons, all $p < 0.05$, LR). Such 'universal' effects of reinforcement may be due to females recognizing and preferring males from their own population based on a 'population-specific' trait (i.e. rather than a 'host-specific' trait) (Kelly & Noor 1996; Higgie *et al.* 2000). Similar findings have been reported in *Drosophila mojavensis*, where females from mainland populations discriminate against conspecific males from other regions and evidence is presented that this discrimination is a by-product of selection for sexual isolation between mainland *mojavensis* and its sympatric sibling species *D. arizonensis* (Zouros and D'Entremont 1980). If such 'universal' effects of reinforcement are common, then reinforcement will contribute to speciation between both ecologically similar and ecologically divergent pairs of populations, and between conspecific populations.

Our findings have broad implications for the study of speciation. First, our results indicate that the outcome of reinforcement depends on the spatial distribution of populations and on relative population sizes. These results concord with recent theoretical developments indicating that ecological interactions between populations are likely to drive speciation (Doebeli and Dieckmann 2003). Second, our results indicate that even within a single species, natural selection can favor the evolution of reproductive isolation in two distinct ways, via effects of ecological adaptation, and via selection for increased premating isolation. The greatest progress towards speciation occurs when both processes operate.

10.6 Acknowledgements

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Figure 10.1 Representative examples of the four 'types' of between population crosses, where C = *Ceanothus* population; A = *Adenostoma* population. Each square represent a homogenous patch of a single host plant and it may or may not have a neighbouring population using the alternative host plant (parapatric and allopatric populations respectively). 'Study' populations used in mating trials are unfilled boxes, and black boxes represent populations that are adjacent to a study population but were not used in mating trials. In the figure, males from allopatric *Ceanothus* populations are used as an illustrative example; in the mating trials, both directions of crosses were actually conducted, such that males from each of twelve study populations were used (i.e. males from allopatric and parapatric populations of each host). 1) Female parapatric, male from the same host. 2) Female allopatric, male from the same host. 3) Female parapatric, male from the alternative host. 4) Female allopatric, male from the alternative host.

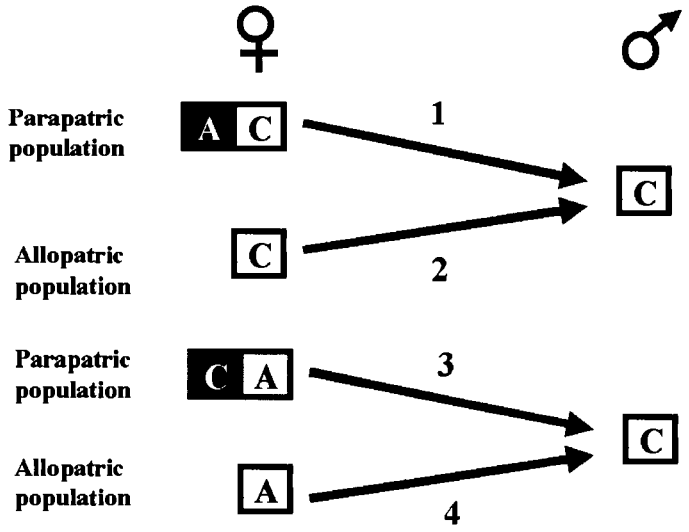


Figure 10.2 The frequency of the intermediate colour-pattern morph declined between age classes within a sample period and between successive sample months.

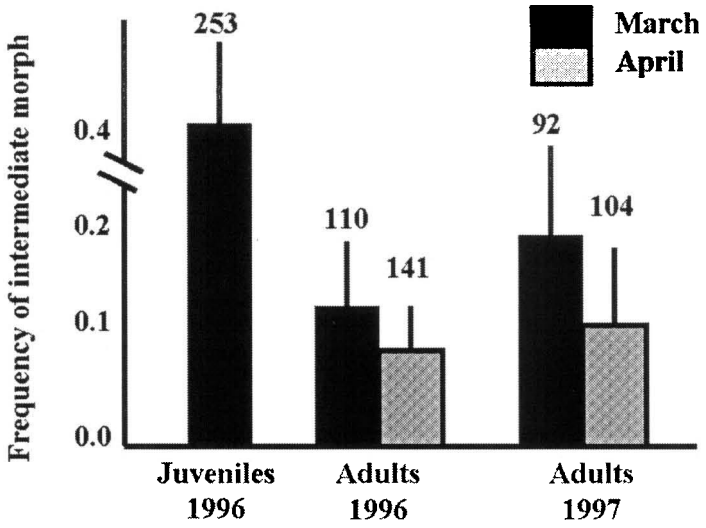


Figure 10.3 Copulation frequencies for male / female pairs of *T. cristinae* walking sticks.

Consistent with reinforcement, between-population copulation frequencies were higher for mating trials involving females from allopatric populations than for trials involving females from parapatric populations ($p < 0.001$). The increased mating discrimination of parapatric females was independent from host-specific sexual isolation (male host x female host, LR = 11.70, d.f. = 1, $p < 0.001$; all other interactions, $p > 0.15$). Thus reproductive isolation has evolved via both reinforcement and as a by-product of adaptation to different habitats. Numbers of mating trials for each comparison are shown above each 95% confidence interval. The numbers above the x-axis refer to the type of between-population mating trial outlined in Figure 10.1. All combinations of mating trials were conducted, such that some females from parapatric populations and from allopatric populations were tested with males from their own population, some with males from different populations using the same host, and some with males from different populations using the alternate host.

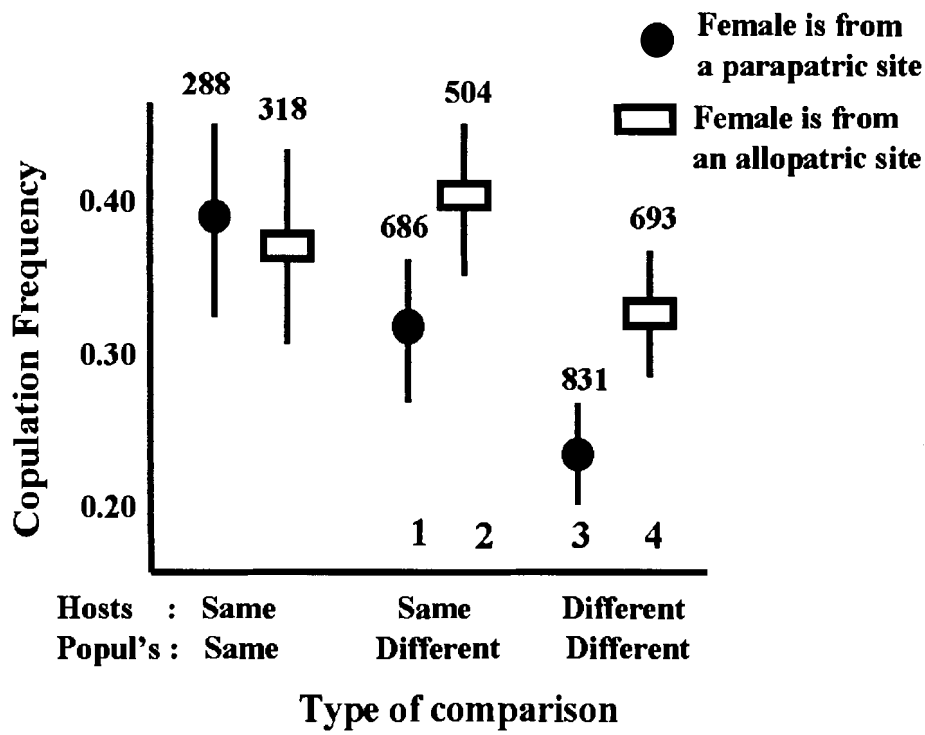
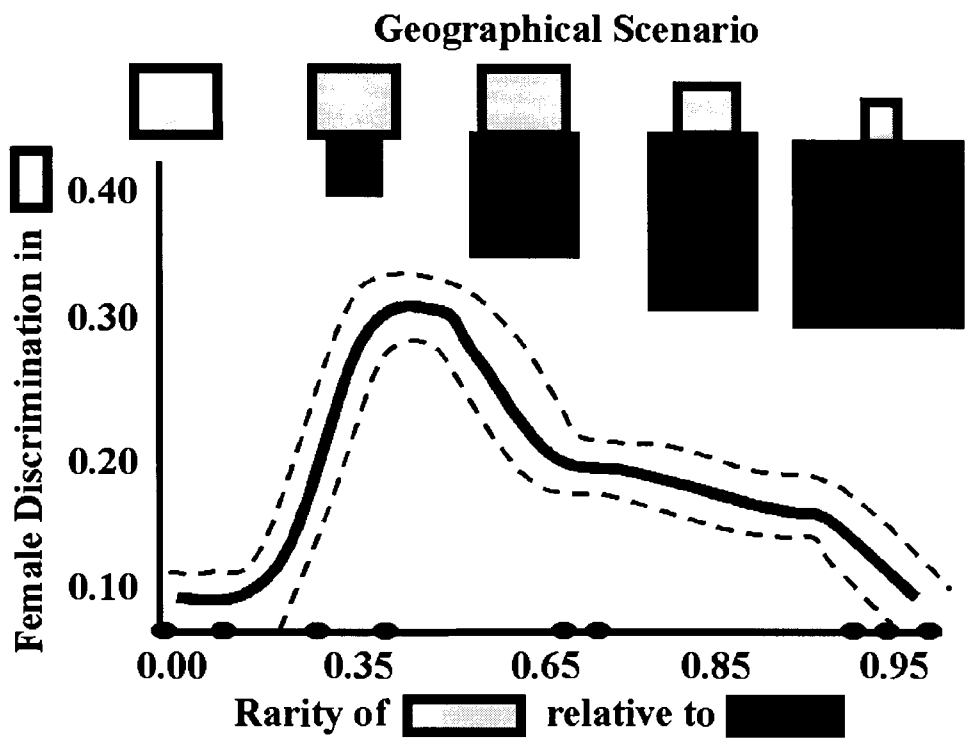


Figure 10.4 Female mating discrimination against males from other populations is strongest when the rarity of the study population is intermediate (males from the alternative and same host respectively; t = -2.37, -2.25, both p < 0.05, quadratic term in regression model including both linear and quadratic terms; r^2 change between a linear and quadratic model = 0.37, 0.33, both p < 0.05, partial F-test).
Shown here is the relationship between the rarity of a study population (relative to its neighbouring population of the alternative host; values for each study population denoted by black circles on the x-axis) and female mating discrimination against foreign males that use the alternative host (absolute value of mean copulation frequency with foreign males minus mean copulation frequency with resident males, for each of the 12 study populations). Boxes illustrate the different geographical scenarios, where the grey box denotes the study population and the black box denotes the neighbouring population. The curve was estimated using the non-parametric cubic spline (dashed lines show standard errors from 1000 bootstrap replicates) (Schluter 1988).



CHAPTER 11.
NATURAL SELECTION AND DIVERGENCE IN MATE PREFERENCE DURING SPECIATION*

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11.1 Abstract

Sexual isolation can evolve due to natural selection against hybrids (reinforcement). However, many different forms of hybrid dysfunction, and selective processes that do not involve hybrids, can contribute to the evolution of sexual isolation. Here we review how different selective processes affect the evolution of sexual isolation, describe approaches for distinguishing among them, and assess how they contribute to variation in sexual isolation among populations of *Timema cristinae* stick-insects. Pairs of allopatric populations of *T. cristinae* living on different host-plant species exhibit greater sexual isolation than those on the same host, indicating that some sexual isolation has evolved due to host adaptation. Sexual isolation is strongest in regions where populations on different hosts are in geographic contact, a pattern of reproductive character displacement that is indicative of reinforcement. Ecological costs to hybridization do occur but traits under ecological selection (predation) do not co-vary strongly with the probability of between-population mating such that selection on ecological traits is not predicted to produce a strong correlated evolutionary response in mate preference. Moreover, hybrid egg inviability is lacking and the factors contributing to reproductive character displacement require further study. Finally, we show that sexual isolation involves, at least in part, olfactory communication. Our results illustrate how understanding of the evolution of sexual isolation can be enhanced by isolating the roles of diverse ecological and evolutionary processes.

11.2 Introduction

Speciation involves the evolution of reproductive isolation between diverging populations. Understanding speciation thus requires determining which reproductive barriers initially reduced gene flow between populations and the evolutionary forces producing them (Mayr 1947, 1963; Coyne and Orr 2004). Barriers to gene exchange can occur before or after mating, and different forms of reproductive isolation are not necessarily independent. For example, selection against hybrids (postmating isolation) can drive the evolution of increased premating isolation (i.e. reinforcement; Dobzhansky 1937; Servedio and Noor 2003). The evolution of premating isolation caused by divergent mating signals and preferences (sexual isolation hereafter) appears to be an important component of speciation in many taxa (Coyne and Orr 2004). Many selective processes can affect the evolution of sexual isolation, but their relative contributions are poorly understood. In this paper, we describe selective processes which can promote the evolution of sexual isolation, present methods for distinguishing among them, and apply the methods to explain variation in sexual isolation among populations of walking-stick insects.

Reproductive isolation can evolve simply as a by-product of populations adapting to different ecological environments (Funk 1998; Schluter 2000; Podos 2001; Jiggins et al. 2001, 2004; Etges 2002; Rundle and Nosil 2005; Funk et al. 2006; Vines et al. 2006). Divergent natural selection acts on ecologically-important traits, resulting in population divergence in ecological traits. If these traits, or ones genetically-correlated with them, incidentally affect mate choice, then sexual isolation evolves as a by-product of local adaptation. This is the classic 'by-product' model of allopatric speciation (Muller 1942), but it applies to any geographical scenario. Notably, by-product speciation invokes selection on ecological traits, and the resulting selection on mating traits can be either direct or indirect (Rundle and Nosil 2005). If the loci affecting selected and mating traits are the same (i.e. due to pleiotropy; Kirkpatrick and Ryan 1991), then direct selection occurs. If the loci are physically different, then selection acts indirectly on mate preference alleles through their genetic association (i.e. linkage disequilibria) with alleles at other loci which are under selection (Barton and Turelli 1991; Kirkpatrick 1996;

Kirkpatrick and Barton 1997; Kirkpatrick and Servedio 1999; Servedio 2001, 2004). Such indirect selection acting through imperfect genetic associations is thought to be less effective at driving speciation than is direct selection (Kirkpatrick and Ravigne 2002).

Sexual isolation can also evolve due to direct selection on actual mate preferences, rather than selection on ecological traits per se (Servedio 2001). Selection on a preference is direct when the preference allele affects fitness independent of the genetic background in which it is found (Kirkpatrick and Ryan 1991; Kirkpatrick 1996; Kirkpatrick and Ravigne 2002). Simple examples are where preferences for detectable signals accrue high fitness or where preference results in greater parental investment from mating partners. If divergent environments differ in their signal transmission properties, direct and habitat-specific selection may be imposed on sensory systems and preferences (i.e. 'sensory drive' – Morton 1975; Ryan et al. 1990; Endler 1992; Boughman 2002; Slabbekoorn and Smith 2002; Patten et al. 2004; Seddon 2005; Fuller et al. 2005 for review). Preference evolution arising from such habitat-specific and direct selection on preferences can be thought of as a form of local adaptation. Thus both habitat-specific selection on preferences and the by-product model predict greater sexual isolation between ecologically-divergent pairs of populations than between ecologically-similar pairs of similar age.

Selection against hybrids can also result in the evolution of sexual isolation ('reinforcement' Dobzhansky 1937). During reinforcement, selection acts directly on genes causing low hybrid fitness, and mating preferences evolve via their genetic association with genes causing low hybrid fitness (i.e. via indirect selection). When populations or species hybridize, both selection against hybrids and the genetic association (disequilibria) between loci may be large, such that even indirect selection acting thru imperfect genetic associations is relatively strong (Kirkpatrick and Ravigne 2002). Initial work on reinforcement focused on intrinsic postmating isolation (hybrid inviability or sterility) resulting from between-locus genetic incompatibilities (Bateson 1909; Dobzhansky 1936, 1937; Orr and Turelli 2001; Servedio and Noor 2003; Gavrilets 2004). However, selection against hybrids can also occur without intrinsic postmating isolation. Hybrids may exhibit a poor ecological fit to the niches of parental species and such 'extrinsic hybrid inviability' can drive reinforcement (Fisher 1930; Hatfield and

Schluter 1999; Kirkpatrick 2001; Rundle and Whitlock 2001; Rundle 2002; Servedio 2004). Likewise, sexual selection against hybrids can contribute to reinforcement (Phelan and Baker 1987; Noor 1997; Vamosi and Schluter 1999; Naisbit et al. 2001). Finally, costs to hybridization that do not involve hybrids can affect preference evolution. For example, hybridization can reduce the survival or fertility of females themselves, favoring the evolution of sexual isolation (i.e. these are the postmating, prezygotic incompatibilities of Howard and Gregory 1993; Servedio 2001).

These considerations indicate that numerous selective processes can contribute to the evolution of sexual isolation, and the relative importance of different processes is a major outstanding question in speciation research. We do not exclude a role for processes that were not mentioned, such as genetic drift, sexual selection, or sexual conflict (Ryan and Rand 1993; Panhuis et al. 2001; Shaw and Parsons 2002; Gavrilets 2004). Rather, we focus on local adaptation and selection against hybridization because they 1) appear common in nature (Coyne and Orr 2004), 2) are the topic of recent yet generally untested theory, and 3) apply to our study system, *Timema cristinae* walking sticks (drift and environment-independent sexual selection have been examined, but evidence suggests they do not contribute strongly; Table 11.1). Speciation is often a continuous process, with different 'stages' corresponding to the magnitude of reproductive isolation that has evolved. Ecology and geography dictate which processes act, and genetics influences the response to selection (Fig. 11.1).

11.2.1 Distinguishing among selective processes involved in speciation

A first step towards distinguishing among processes is to compare sexual isolation among populations with varying degrees of ecological divergence (a proxy for local adaptation) and geographic potential for reinforcement (Funk et al. 2002). Further steps involve elucidating the costs to hybridization potentially driving reinforcement, quantifying the association between selected traits and mating preferences (which affects the strength of indirect selection transmitted to mate preferences), and determining the traits upon which sexual isolation is based. We describe these steps in more detail below, and apply them to populations of *Timema* walking-sticks. Some of our inferences are based upon indirect evidence but we nonetheless dissect the role of multiple selective

processes in the evolution of sexual isolation in the context of an explicit body of theory (Table 11.1 for summary).

11.2.2 Timema walking-stick study system

Timema walking-sticks are wingless, phytophagous insects inhabiting the chaparral of Southwestern North America (Crespi and Sandoval, 2000). Individuals feed and mate exclusively on the hosts upon which they rest. We focus on *T. cristinae,* a species feeding upon two different host-plant species (*Ceanothus spinosus* and *Adenostoma fasciculatum*). We define a 'population' of walking-sticks as all the individuals collected within a homogenous patch of a single host species (as in Nosil *et al.* 2002, 2003). Thus 'parapatric' insect populations are in contact with an adjacent population using the alternative host, whereas 'allopatric' populations are separated from all other populations adapted to the alternative host by distances > 50 times the 12m per-generation gene flow distance (Sandoval 1993). Sample sites with both hosts were chosen such that each population had only one adjacent population on the alternate host. For simplicity, we use the term 'hybridization' to refer to interbreeding between populations on different hosts, but do not imply that the host forms have achieved species status.

In this study we present much new data, but also re-analyze and re-evaluate some previously published data in order to synthesize the collective findings. The data novel to this paper include: 1) analyses of pheromones and behavioural responses to them, 2) genetic crosses examining hybrid egg inviability, 3) field collections examining the fitness of different mating-pair types in nature, and 4) rearing experiment testing for a heritable basis to population divergence in color-pattern. By contrast, all mate preference data stem from previously published mate-choice experiments and the examination of covariance between color-pattern and mate preference in the current study involves a novel analysis of these data (Nosil et al. 2002, 2003). Collectively, the different types of data provide insight into the divergence of mating preferences during the early stages of speciation. We first review previous evidence for the role of host-adaptation and reinforcement in the evolution of sexual isolation. We then present new results on the costs to hybridization potentially driving reinforcement and the traits affecting sexual isolation.

11.2.3 Host-adaptation and the evolution of sexual isolation

If local adaptation drives divergence, then sexual isolation is predicted to be greater between ecologically-divergent pairs of populations than between ecologically-similar pairs of similar age. Several studies have provided evidence for such a pattern (Funk 1998; Rundle et al. 2000; Cruz et al. 2004; McKinnon et al. 2004; Funk et al. 2006; Vines et al. 2006). In *T. cristinae*, a role for host-adaptation is implicated by the observation that sexual isolation is stronger between geographically-separated pairs of populations using different host-plant species than between geographically-separated pairs of populations using the same host (Nosil et al 2002). Conversely, sexual isolation is uncorrelated with divergence in mitochondrial and nuclear DNA (COI and ITS-2 respectively) between these populations. Thus divergence in host plant use, rather than neutral differentiation via genetic drift, is the predictor of sexual isolation. The weak sexual isolation between populations using the same host also indicates that environment-independent forms of sexual selection do not strongly affect sexual isolation. Because selection against hybridization cannot occur when populations are geographically-separated, a role for local adaptation is inferred. We note that sexual isolation likely has a strong heritable genetic basis because it is unaffected by rearing environment (i.e. *Ceanothus* versus *Adenostoma*, Nosil et al. 2003).

11.2.4 Reinforcement and reproductive character displacement

Reinforcement predicts reproductive character displacement: increased sexual isolation in geographic regions where hybridization occurs (sympatry / parapatry) relative to regions where it does not (allopatry; but see Lemmon et al. 2004). Several cases of reproductive character displacement have been documented (Wasserman and Koepfer 1977; Zouros and d'Entremont. 1980; Noor 1995; Saetre et al. 1997; Rundle and Schluter 1998; Higgie et al. 2000; Hobel and Gerhardt 2003; Albert and Schluter 2004; Hoskin et al. 2005), yet few studies have distinguished among alternative hypotheses to reinforcement, or the role of different types of low hybrid fitness (Butlin 1995; Servedio and Noor 2003 for review). This is not a trivial task because different processes are not mutually exclusive and often generate overlapping predictions (Day 2000; Servedio and Noor 2003). For example, novel signals might be favored in a new environment to reduce

overlap with signals in the ancestral environment (Wasserman and Koepfer 1977). In this scenario, 'competition' among signals occurs only in parapatry such that direct selection on the preference (rather than reinforcing selection) would generate reproductive character displacement (Servedio 2001; Boughman 2002; Slabbekoorn and Smith 2002).

In addition to the effects of host adaptation, there is evidence for reinforcement in *T. cristinae*. Female mating discrimination against males from the alternative host is stronger when populations from different hosts are in geographic contact than when they are fully allopatric (Nosil et al. 2003). This pattern of reproductive character displacement could have evolved in response to maladaptive hybridization between the host-forms because 1) gene flow and interbreeding occurs, 2) alternative explanations such as ecological character displacement, differential fusion of populations, and population ancestry were examined, but are unsupported, and 3) indirect evidence suggests that hybrids will suffer low fitness due to high rates of visual predation. However, costs to hybridization have not been systematically evaluated in previous studies. Moreover, the association between selected traits and mating preferences affects the potential for reinforcement, but has also not been examined. In this paper, we present preliminary data on these two factors, and discuss their potential role in driving divergence in parapatric populations.

11.3 Costs to Hybridization and Reinforcement

A clear step beyond simply documenting reproductive character displacement is to ascertain which forms of selection drove preference evolution. Multiple forms of postmating isolation are measured, as well as costs to hybridization for individuals themselves. If individual or hybrid fitness is not reduced by a particular mechanism, a critical role for this process is unlikely. Here we estimate two costs to hybridization, intrinsic hybrid egg inviability and reduced survival of hybridizing females due to predation. We also evaluate the potential for extrinsic reductions in hybrid fitness.

11.3.1 Intrinsic hybrid egg inviability (hatching success)

We tested for intrinsic F1 hybrid egg inviability using within-population and between-population crosses (Table 11.2 for population pairings; n = 607 crosses). Nymph

T. cristinae were field-collected from 28 populations in the Santa Ynez Mountains, California in spring 2003 and 2004 using sweep nets. They were reared in glass jars (20° C) with 10-15 individuals per jar at the University of California at Santa Barbara. All individuals used in the crosses were sexually-immature instars that were reared to sexual maturity on *Ceanothus* cuttings. Individuals from different populations, and males and females, were kept separate during rearing. Within two days of attaining sexual maturity, a single virgin male and a single virgin female were housed together in a Petri dish and observed until they mated. Then they were fed *Ceanothus* cuttings every second day until the female died (females lay eggs singly).

Hatching success was quantified from egg shell (n = 30, 958 eggs) characteristics. Hatching nymphs left the egg shell fully intact except for a small opening at one end. Thus the proportion of hatched eggs within a brood can be calculated by counting the number of eggs with or without a small opening at one end. Hatching success measured this way was highly correlated with hatching success measured by monitoring broods daily for newly hatched nymphs ($r = 0.99$, $p < 0.001$, $n = 12$). The repeatability of hatching success estimates based on egg case characteristics was also estimated by recounting some broods in the same year and in a subsequent year, to estimate repeatability. Statistical analyses were conducted on egg number from the year of initial count, in case some eggs were lost or damaged. This does not affect our results because hatching success was highly repeatable both within years ($r = 0.97$, $p < 0.001$, $n = 28$) and between years ($r = 0.95$, $p < 0.001$, $n = 107$).

In *T. cristinae*, most viable eggs overwinter in diapause and hatch the year after mating occurred. Thus our analyses focus on hatching success following one over-wintering. However, a small portion of eggs diapause over one additional winter. There is no reason to suspect that variation in the duration of diapause would differ for within-population versus between-population crosses, and thus hatching success following one over-wintering likely provides an unbiased measure of egg viability. However, to ensure that our data are not affected by variable diapause, we estimated hatching success for 104 broods after a 2-year (2003-2005), instead of 1-year (2003-2004), diapause. These data are also presented.

Two types of ANOVA were used to analyze hatching success. The first analysis pools data among different population pairs and examines whether hatching success varied among the four main types of crosses (within-population, between populations using the same host, between parapatric populations using different hosts, and between allopatric populations using different hosts). Year (2003 or 2004) was also included as a factor. Mean hatching success was similar for all types of crosses (within population crosses = 49%, s.d. = 31; between populations using the same host = 40%, s.d. = 28; between parapatric populations using different hosts = 45%, s.d. = 35; between allopatric population using different hosts = 51%, s.d. = 39). Thus there was no evidence for reduced hatching success of F1 individuals from between-population crosses (main effects of cross-type $F_{3,607} = 1.22$, $p = 0.30$, main effects of year $F_{1,607} = 0.07$, $p = 0.80$, year x cross-type interaction $F_{3,607} = 1.10$, $p = 0.35$; main effects of cross type in an analysis excluding the interaction term $F_{3,607} = 0.54$, $p = 0.65$). Congruent results were obtained when hatching success was calculated after two, rather than one, over-wintering (within population crosses = 70%, s.d. = 21, n = 56; between parapatric populations using different hosts = 72%, s.d. = 23, n = 48; main effects of cross-type $F_{1,103} = 0.36$, $p = 0.55$).

The analyses above pool the results among population pairs and are general and useful because they yield very large sample sizes for analysis and do not assume independence of population pairs. However, analyses on pooled data might obscure trends in individual population pairs and do not treat population pairs as the unit of replication. The second set of analyses alleviates these problems in two ways. First, we conducted a separate ANOVA for each of the 14 population pairs. The model for each individual population pair included male population, female population, and the interaction between male and female population. We are interested primarily in the interaction term which tests whether hatching success is dependent on population of origin for the two sexes (e.g. the same population or not). Examination of individual pairs of populations confirmed the absence of F1 hybrid egg inviability (Table 11.2). The interaction between male population and female population was significant ($p = 0.01$) for only one of the 14 population pairs, and this difference is insignificant following Bonferroni correction. Second, we examined trends among population pairs (i.e. using

population pairs as the unit of replication). There were no directional trends evident such that only 4 of 14 pairs showed reduced hatching success in between-population versus within-population crosses ($p = 0.82$, Binomial test). Thus strong, F1 hybrid egg inviability was not detected in our study, and is therefore unlikely to strongly contribute to reinforcement in this system.

Intrinsic inviability might be more pronounced in F2 or backcrossed individuals, which were not examined. Furthermore, hybrid sterility generally evolves prior to inviability (Coyne and Orr 2004) and was also not examined. However, even if intrinsic inviability occurs in advanced hybrids, or if hybrid sterility occurs, it is likely incomplete because there is evidence for ongoing gene flow between the host forms (Nosil et al. 2003). Thus, at the very least, our data indicate that F1 hybrids do not show intrinsic inviability as represented by hatching success. This is the level of hybrid breakdown examined in most comparative studies of the evolution of reproductive barriers (reviewed in Coyne and Orr 2004; see also Mendelson 2003). Whether there has been sufficient time for hybrid egg inviability to evolve cannot be answered without studying more divergent taxa in the genus *Timema* (Crespi and Sandoval 2000; Sandoval and Nosil 2005). We note that there has been sufficient time for other forms of reproductive isolation to evolve in this species and that allopatric pairs of populations of *T. cristinae* exhibit substantial divergence in mitochondrial and nuclear DNA, indicative of reasonably ancient divergence times (Nosil et al. 2002).

11.3.2 Ecological costs to hybridization I – survival of females

Low survival of females that mate with males from the alternative host could also select for avoidance of between-host mating. The survival of females could be affected by the color-pattern of the males riding on their back. *T. cristinae* exhibits two main color-pattern morphs. Both morphs occur on both host species, but relative frequencies have diverged between hosts such that the unstriped morph is more common on *Ceanothus* (mean frequency = 81%), whereas the striped morph is more common on *Adenostoma* (mean frequency = 72%). Thus populations on different hosts show consistent, but not fixed, differences in morph frequency. Population divergence has occurred via differential visual predation: the unstriped color-pattern confers high

survival on *Ceanothus* but low survival on *Adenostoma*, and vice versa for the striped pattern (Sandoval 1994a,b; Nosil 2004).

Population divergence in color-pattern morph frequencies can result in a cost to between-population mating, particularly for females. In *T. cristinae*, males ride on the back of the female during and following mating. This period where a male rides on a female's back lasts at least several hours in the field (Nosil unpublished) and several days under laboratory conditions (Nosil et al. 2003). Most individuals within a population are the locally cryptic color-pattern morph. Conversely, many immigrant males from the adjacent population on the alternative host are the locally less-cryptic morph. Thus females that mate with and carry less-cryptic immigrant males on their back are likely to suffer higher rates of visual predation than females who mate with cryptic males from their own population. This reduction in survival of hybridizing females could favor the evolution of increased mating discrimination against males from the alternative host.

The scenario outlined above requires reduced survival of females mating with less-cryptic males. Studies of individuals (rather than mating pairs) suggest that this will occur (Sandoval 1994a,b; Nosil 2004; Nosil et al. 2005), but the fitness of mating pairs themselves has not been examined. We tested for selection against less-cryptic mating pair types in natural populations. If selection against a pair type occurs, its relative frequency should decline through time (i.e. between sequential sample periods; Endler 1986). In our analyses, the sequential sample periods are successive months. We have used these procedures previously to test for selection against the rare, intermediate morph (Nosil et al. 2003). In 2004, we collected mating pairs at one site that contained a population on each host (Refugio 12). We sampled adult insects in April and May 2004 (which represent periods before and after selection, respectively), recording the color-morph combination of each mating pair (total n = 174 mating pairs). For each host separately, we assessed whether the frequency of pair-types differed between months (i.e. before and after selection) using chi-square tests. We also tested whether the change in pair types was dependent on host species, using a loglinear analysis. This analysis used a partial chi-square value to assess the significance of the three-way interaction between pair-type, month and host (Norusis 1993).

We detected evidence for natural selection against less-cryptic mating pairs in natural populations (Fig. 11.2). Thus the relative frequency of the four different male/female pair types changed between months (i.e. before versus after selection) and, as expected, the nature of these changes was significantly dependent on host-plant species (pair-type x month x host interaction, chi-square = 8.94, d.f. = 3, $p < 0.05$, loglinear). On both hosts, mating pairs where both sexes were the cryptic morph increased after selection (thus striped/striped increased on *Adenostoma* and unstriped/unstriped increased on *Ceanothus*). Conversely, pairs where both sexes were the less-cryptic morph decreased after selection. However, differences before and after selection were statistically significant only for *Ceanothus* (chi-square = 8.78, d.f. = 3, $p < 0.05$, n = 97; for *Adenostoma*, chi-square = 5.50, d.f. = 3, $p = 0.14$, n = 77). Selection against less-cryptic mating pairs likely occurs and represents a cost to hybridization potentially contributing to reproductive character displacement.

Although our method was the only way to assess selection using undisturbed, natural populations, it potentially confounds differential survival with differential dispersal (Endler 1986). We note, however, that the results of manipulative experiments have demonstrated selection against less-cryptic individuals (Sandoval 1994a; Nosil 2004), increasing the likelihood that our current results represent selection against less-cryptic mating pairs. The reduced survival of hybridizing females is more likely indirect than direct selection, because the cost to females depends on the traits (color-pattern in this case) males and females carry (Servedio 2001, p. 1913 for explicit discussion of this issue). Thus, genetic covariance between color-pattern and mate preference is required for mate preference evolution due to direct selection on color-pattern. This covariance is assessed in a subsequent section.

11.3.3 Ecological costs to hybridization II – survival of hybrids

Even if hybridizing females survive and produce offspring, ecologically-based natural selection against hybrids could drive reinforcement. We were unable to assess directly the fitness of hybrids in the wild. Instead, we inferred the possibility of ecological selection against hybrids using estimates of selection on color-pattern and inferences about the color-pattern of hybrids. As noted, populations on different hosts

exhibit divergent color-pattern morph frequencies such that each morph is more common on the host upon which it has higher survival. If population divergence in color-pattern has a strong genetic basis, then it is likely that hybrids will suffer reduced fitness due to exhibiting maladaptive color-patterns. Under additive inheritance of color-pattern, hybrids will exhibit low survival because intermediate morphs (which bear a faint stripe and occur at low frequency in the wild) have lower survival on both hosts than does the locally-cryptic morph (Nosil et al. 2003; Nosil 2004). Under dominance, hybrids will still exhibit lower survival on average, because hybrid broods have a higher tendency to be 'mixed' such that they exhibit both color-pattern morphs (rather than only the locally-cryptic one; Sandoval 1993; Nosil et al. 2003). It is unclear which scenario applies because dominance in within-population genetic crosses is incomplete and between-population crosses are lacking. Because of this ambiguity, we cannot yet provide a quantitative estimate of hybrid fitness. Rather, we demonstrate that population divergence in color-pattern has a strong heritable basis and note that population divergence in color-pattern is predicted to result in low hybrid fitness under both modes of inheritance. To the extent that hybrids are intermediate, or to which hybrid broods contain both color-pattern morphs, the fitness of hybrid offspring will be reduced relative to offspring derived from within-population mating.

We tested for a heritable basis to population divergence in color-pattern using a reciprocal rearing experiment. For nine populations, we raised field-collected first instar nymphs (n = 1859; 4-6 weeks of rearing) to sexually mature adults on their native or alternative host (Fig. 11.3 for population-specific sample sizes). We used logistic regression analyses to test whether color-pattern (striped versus unstriped individuals) in these populations was affected by genotype, rearing environment (host plant reared on) or a genotype by environment interaction (assessing significance using likelihood ratio tests (LR)). We conducted two analyses, one using population of origin as the genotype term and one using host of origin as the genotype term. We report the results from a full model that included both factors and the interaction between them as well as the results from a reduced regression model derived using backward elimination (initial model included both factors and the interaction term but then removed all terms for which the significance of $-2 \log LR$ was > 0.10).

The reciprocal-rearing experiment revealed that population divergence in color-pattern has a strong heritable basis (Fig. 11.3). A strong effect of genotype occurred when host of origin was used as the genotype term (full model, host of origin -2LR = 124.71, $p < 0.001$; host reared upon -2LR = 17.05, $p < 0.001$; interaction -2LR = 12.82, $p < 0.001$) and when population of origin was used as the genotype term (full model, population of origin -2LR = 10.15, $p < 0.01$; host reared upon -2LR = 49.12, $p < 0.001$; interaction term -2LR = 2.32, $p = 0.13$; reduced model, population of origin -2LR = 62.91, $p < 0.001$; host reared upon -2LR = 56.27, $p < 0.001$; interaction term removed). Although rearing effects were also detected, genotypic effects were consistent and large in magnitude. Heritable population divergence in color-pattern suggests that hybrid offspring will be less-cryptic such that selection against them could contribute to reinforcement. Evidence for reduced survival of hybridizing females themselves was presented above. Thus ecological costs to hybridization likely occur. The potential for these costs to drive the evolution of divergent mate preferences depends on the association between ecologically-selected traits and mating preferences.

11.4 The Association Between Selected Traits and Mate Preferences

Direct selection on a trait generates indirect selection on correlated traits, and potentially a correlated evolutionary response. Another step in understanding preference evolution is to examine the magnitude of the association between traits under direct selection (e.g. ecological selection) and mate preference. Evolution of mate preferences by indirect selection depends on the magnitude of this genetic association. If the association is weak, then direct selection on mate preferences themselves may be a more likely cause of preference evolution (Kirkpatrick and Barton 1997).

In *T. cristinae*, for selection on color-pattern to cause the evolution of between-population mate preference, a genetic association must exist between color-pattern and the probability of between-population mating. We have previously shown that the probability of between-population mating is independent from whether both sexes are the same or different morphs (Nosil et al. 2002). These analyses test for assortative mating by color-pattern, but do not explicitly test for a genetic association between color-pattern and mate preference per se. A genetic association between these traits would be indicated

by a difference between color-pattern morphs within each sex in the probability of between-population mating (i.e. a difference in whether each morph accepts or rejects individuals from the alternate host). We would expect that the more-cryptic morph is less-likely to hybridize in between-population mating trials. These predictions have not been tested previously, and are examined here using the mating trials from Nosil et al. (2002, 2003).

The previous studies examined mate choice using no-choice mating trials. A single male and a single female were housed in a Petri dish and whether copulation occurred within a one hour period was recorded. We employed logistic regression to test whether the probability of copulation in these trials was dependent on color-pattern morph. Our prediction concerns the probability that different color-pattern morphs within populations will hybridize with individuals from other populations. Thus, our analyses are restricted to the between-population mating trials and we conducted separate analyses for males and for females within each of the 12 populations from Nosil et al. (2002, 2003). We detected little or no evidence for a phenotypic association between color-pattern and mate preference. Thus color-pattern morphs did not tend to differ in the probability of between-population copulation when either males or females were considered (Table 11.3). Significant differences between color-pattern morphs were detected in only two of 24 cases, one was in the direction opposite to that predicted (i.e. cryptic morph more likely to hybridize), and neither was significant following Bonferroni correction (i.e. $p = 0.024, 0.032$ before correction for 12 comparisons within each sex).

Because the association between color-pattern and mate preference is weak, selection on color-pattern (in hybrids or hybridizing parental forms) is not predicted to cause a strong correlated evolutionary response in mate preference. Indirect selection on mate preference, generated by direct selection on color, will be weak. Thus how strongly the observed ecological costs to hybridization contribute to reproductive character displacement remains unclear. The observed lack of covariation between colour-pattern and between-population mate preference is surprising because migration between populations should generate non-random association between alleles at different loci (i.e. linkage disequilibrium), even when they are physically-unlinked (Nei et al. 1973; Kirkpatrick et al. 2002). Notably, this possibility of genetic covariance generated by

migration applies only to parapatric populations of *T. cristinae* (Nosil et al. 2006b). The current study examined the phenotypic covariance between color-pattern and mate preference, whereas the most relevant parameter for evolutionary response is genetic covariance. The degree of correspondence between phenotypic and genetic covariance remains a topic of debate (Cheverud 1988; Willis et al. 1991; Arnold 1992; Schluter 1996). If the phenotypic covariance is not representative of the underlying genetic covariance, an undetected association at the genetic level could occur and have driven reinforcement. Moreover, recombination erodes genetic covariance caused by linkage disequilibrium, potentially accounting for the lack of covariance observed. Although further work is required, examining the association between color-pattern and mate preference was useful because it prevented us from concluding prematurely that ecological costs to hybridization (which appear strong) clearly drove reproductive character displacement.

Given that the available evidence suggests that indirect selection on mate preference will be weak, a role for direct selection on mate preference in the reproductive character displacement of mate preferences becomes highly plausible (Kirkpatrick and Barton 1997). Further studies in *Timema* could focus on direct selection on preference and signals, particularly now that candidate signals (pheromones) are known.

11.5 Traits Affecting Sexual Isolation

A different approach to understanding sexual isolation, which is complementary to studies of geographic variation, is to identify the traits affecting mate choice (Nagel and Schluter 1998; Jiggins et al. 2001). Determining such traits allows subsequent studies of how selection acts on them, and thus how selection affects the evolution of sexual isolation (Hobel and Gerhardt 2003; Boughman et al. 2005). Surprisingly, there are relatively few systems where both geographic variation and the key traits affecting sexual isolation have been studied (reviewed in Coyne and Orr 2004; but see Higgie et al. 2000; Cruz et al. 2004; McKinnon et al. 2004; Boughman et al. 2005 for some exceptions).

Previous studies did not identify the traits affecting sexual isolation in *T. cristinae*. For example, between-population mating probability is independent of

differences between the sexes in color-pattern, body size, and body shape (Nosil et al. 2002; Nosil and Crespi 2004). Here we present evidence from pheromone analyses and behavioural experiments that sexual isolation between populations stems, at least in part, from divergence in pheromones and responses to them. Successful copulation in *T. cristinae* involves at least two distinct stages. First, the male must approach the female and attempt to mount her. Second, the female must allow the male to mount and copulate with her, as males cannot force copulation. Here we test whether pheromonal communication affects the initial attraction of males to females, and thus has a potential role in sexual isolation.

We focus on two population pairs comprised of three populations. Thus a population adapted to *Adenostoma* (HVA) is compared to a population feeding on the same host plant (OUTA) and to a population feeding on *Ceanothus* (PR). The pair of populations using different hosts exhibits stronger sexual isolation than the pair using the same host (Nosil et al. 2002, 2003 for details). For example, using an index of sexual isolation (I_{PSI}) that ranges from –1 to +1 (with zero and one indicating random mating and complete assortative mating, respectively; Rolan-Alavarez and Cabarello 2000), I_{PSI} = 0.20 for HVA x PR versus I_{PSI} = 0.11 for HVA x OUTA. Averaged across numerous pairs of populations, mean I_{PSI} = 0.24 and 0.08 for pairs on different versus the same host, respectively.

11.5.1 Methods for analyzing pheromones and behavioral responses

Our examination of pheromonal communication contained two main components; analytical procedures aimed at pheromone identification and behavioural experiments. To obtain pheromone, 16-24 male or female HVA or PR were placed in separate Pyrex glass chambers (5 × 10 cm) maintained at a photoperiod of 14L:10D and a temperature of 23-25°C. A water aspirator was used to draw humidified, charcoal-filtered air at a rate of 80 ml/min through the chamber and through a glass column (6 x 30 mm) filled with Porapak Q (50-80 mesh, Waters Associated Inc. Milford, MA, USA). After 40 hr, absorbed volatiles were desorbed with 2 ml of redistilled pentane.

Pheromone extract was analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry (MS). Aliquots (3 insect-hour equivalents) of Porapak Q extract were analyzed by GC-EAD (Arn et al. 1975; Gries et al. 2002), employing a Hewlett-Packard (HP) 5890 gas chromatograph fitted with a GC column (30 m x 0.25 or 0.32 mm ID) coated with DB-5, DB-17, DB-23, or DB-210 (J&W Scientific, Folsom, California, USA). The use of four GC columns with different retention characteristics allowed us to calculate the retention indices (RI) (Van den Dool and Kratz 1963) and intercolumn IR differentials of the antennal stimulatory compounds. This analytical method helped reveal the chemical functionality (e.g., secondary alcohol) of these compounds, and contributed to their identification (Nosil et al. unpublished data).

For GC-EAD recordings, an antenna was carefully removed from an insect's head, and its base placed into the opening of a glass capillary electrode (1.0 x 0.58 x 100 mm) (A-M Systems, Inc., Carlsborg, Washington, USA) filled with saline solution (Staddon and Everton 1980). The distal end of the antenna with its tip removed by spring micro-scissors (Fine Science Tools Inc., North Vancouver, British Columbia, Canada), was inserted into the recording capillary electrode. Coupled GC-MS analyses of pheromone extract employed a Varian Saturn 2000 Ion Trap GC-MS fitted with the DB-5 or DB-17 column.

GC-EAD analyses of female *T. cristinae* effluvia revealed several volatiles that elicited strong responses from male antennae (Fig. 11.4). The volatile and antennal response profiles were very similar for HVA and PR populations. GC-MS analyses confirmed that the same components were EAD-active in HVA and PR populations. The mass spectrum and retention indices (RI) (Van den Dool and Kratz 1963) of the most EAD-active component (DB-5: RI =1258; DB-17: RI=1472; DB-23: RI=1909; DB-210: RI=1476) in both populations were strongly indicative of a secondary alcohol.

We then performed no-choice experiments using static-air olfactometers (Vet 1993, Takács and Gries 2001) to assess whether pheromonal cues contribute to mate discrimination. These experiments provide evidence for olfactory-based discrimination independent of the molecular structure of the pheromone component(s). The

olfactometers' three chambers (each 10 cm diameter x 3.5 cm high) were linearly interconnected by a glass tube (each 1cm diameter x 2.5 cm long). A no-choice design was used because it allowed a direct comparison to the no-choice experiments used to quantify sexual isolation (Nosil et al. 2002, 2003), and it avoids confounding mating propensity with mate preference (Rolan-Alavarez and Cabarello 2000). Thus for each trial a male was tested against a single female that was from either the males own population or from the alternative population. The experiment tests whether a male exhibits no response (stays in the central release chamber), responds to the test stimulus (by approaching the female), or responds to the control stimulus (approaches the empty chamber).

The experiment proceeded in four steps. First, olfactometers were randomly assigned to locations on the laboratory bench. Second, females were randomly assigned to individual olfactometers and to one of the two side chambers. Females were confined within a glass tube covered with wire mesh to prevent physical contact of the bioassayed male with a female as the test stimulus. The control chamber contained an empty glass tube of the same type. Third, a male was released in the central chamber. Fourth, all activity of the male was recorded for two hours. Specifically, we recorded when the male moved, and which chamber it entered. We analyze the first movement of each male because multiple movements occurred very rarely (5% of trials). Between trials run every other day, olfactometers were rinsed with 70% ethanol, thoroughly washed with soap, and left to dry 24 hours (i.e. experiments were run every second day). A total of 318 trials were conducted (Experiment 1: 192 trials for HVA x PR; Experiment 2: 126 trials for HVA x OUTA).

We performed three statistical analyses for each experiment, predicting larger between-population discrimination in the different host experiment (Experiment 1). The first logistic regression analysis uses data from all the trials for a particular experiment. It tests whether a male's approach towards the female (binary dependent variable – approached occurred or not) was dependent on the male population, female population or the interaction between the male and female population. We are interested in the interaction term, which tests whether the response of a male from a particular population is dependent on the population of female. We also included male colour morph, male age

upon capture (sexually immature versus mature), female colour morph and female age upon capture as factors in the analysis. Models not including these factors yielded similar results and are not shown. Significance was assessed using likelihood-ratio tests from full and reduced models. The second analysis was the same as the first, with one modification; we excluded all the trials where a male remained in the central release chamber for the entire trial. This exclusion allowed us to test whether the results of the first analyses are confirmed when only active males are considered. The third analysis examined only those trials where a female was chosen, and tested which factors predict the time period until the female is chosen. The time until a female was chosen is continuously-distributed such that all the terms described in the other two analyses were included, but analyzed using ANOVA.

11.5.2 Evidence for pheromone-based discrimination

We detected evidence for olfactory-based behavioural discrimination for the population pair using different hosts, but not for the pair using the same host (Table 11.4 for statistics and Fig. 11.5 for data). Thus for the HVA x PR comparison, the probability that a female was approached was significantly greater for within-population trials than for between-population trials. This pattern occurred both when all trials were considered, and when only trials where a male entered a side chamber were considered ($p < 0.05$ in both cases, see Fig. 11.5 A-C and Table 11.4). Additionally, when only the trials where a female was approached were examined, the time until a female was approached was significantly lower for within-population versus between-population trials, as expected if there was between-population discrimination based upon olfactory cues. In contrast to the results with the pair using different hosts, there was little or no evidence for olfactory-based discrimination in the trials using the population pair on the same hosts. For the HVA x OUTA comparison, significant differences for within-population versus between-population trials were not detected in any case (all $p > 0.25$). In fact, trends were sometimes in the direction opposite to that expected if there was between-population discrimination. For example, the time taken for a male to approach a female tended to be greater when the female was from the same population (Fig. 11.5 D-F and Table 11.4).

The results suggest that sexual isolation between populations of *T. cristinae* that use different host plants involves, at least in part, pheromonal communication. Behavioral experiments revealed evidence for discrimination based upon pheromonal cues, and several candidate pheromone components in the insects' effluvium elicited antennal responses. Subtle, rather than large, differences in the insects' volatile effluvia and antennal response pattern were detected. One hypothesis is that the host forms of *T. cristinae* differ in some qualitative aspect of pheromones, such as enantiomer composition, and that discrimination is based on enantiospecific responses. The most antennally active candidate pheromone component indeed can exist as two enantiomers (Nosil et al. unpublished data) which may or may not be so for the as yet unidentified candidate pheromone components. Enantiospecific pheromone production and differential attraction to specific blends of enantiomers has been reported in other insects (Miller et al. 1996; Millar et al. 1990, 1991; Gries et al. 1999, 2003). Such enantiospecific responses have been shown to result in assortative mating (Teale et al. 1994) and partial barriers to gene flow between pheromone races (Cognato et al. 1999). Further work could examine whether the enantiomer ratio of major pheromone components differs between populations. We also do not know how strongly pheromone production in *T. cristinae* is affected by environmental (i.e. host-plant reared upon) versus genetic factors. However, sexual isolation itself is unaffected by rearing environment (Nosil et al. 2003), and pheromonal discrimination in our behavioural experiments was the same for field-caught adults and for nymphs reared to maturity in the lab in a common environment (i.e. on *Ceanothus*). Other studies of insect pheromones have detected a strong genetic basis to their composition (Seybold et al. 1995; Hager and Teale 1996).

We examined male responses to female pheromones, because sexual isolation between allopatric pairs of populations involves preference divergence of both sexes, and the male approach to females is an important component of sexual isolation (Nosil et al. 2002). It is not yet possible to determine conclusively whether divergence in pheromonal communication is linked to adaptation to different host plants, because only two population pairs have been examined. However this level of replication is highly typical of studies of the traits underlying sexual isolation (Ryan et al. 1990; Nagel and Schluter

1998; Cruz et al. 2001; Jiggins et al. 2001; Fordyce et al. 2002; Vines et al. 2006). Two lines of evidence are highly suggestive. First, pheromonal discrimination was detected between the population pair on different hosts, but not between the pair on the same host. Second, male mating preferences are not reinforced in parapatry, such that discrimination by males based upon pheromonal cues is more likely to involve local adaptation than reinforcement (Nosil et al. 2003).

Sexual isolation due to local adaptation versus reinforcement need not involve divergence in the same traits. In *T. cristinae*, it appears that preferences of both sexes diverge due to local adaptation, with only female preferences reinforced in parapatry. Overall variation in sexual isolation could thus involve multiple traits, as has been observed in *Drosophila*. Sexual isolation between allopatric populations of *D. pseudoobscura* and *D. persimilis* involves divergence in courtship song (Williams et al. 2001), whereas genomic regions affecting reinforced mating discrimination contain genes involved in pheromonal communication (Ortiz-Barrientos et al. 2004; see also Boughman et al. 2005 for composite mating traits in stickleback). Future studies of *T. cristinae* could examine the possibility of composite mating traits, particularly because courtship behavior occurs but has not been examined.

Finally, we note that few studies of sexual isolation have examined mating preferences and also determined the traits (e.g. color-patterns, body size, pheromones, behavior; but see Boughman et al. 2005) upon which mate preferences are based. In the few cases of reinforcement where both have been examined, preferences often exhibit greater displacement than the mating traits themselves, suggesting that selection acts mostly on receiver selectivity (Hobel and Gerhardt 2003). In cases of local adaptation, interactions between natural and sexual selection are often involved in the divergence of traits and preferences (Naisbit et al. 2001; Leal and Fleishman 2004; Boughman et al. 2005). It would be of interest to further quantify geographic variation in pheromones and other potential mating traits among populations of *T. cristinae,* and to compare this variation to geographic variation in preferences. Determining that phermonal communication is involved in sexual isolation is a major step because it will allow such studies.

11.6 General Discussion

Our approach to examining the contribution of different selective processes to the evolution of sexual isolation may be applied to other systems, and to other forms of preference (e.g. habitat preferences, temporal isolation). Many studies have examined the effects of one or two processes on the evolution of sexual isolation (e.g. herbivorous insects – Funk et al.1998; *Drosophila* – Etges 2002; flowering plants – Bradshaw and Schemske 2003; stickleback fishes – Rundle et al. 2000; McKinnon et al. 2004; butterflies - Jiggins et al. 2001; Fordyce et al. 2002; intertidal snails - Rolan-Alvarez et al. 1997, Cruz et al. 2004). Outstanding questions concern the relative importance of distinct evolutionary forces such as ecological selection, reinforcing selection and gene flow, and the traits involved. Ascertaining these factors will require detailed studies of individual systems. For example, in the cricket frog (*Acris crepitans*) environmental selection for transmission efficiency must be integral for call divergence because alternative hypotheses such as reinforcement and pleiotropic effects of body size divergence are ruled out (Ryan et al. 1990; Ryan and Wilczynski 1991).

In cases of reproductive character displacement, very few systems have examined multiple costs to hybridization or the potential role of gene flow (Servedio and Noor 2003). In *T. cristinae,* the effects of reinforcing selection are greatest under intermediate migration rates, where encounters between populations are common enough to promote reinforcement, but low enough to prevent gene flow from eroding divergence (Nosil et al. 2003; see also Noor 1995). Additionally, although *T. cristinae* females are selected to be more discriminating against males from an adjacent population, this selection has indirectly resulted in increased mating discrimination against foreign males from multiple other populations that use either host (Nosil et al. 2003). Such 'universal' effects of reinforcement may be due to females recognizing and preferring males from their own population based on a 'population-specific' trait instead of a 'host-specific' or 'species-specific' trait) (Kelly and Noor 1996; Higgie et al. 2000; see also Zouros and D'Entremont 1980; Hoskin et al. 2005). If such 'universal' effects are common, then reinforcement could contribute to speciation between ecologically-similar pairs of populations, between populations that are geographically-separated from one another

(Hoskin et al. 2005), and between conspecific populations. Notably, preference for population-specific traits makes reinforcement theoretically more likely in an island-continent scenario (Servedio 2000), perhaps explaining why the effects of reinforcement are evident in *T. cristinae* even in causes of highly asymmetric gene flow between adjacent populations.

Although the current study focused on premating isolation, it also presents some data on postmating isolation. We detected no evidence for reduced F1 hatching success in between-population versus within-population crosses. Our data do not allow us to disentangle the effects of differential fertilization (e.g. gametic isolation, Palumbi 1998; Swanson and Vacquier 2002) from differential mortality of embryos. However, we detected no differences in hatching success, suggesting that neither process occurs. An alternative explanation, which is somewhat contrived, is that fertilization success and embryonic death work in opposite directions in different types of crosses such that they exactly cancel each other out (yielding equal hatching success in all cross types). More likely, neither differential fertilization nor differential embryonic mortality occurs. A lack of intrinsic F1 hybrid inviability between sister species has certainly been observed in nature (Schluter 2000 for review; Saldamando et al. 2005), but examples where it does occur are also common (Coyne and Orr 2004).

Most work on speciation via natural selection ('ecological speciation') has not examined gametic isolation and intrinsic inviability (but see Lu and Bernatchez 1998), perhaps because these barriers can evolve via any form of selection or by genetic drift (Rundle and Nosil 2005 for review). Previous work on other systems has implicated a role for selection in both the evolution of gametic isolation and intrinsic reductions in hybrid fitness. Reproductive proteins involved in fertilization often evolve rapidly via selection (Vacquier et al. 1997; Swanson and Vacquier 2002). Likewise, the three *Drosophila* genes causing intrinsic postzygotic isolation which have been identified exhibit a history of evolution via positive selection (*Hmr*, Barbash et al. 2004; *Nup96*, Presgraves et al. 2003; *OdsH*, Ting et al. 1998; Wu and Ting 2004 for review; Shuker et al. 2005 for a potential counterexample). However, the causes of selection (e.g. ecological or not) cannot be determined from these data alone. Gene flow and population subdivision are also predicted to affect the evolution of genetic incompatibilities (Orr and

Orr 1996; Church and Taylor 2002; Gavrilets 2004). The current study examined scenarios where ecology and gene flow varied, but the overall lack of inviability precludes a strong test of the effects of either factor. Further studies are clearly warranted, because the processes driving the evolution of genetic incompatibilities remain poorly understood.

Barriers to gene flow that evolve before reproductive isolation is complete can provide particular insight into speciation, and thus the temporal order of evolution of different forms of isolation is important. A few comparative studies indicate that sexual isolation can evolve before intrinsic postmating isolation but the generality of this finding is unknown (Coyne and Orr 1997; Mendelson 2003; Ramsey et al. 2003). Every form of reproductive isolation examined previously in *T. cristinae* was detected (Table 11.1). Populations on different hosts are partially reproductively isolated by divergent host preferences (Nosil et al. 2006a,b), low survival of between-host migrants (Nosil 2004), sexual isolation (Nosil et al. 2002, 2003), and postmating, prezygotic incompatibilities (Nosil and Crespi 2006). The current study focused on the same populations examined in previous work such that in these populations premating isolation has evolved earlier than intrinsic F1 hybrid egg inviability.

Speciation is often a continuous process whereby populations diverge from randomly mating units to reproductively isolated species. Theory clearly demonstrates that the evolution of sexual isolation can involve many selective processes, and our study shows how their contributions might be examined. The results show that multiple processes can act simultaneously, with a central role for ecology and geography. The host-associated forms of *T. cristinae* are unlikely to have achieved species status, as indicated by only partial barriers to gene flow at the premating level and weak mtDNA differentiation between adjacent populations on different hosts due to ongoing gene flow (Nosil *et al.* 2003). The host forms represent either an ongoing speciation event or population divergence that has reached equilibrium such that we have examined the early stages of the speciation process. Studies of more divergent taxa are required to test how our findings apply to the latter stages of speciation (Sandoval and Nosil 2005). The degree to which the same traits and processes are involved at different stages of speciation is poorly understood (Claridge and Morgan 1993; Ryan and Rand 1993; Mallet

et al. 1998; Boake et al. 1997; Bordenstein et al. 2000; Jiggins et al. 2004). Studies examining both traits and preferences, and multiple selective processes at different stages of divergence, will likely provide the most complete picture of the entire speciation process.

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Figure 11.1 A scenario for how speciation may progress from its earliest to latest stages (magnitude of reproductive isolation is depicted by the wavy, black line).

The scenario shown here applies to the evolution of sexual isolation, and lists the potential processes involved. Populations are initially allopatric, but secondary contact can occur at any point and results in additional processes which can drive divergence. In the particular case shown here, secondary contact occurs roughly midway through speciation – depicted by the dashed vertical line. When indirect selection occurs, the genetic covariance between traits affecting mate preference and traits under selection is important for evolution. See the Introduction for details and Table 11.1 for processes that have been most important for the evolution of sexual isolation among populations of *T. cristinae* walking-sticks. RCD refers to reproductive character displacement.

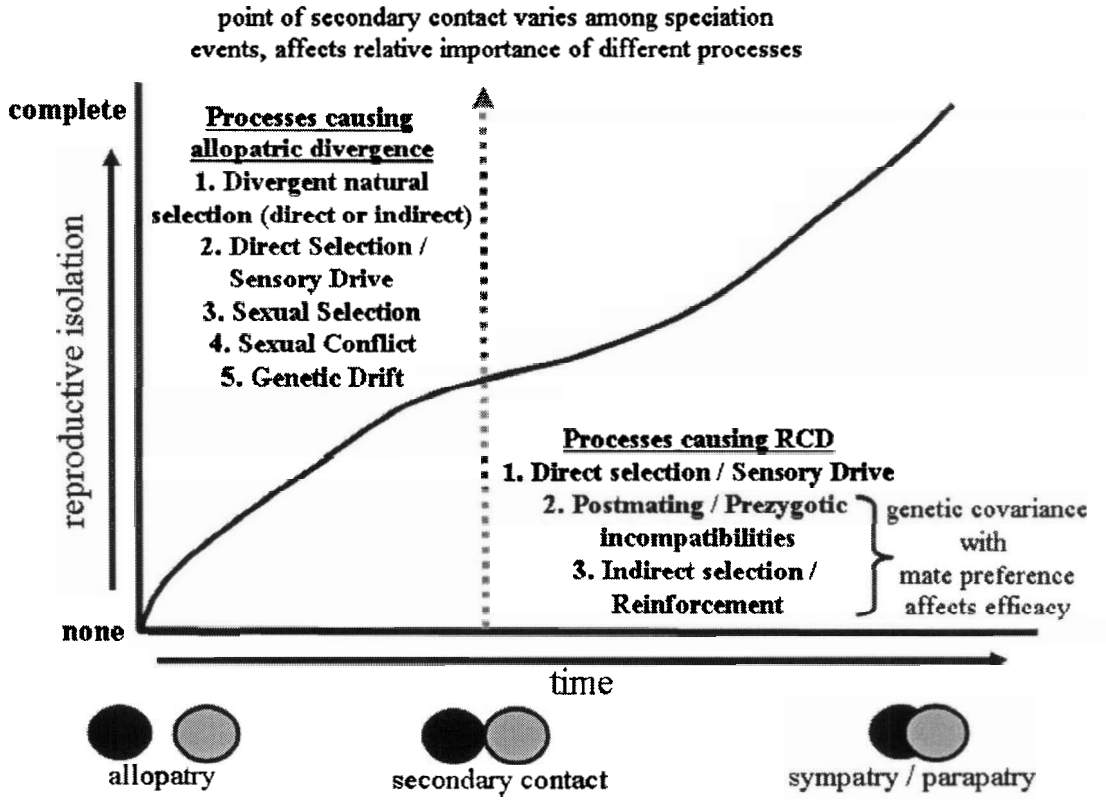


Figure 11.2 Analyses of natural selection using *T. cristinae* mating pairs in the wild show selection against the locally less-cryptic mating pairs; on both hosts, mating pairs with a cryptic male and cryptic female morph increased after selection (i.e. between successive sample months). Thus "striped/striped" pairs increased on *Adenostoma* and "unstriped/unstriped" pairs increased on *Ceanothus*. Conversely, pairs with a less cryptic male and female morph decreased. UU = both sexes unstriped. US = unstriped male on striped female. SU = striped male on unstriped female. SS = both sexes striped.

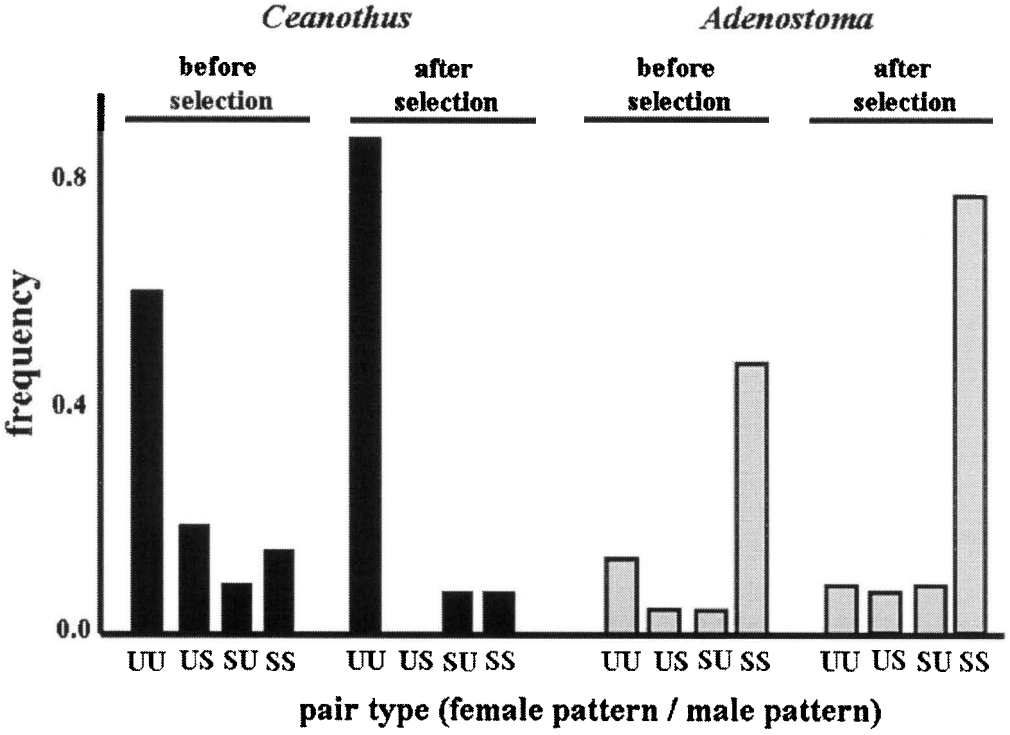


Figure 11.3 Reciprocal rearing experiment demonstrates a heritable basis to population divergence in color-pattern.
Shown is the proportion of striped adults (± 95% C.I) observed when field-caught first instar *T. cristinae* were reared to adults during 4-6 weeks on *Ceanothus* (C) or *Adenostoma* (A). Populations adapted to *Adenostoma* are depicted on the left, populations adapted to *Ceanothus* are on the right. Genotypic effects (population and host of origin) were large and highly statistically significant (see Results for statistics). Values below the x-axis refer to number of individuals.

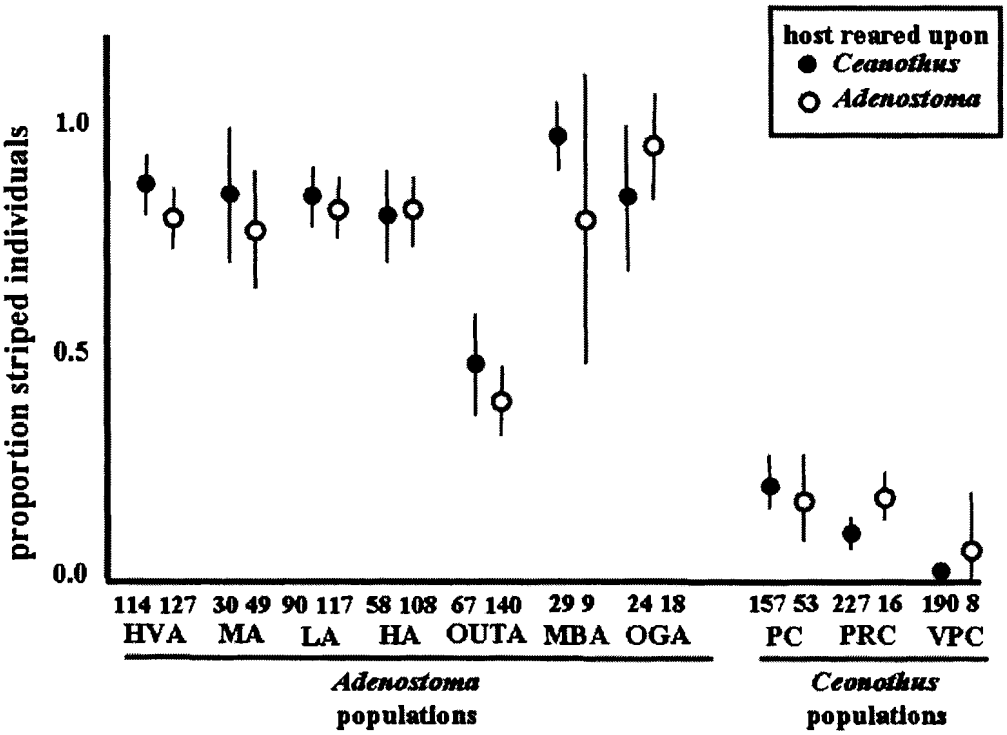


Figure 11.4 Flame ionization detector (FID) and electroantennographic detector (EAD: male *Timema cristinae* antenna) responses to effluvium volatiles from female HVA (*top*) and female PR (*bottom*).
Chromatography: DB-5 GC column (0.32 mm ID x 30 m); temperature program: 50° C (1 min), 10° C per min to 200° C, then 25° C per min to 280° C. Most antennally active compound marked by an arrow.

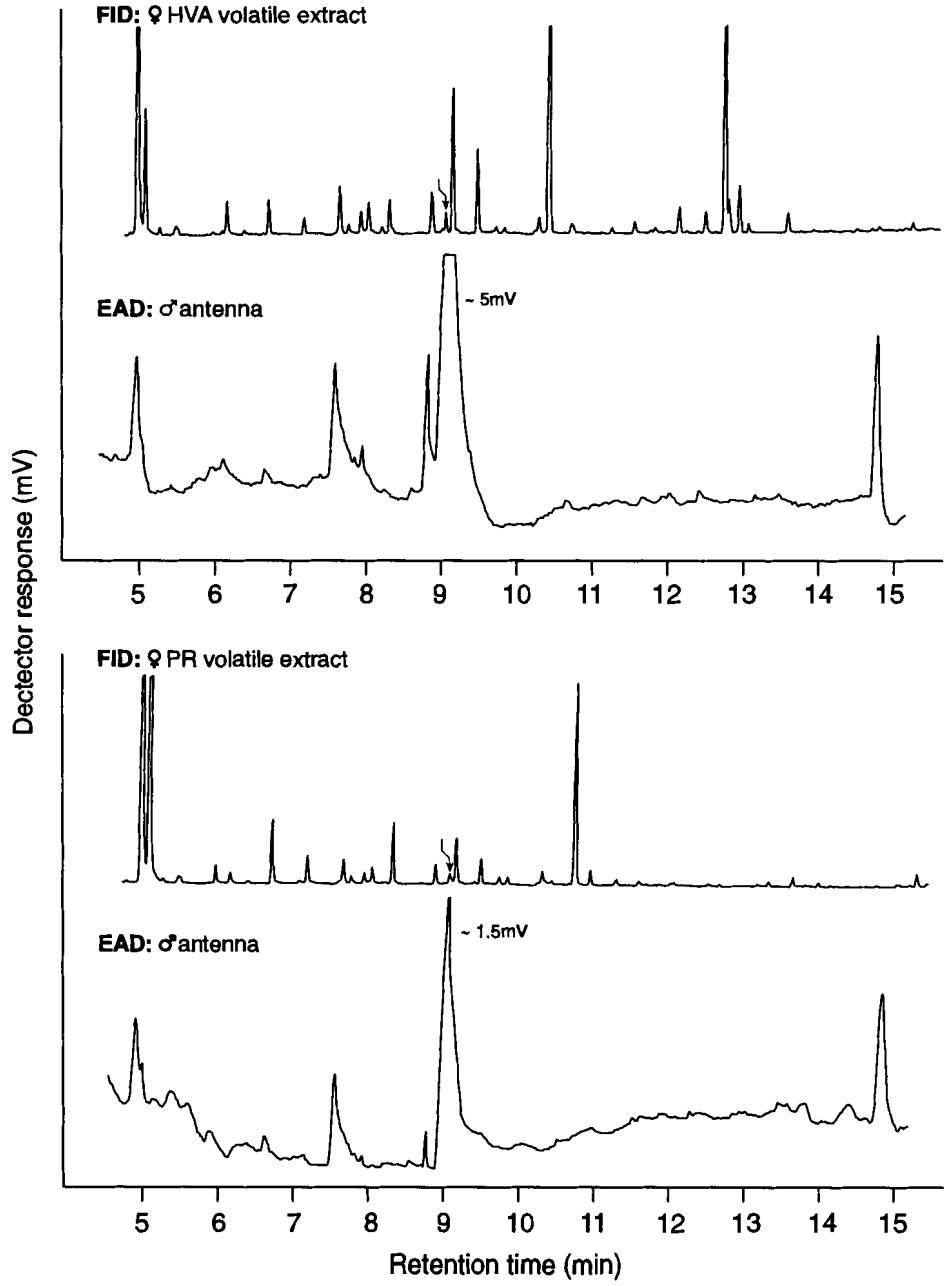
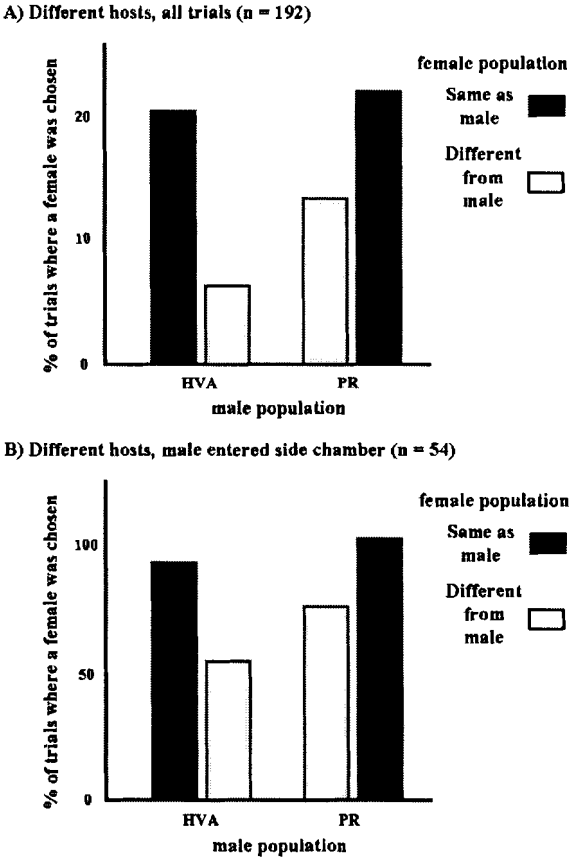
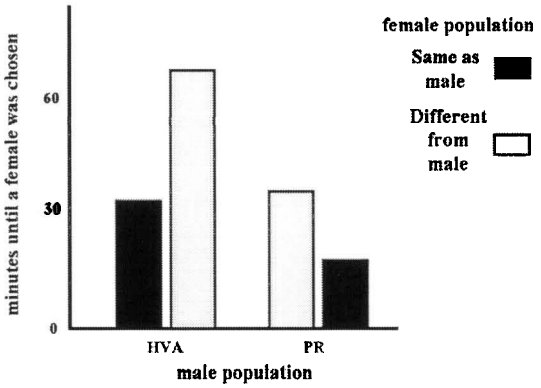


Figure 11.5 Olfactometer experiments showing that male discrimination based on olfactory cues occurs only for the *T. cristinae* population pair using different hosts.

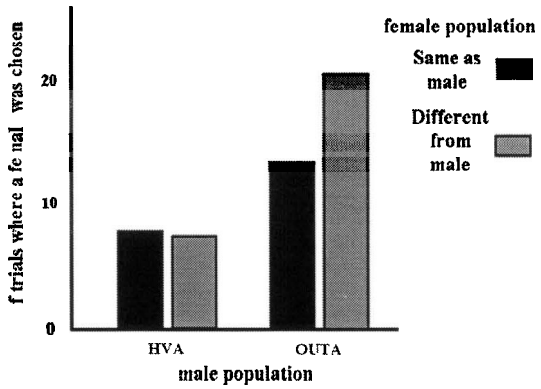
Panels A-C show the results from a population pair that uses different hosts (HVA x PR), where discrimination based on olfactory cues was detected (all $p < 0.05$). Panels D-F show the results from a population pair that uses the same host (HVA x OUTA), where discrimination based on olfactory cues was not detected (all $p > 0.15$).



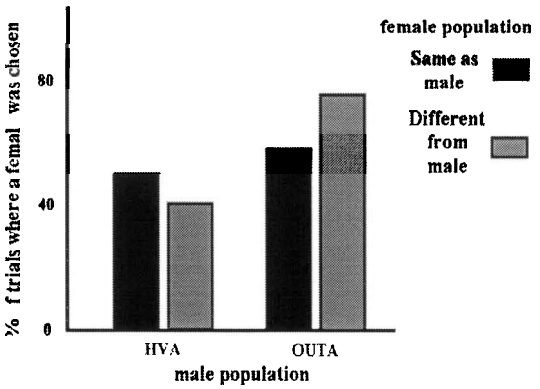
C) Different hosts, female was chosen only (n = 30)



D) Same hosts, all trials (n = 126)



E) Same hosts, male entered side chamber (n = 26)



F) Same hosts, female was chosen only (n = 15)

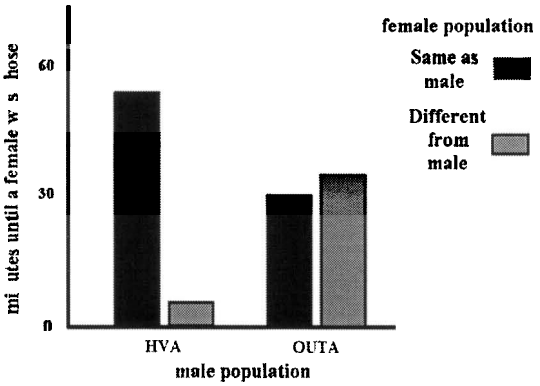


Table 11.1 Summary of the Evolutionary processes involved in the evolution of sexual isolation among populations of *T. cristinae* walking-stick insects. Shown is whether an evolutionary process occurs at all, and then if it contributes to the evolution of sexual isolation.

Evolutionary Process	Occurs?	Contributes?	Explanation
Local Adaptation	Yes	Yes	Allopatric populations using different hosts show greater sexual isolation than those using the same host
Direct Selection / Sensory Drive	Possible, evidence lacking	Possible	Trait causing postmating incompatibilities does not covary strongly with mate preference, leaving a potential role for direct selection
Genetic Drift	Yes	No (at least not in isolation)	Levels of sexual isolation are uncorrelated with divergence in neutral markers
Environment-independent sexual selection	Unknown	No (at least not in isolation)	Different populations which use the same host exhibit little or no sexual isolation
Postmating, prezygotic incompatibilities			
Female survival	Yes	Possible	Selection occurs, but male trait reducing female survival does not covary strongly with preference
Female fecundity	Maybe	Maybe	Not certain if selection occurs
Selection against hybrids			
Intrinsic F1 egg inviability	No	No	Does not occur, so unlikely to contribute strongly
F1 sterility, F2 and backcross breakdown	Unknown	Unknown	Not examined
Natural selection against hybrids	Yes	Possible	Selection occurs, but trait reducing hybrid survival does not covary strongly with preference
Sexual selection against hybrids	Likely	Unknown	Never been measured, but likely given divergent mate preferences / mating traits of parental forms

Table 11.2 Hatching-success was not reduced for between-population versus within-population crosses.

The table shows mean % (s.d.) hatching success for between-population and within-population crosses for 14 different pairs of *T. cristinae* walking-stick populations. Also shown are the number of crosses for each population pair (n) and the test statistic for the male population by female population interaction term (this F-ratio stems from a separate ANOVA analysis for each population pair such that the degrees of freedom are $F_{1,n}$ where n refers to the total sample size).

Population pair	Within-population	Between-population	n	F-ratio	p-value
Allopatric same hosts					
P x PR	32 (20)	34 (29)	56	0.38	0.54
PE x WCC	50 (33)	65 (25)	49	2.47	0.12
LOG x BT	41 (55)	42 (32)	11	0.11	0.74
Parapatric different hosts					
HVA x HVC	48 (34)	54 (29)	72	0.09	0.77
MA x MC	23 (26)	49 (30)	30	10.26	0.004
HA x HC	41 (28)	57 (18)	14	0.98	0.34
OUTA x OUTC	44 (32)	53 (30)	51	1.90	0.17
R12A x R12C	40 (40)	44 (35)	55	0.11	0.74
MBOXC x MBOXA	45 (37)	44 (24)	38	0.01	0.92
OGC x OGA	54 (42)	46 (28)	28	0.03	0.88
VPA x VPAC	54 (33)	25 (34)	44	7.36	0.01
Allopatric different hosts					
LA x VPC	65 (35)	49 (34)	50	1.11	0.30
R6C x R23A	40 (41)	47 (30)	66	0.49	0.49
SC x LRN	53 (39)	65 (26)	43	0.40	0.53

Table 11.3 The proportion of between-population mating trials resulting in copulation for different color-pattern morphs of *T. cristinae*.
The probability of copulation did not tend to differ between color-pattern morphs. PC, VPC, PRC and LA are allopatric populations, whereas the others are parapatric. Also shown is the likelihood ratio from likelihood ratio tests (all d.f. = 1).

Population	Unstriped Mean (s.d.)	Striped Mean (s.d.)	Unstriped n	Striped n	LR	p-value
Males						
PC	0.32 (0.47)	0.29 (0.46)	156	58	0.15	0.70
HVC	0.26 (0.44)	0.36 (0.48)	54	59	1.24	0.27
VPC	0.30 (0.46)	0.13 (0.35)	223	15	2.19	0.14
PRC	0.29 (0.45)	0.00 (0.00)	168	1	0.67	0.41
MBOXC	0.27 (0.45)	0.29 (0.46)	26	174	0.06	0.80
OGC	0.18 (0.39)	0.17 (0.38)	78	86	0.01	0.93
HVA	0.49 (0.50)	0.35 (0.48)	98	145	4.61	0.03
VPA	0.33 (0.47)	0.29 (0.46)	148	66	0.40	0.53
MA	0.25 (0.44)	0.26 (0.44)	48	121	0.01	0.93
LA	0.23 (0.43)	0.26 (0.44)	52	162	0.17	0.68
HA	0.35 (0.48)	0.36 (0.48)	48	192	0.01	0.95
OUTA	0.29 (0.45)	0.34 (0.48)	153	73	0.69	0.41
Females						
PC	0.30 (0.46)	0.35 (0.48)	263	54	0.63	0.43
HVC	0.19 (0.40)	0.22 (0.42)	27	46	0.11	0.74
VPC	0.33 (0.47)	0.31 (0.47)	282	29	0.06	0.80
PRC	0.36 (0.48)	0.50 (0.71)	225	2	0.16	0.69
MBOXC	0.15 (0.36)	0.19 (0.40)	98	84	0.45	0.50
OGC	0.30 (0.46)	0.20 (0.40)	69	97	2.57	0.11
HVA	0.25 (0.44)	0.31 (0.46)	92	173	0.95	0.33
VPA	0.33 (0.47)	0.30 (0.46)	147	57	0.15	0.70
MA	0.00 (0.00)	0.23 (0.42)	11	48	5.09	0.02
LA	0.46 (0.50)	0.38 (0.49)	69	128	1.46	0.23
HA	0.34 (0.48)	0.32 (0.47)	38	135	0.08	0.78
OUTA	0.36 (0.48)	0.31 (0.47)	145	89	0.48	0.49

Table 11.4 Statistical analyses showing that responses of male *T. cristinae* to olfactory cues are dependent on an interaction between male population and female population (i.e. male population x female population interaction term), but only for the population pair using different hosts (i.e. Experiment #1).

Tests 1 and 2 use logistic regression analyses (test-statistic is -2LR) and test 3 uses ANOVA (test statistic is F-ratio). The 'full model' included all the factors examined and the interaction term, and 'reduced model' removed all terms for which $p > 0.10$ (Materials and Methods for details).

Test	n	Model	test-statistic	p-value
Experiment #1 - Populations on different hosts				
1. Was a female chosen?	192	Full	5.01	0.024
		Reduced	7.29	0.007
2. If the male left the center, was a female chosen?	54	Full	8.14	0.004
		Reduced	9.07	0.003
3. When a female was chosen, how long did it take?	30	Full	4.34	0.051
		Reduced	4.77	0.019
Experiment #2 - Populations on the same host				
1. Was a female chosen?	126	Full	0.20	0.65
		Reduced	Removed	>0.10
2. If the male left the center, was a female chosen?	26	Full	0.55	0.46
		Reduced	Removed	>0.10
3. When a female was chosen, how long did it take?	15	Full	1.65	0.29
		Reduced	Removed	>0.10

CHAPTER 12.
ECOLOGICAL DIVERGENCE PROMOTES THE EVOLUTION OF CRYPTIC REPRODUCTIVE ISOLATION*

*A version of this chapter appears as Nosil, P., and Crespi, B.J. 2006. Ecological divergence promotes the evolution of cryptic reproductive isolation. Proceedings of the Royal Society of London B 273: 991-997. Reprinted with permission from the Royal Society of London.

12.1 Abstract

Speciation can involve the evolution of 'cryptic' reproductive isolation that occurs after copulation but before hybrid offspring are produced. Because such cryptic barriers to gene exchange involve post-mating sexual interactions, analyses of their evolution have focused on sexual conflict or traditional sexual selection. Here we show that ecological divergence between populations of herbivorous walking-sticks is integral to the evolution of cryptic reproductive isolation. Low female fitness following between-population mating can reduce gene exchange between populations, thus acting as a form of cryptic isolation. Female walking-sticks show reduced oviposition rate and lower lifetime fecundity following between-population versus within-population mating, but only for mating between populations using different host-plant species. Our results indicate that even inherently sexual forms of reproductive isolation can evolve as a by-product of ecological divergence and that post-mating sexual interactions do not necessarily evolve independently of the ecological environment.

12.2 Introduction

Speciation involves the evolution of barriers to gene exchange (reproductive isolation) between diverging populations. Understanding speciation thus involves two major tasks: determining which reproductive barriers were involved in the initial reduction in gene flow between populations and understanding which evolutionary forces produced them (Coyne & Orr 2004).

Until recently, forms of reproductive isolation that act after copulation but before hybrids are produced have been less-explored than other types of barriers. Examples include poor transfer or storage of sperm (Price et al. 2001), failure of fertilization when gametes contact each other (Palumbi 1998; Swanson & Vacquier 2002), and reduced oviposition rates because foreign ejaculate or courtship behaviour fails to stimulate oviposition (Fuyama 1983; Gregory & Howard 1993; Price et al. 2001). These processes can lead to reduced reproductive output for between-population versus within-population matings, thereby decreasing gene exchange between populations. Such reproductive barriers are sometimes referred to as 'cryptic reproductive isolation' because they cannot be detected from mating probabilities or by examining hybrid fitness (Coyne & Orr 2004; Price et al. 2001). Despite recent examples of cryptic reproductive isolation, the processes that drive their evolution remain obscure (Coyne & Orr 2004). Selection has been implicated, but it remains unclear what forms of selection are involved.

Three main processes have been hypothesized to drive the evolution of cryptic reproductive isolation: sexual selection, reinforcing selection, and natural selection (Coyne & Orr 2004). Sexual selection may be particularly likely to cause cryptic reproductive isolation because such isolation involves sexual interactions. Most work on speciation via sexual selection has focused on divergence in mate preferences, but the same predictions generally apply to cryptic isolation as well (Parker & Partridge 1998). In particular, sexual conflict between males and females has received much attention as a mechanism driving the evolution of forms of reproductive isolation that involve sexual interactions (Parker & Partridge 1998; Rice 1998; Gavrilets 2000; Martin & Hosken 2003; Knowles et al. 2004). However, the role of sexual conflict is controversial because it may or may not promote reproductive isolation (Parker & Partridge 1998; Rowe et al.

2003). Furthermore, traditional sexual selection can generate similar predictions and can also lead to the evolution of reproductive isolation (Lande 1981; Panhuis et al. 2001; Pizzari & Snook 2003; Arnqvist 2004). However, because both sexual conflict and many forms of sexual selection are expected to operate independently from the ecological environment, divergence driven by either process leads to the expectation that reproductive isolation should occur for crosses between both ecologically-similar and between ecologically-divergent populations.

Second, a reinforcement-like process may cause cryptic reproductive isolation via selection against maladaptive hybridization (Coyne & Orr 2004). Under this scenario, greater divergence is expected between populations in geographic contact versus geographically-separated populations, because maladaptive hybridization occurs only in the former situation (Servedio & Noor 2003).

Third, speciation may occur via divergent natural selection when reproductive isolation evolves as a pleiotropic by-product of populations adapting to different ecological environments (Schluter 2000; Funk et al. 2002; Rundle & Nosil 2005). The central prediction of this 'ecological speciation' hypothesis is that ecologically-divergent pairs of populations will exhibit greater reproductive isolation than ecologically-similar pairs. If natural selection drives the evolution of cryptic reproductive isolation, then reproductive success should be reduced following between-population mating, but only for pairs of populations that are ecologically-divergent. Research on ecological speciation has focused on habitat and mate preference, or the ecological fit of hybrids to the niches of parental species (Schluter 2000; Funk et al. 2002; Rundle & Nosil 2005). Cryptic reproductive isolation has received almost no attention in studies of ecological speciation, perhaps because it is assumed that this inherently sexual form of isolation evolves via non-ecological sexual selection or sexual conflict (but see Knowles et al. 2004). We note that ecological speciation can involve sexual selection, but only those forms that depend on the ecological environment (Endler 1992; Schluter 2000; Boughman 2002; see Discussion).

One approach to addressing which processes drive the evolution of reproductive isolation is to quantify reproductive isolation in crosses between populations under

different ecological and geographic scenarios (Schluter 2000; Funk et al. 2002). However, this approach has yet to be systematically applied to the analysis of cryptic reproductive isolation. Here, we conducted crosses within and between pairs of *Timema* walking-stick populations to test for the presence and causes of forms of cryptic reproductive isolation. Because these populations differ in both ecology (host plant use) and in geography (allopatry versus parapatry), their study allows partitioning of the effects of ecology and geography on reproductive isolation. The results show that that ecological divergence between populations is integral to the evolution of cryptic reproductive isolation.

Timema are wingless, phytophagous insects inhabiting the chaparral of southwestern North America (Crespi & Sandoval 2000). Here we focus on *T. cristinae*, a species which feeds on two different host-plant species (*Adenostoma fasciculatum* and *Ceanothus spinosus*). The current study examines divergence between populations, where a 'population' is defined as all of the walking-sticks collected within a homogenous patch of a single host-plant species (as in Nosil *et al.* 2002, 2003). Pairs of populations on different hosts are considered ecologically-divergent, whereas those on the same host are considered ecologically-similar. Additionally, populations can be parapatric (i.e. in geographic contact with a population of insects adapted to the alternative host) or allopatric (geographically-separated from all populations adapted to the alternative host).

We calculated total lifetime fecundity (number of eggs laid), longevity and oviposition rate of females used in between-population versus within-population crosses (n = 31,369 eggs from 689 crosses; Table 12.1). A cross is a mating between a male and a female, thus when referring to female fitness we refer to the females used in the actual crosses (not their F1 offspring). Reduced female fecundity, longevity or oviposition rate in between-population versus within-population crosses represents a partial barrier to gene exchange because reproductive output is lower for between-population matings. Lifetime fecundity and longevity are standard measures of fitness. In *T. cristinae*, oviposition rate following mating likely also represents an important component of fitness. Females that mate with males from the alternate host likely suffer high rates of visual predation due to having less-cryptic males riding on their back for several days following copulation (Sandoval 1994a,b; Nosil et al. 2002, 2003; Nosil 2004). Thus

females engaging in between-host mating may live only a short time in nature such that a low rate of oviposition (i.e. fewer eggs per unit time) translates into reduced lifetime fitness. We note that even locally-cryptic individuals are preyed upon (albeit at a lower rate than less-cryptic individuals) such that there are potential fitness costs to low oviposition rate even for within-host matings.

We can use the geographic and ecological variation among *T. cristinae* populations to evaluate three alternative predictions concerning the processes driving the evolution of their reproductive barriers. If sexual conflict or sexual selection acting independently from the ecological environment drives evolution, then female fitness should be reduced in between-population crosses even for pairs of populations that are not ecologically-divergent (i.e. those on the same host). If reinforcing selection is important, then female fitness should be most reduced for crosses involving parapatric pairs of populations on different hosts. If divergent natural selection drives evolution, then female fitness should be reduced only for between-population crosses that involve populations using different hosts.

12.3 Materials and Methods

12.3.1 Study system

T. cristinae were captured from 28 populations in the Santa Ynez Mountains, California in spring 2003 and 2004 using sweep nets. A population is all the insects captured within a homogeneous patch of a single host-plant species. 'Parapatric' insect populations are in contact with a population of insects adapted to the alternative host (i.e. they have an adjacent population using the alternative host). 'Allopatric' populations are separated from all other populations adapted to the alternative host by distances > 50 times the 12m per-generation gene flow distance (Sandoval 1993). Allopatric populations were paired together such that the geographic distance between each population pair was comparable (Table 12.1 for population pairings). Sample sites with both hosts were chosen such that there was only one population on each host species (i.e. each parapatric population had only one adjacent population on the alternate host).

12.3.2 Population crosses

Walking-sticks were reared in glass jars at the University of California at Santa Barbara (20 degrees C) with 10-15 individuals per jar. Individuals from different populations and the sexes were kept separate. Within-population and between-population crosses were conducted (the male and the female from the exact same versus from different populations respectively).

All individuals used in the crosses were sexually-immature first instars captured in the field that were reared to sexual maturity on *Ceanothus* cuttings (about 4-6 weeks of rearing). Within two days of achieving sexual maturity, a single virgin male and a single virgin female were housed together in a petri dish until copulation was observed and then fed *Ceanothus* cuttings every second day until the female died (females lay eggs singly). We recorded the longevity of females and the number of eggs laid.

Egg number in some broods was recounted in the same year and in the subsequent year, to estimate repeatability (i.e. measurement error). Further statistical analyses were conducted on egg number from the year of initial count, in case some eggs were lost or damaged. This does not affect our results because egg number was highly repeatable both within ($r = 0.96$, $p < 0.001$, $n = 26$) and between years ($r = 0.95$, $p < 0.001$, $n = 107$). Female longevity was recorded in 652 of the 689 crosses.

Four main types of crosses were examined (within-population, between populations using the same host, between parapatric populations using different hosts, between allopatric populations using different hosts). Female longevity was correlated with lifetime fecundity ($r = 0.75$, $p < 0.001$) and the slope of this relationship did not differ among the four cross-types (cross-type x longevity interaction, $F_{3, 652} = 0.17$, $p = 0.92$; ANCOVA test for homogeneity of slopes). Thus we calculated oviposition rate as the residuals of a regression of lifetime fecundity on female longevity.

12.3.3 Statistical analyses

ANOVA analyses were used to test whether lifetime fecundity, female longevity or oviposition rate differed among the four main types of crosses examined (within-population, between populations using the same host, between parapatric populations

using different hosts, between allopatric populations using different hosts). These analyses test for cryptic reproductive isolation, but are not well-suited for the comparative test of whether ecological divergence drove evolution because individuals (rather than population pairs) are the unit of replication.

We used three analyses to provide an explicit test of the hypothesis that ecological divergence results in greater cryptic reproductive isolation. First, we used one-tailed t-tests to examine whether the reduction in females fitness for between-population versus within-population crosses was greater for allopatric pairs of populations using different hosts than for allopatric pairs using the same host. Second, a permutation test was used to provide a non-parametric test of the same hypothesis. Third, we analyzed the results within each of the three ecological scenarios considered separately (allopatric pairs on the same host, parapatric pairs on different hosts, allopatric pairs on different hosts). Paired t-tests were used to test for significant differences in female fitness for between-population versus within-population crosses, with a separate paired t-test conducted for each of the three ecological scenarios. These paired t-tests still treat pairs of populations within each scenario as the unit of replication, and thus are appropriate for comparing different scenarios.

Two types of further ANOVA analyses were conducted. First, Tukey's post-hoc tests were used to examine which specific pairwise comparisons between cross-types in the overall ANOVA analyses described above were significantly different from one another. Year (2003 or 2004) was also included as a factor in all analyses reported. Interactions between year and cross-type were always statistically insignificant and thus this interaction term is not included in the analyses presented. However, retaining this interaction in did not affect our conclusions in any way (e.g. main analysis with lifetime fecundity; main effects of cross- type, $F_{3, 689} = 3.71$, $p < 0.05$; cross-type x year interaction, $F_{1, 689} = 0.58$, $p = 0.63$). Second, we ran separate ANOVA analyses for each of the fourteen population pairs to test whether female fitness differed for between-population versus within-population crosses within each individual population pair. These analyses are required to examine which individual pairs contributed most strongly to overall trends. The model included male population, female population and the interaction between male and female population. We are interested primarily in the

interaction term because a significant interaction indicates that female fitness is dependent on which population the male originates from (i.e. her own or not).

Our rearing regime represents a common garden experiment (i.e. all insects reared on *Ceanothus*) such that differences detected between populations likely have a strong genetic component (we cannot rule out a partial role for maternal effects or early environmental effects but note that the time in the field is very small relative to the duration of rearing in a common environment). However, this design necessitates that about half of the females (i.e. those originating from *Adenostoma*) are reared on their non-native plant. Potentially, female host of origin could thus contribute to variation in female fitness. We conducted three analyses to test for such an effect, all which indicated it did not occur. First, we conducted the overall ANOVA combined among population pairs including an additional term: the interaction between cross-type and female host of origin (*Ceanothus* or *Adenostoma*). If this interaction is insignificant it indicates that the effects of cross-type were independent from female host of origin. Second, we conducted the paired-test tests restricting the results to only females originating from *Ceanothus* (thus comparing female fitness in between-population versus within-population crosses when all the females originate from the same host). Third, the ANOVA analyses on individual pairs of populations deal with this concern explicitly, because they include the main effects of female population.

12.4 Results

Cryptic reproductive isolation was detected: fecundity and oviposition rate was reduced in females used in between-population versus within-population crosses in some cases (Fig. 12.1; main effects of cross-type; fecundity - $F_{1, 689} = 6.71$, $p < 0.001$; oviposition rate - $F_{1, 652} = 15.27$, $p < 0.001$). These effects of cross-type were independent from which actual host, *Ceanothus* or *Adenostoma*, the female originated from (cross-type x female host interaction; fecundity - $F_{3, 687} = 1.68$, $p > 0.15$; oviposition rate – $F_{3,650} = 0.95$, $p > 0.25$). Female longevity did not differ among cross types, for the results combined ($p > 0.25$) or for individual population pairs (all $p > 0.05$), so is not considered further.

Comparing the results from the different ecological scenarios revealed that ecological divergence promotes the evolution of cryptic reproductive isolation. The reduction in female fitness for between-population versus within-population crosses was significantly greater for allopatric pairs using different hosts than for allopatric pairs using the same host (Table 12.1; $t_4 = 2.45, 5.20$, $p = 0.035, 0.004$ for fecundity and oviposition rate respectively, t-tests). Moreover, the reduction in female fitness for between-population versus within-population crosses was greater for all three allopatric pairs of populations using different hosts than for any of the three pairs using the same host (for fecundity and oviposition rate). The probability of this pattern arising by chance is $(3!)(3!)/(6!) = 0.05$ for each component of fitness. Based on these t-test and permutation analyses, we conclude that there is a statistical association between divergence in host-plant use and the magnitude of cryptic reproductive isolation.

Analyses of population pairs within each ecological scenario confirmed that ecological divergence is required for cryptic reproductive isolation to evolve. Specifically, significant reductions in fecundity and oviposition rate in between-population versus within-population crosses were only observed for pairs of populations using different hosts (Table 12.1 for paired t-tests). For example, crosses between allopatric populations using different hosts exhibited significantly lower female fecundity than did within-population crosses ($p < 0.05$ in the paired t-test and $p < 0.001$ in post-hoc ANOVA). Likewise, crosses between populations using different hosts exhibited significantly lower oviposition rates than did within-population crosses, and this pattern occurred for both allopatric and for parapatric pairs (for both scenarios – $p < 0.05$ in paired t-tests and $p < 0.01$ in post-hoc ANOVA). In contrast to the results for different-host pairs, there was no evidence for reduced fecundity or oviposition rate in between-population versus within-population crosses when pairs of populations using the same host were examined (all $p > 0.35$ in paired t-test and post-hoc ANOVA analyses; Table 12.1).

The results from ANOVA analyses on individual pairs of populations support the conclusion that ecological divergence drove evolution, and demonstrate that the trends are replicated across populations (Table 12.2). Although differences between individual pairs of populations were not always significant, clear and consistent trends were evident,

particularly for allopatric pairs. Female fecundity was significantly reduced in between-population versus within-population crosses for two of the three pairs of allopatric populations using different hosts that were examined ($p = 0.01, 0.01$), the trend in the third population pair was in the same direction ($p = 0.17$), and the overall differences were highly significant when combined among the three population pairs (combined $p < 0.005$). Similar differences for were detected for oviposition rate ($p = 0.04, 0.002, 0.004$ for individual pairs, combined $p < 0.001$). When all the pairs of populations using different hosts are considered (i.e. parapatric populations included), 10 of 11 pairs show reduced oviposition rates for between-population versus within-population crosses (Table 12.2; $p < 0.05$, Binomial test). Conversely, there was no evidence for reduced fecundity or oviposition rate in between-population crosses for any of the three pairs of populations using the same host (all $p > 0.20$; Table 12.2).

12.5 Discussion

We detected cryptic reproductive isolation between pairs of populations of *T. cristinae* walking-sticks, but only between pairs that are ecologically-divergent in host plant use. Thus ecological divergence promotes the evolution of cryptic reproductive isolation. The proximate mechanism by which oviposition rates are reduced has yet to be elucidated, but several hypotheses exist. First, reduced oviposition rates could reflect reduced fertilization rates with only fertilized eggs being laid (Gregory & Howard 1993). Second, they could reflect the relative inability of foreign seminal proteins to stimulate oviposition (Herndon & Wolfner 1995). Third, females might lower their oviposition rates when sperm is limited, and sperm could be most limited in between-host crosses because it is known that copulation within a one-hour period is least-likely in between-host crosses (Nosil et al. 2002, 2003). We note that all the females in our experiment copulated at least once (see Methods). It is clear that the proximate mechanism, although unknown, is linked to adaptation to different hosts.

Female fecundity is consistently reduced in crosses between allopatric populations using alternate hosts. The results for parapatric pairs are in the same direction, but less definitive and relatively heterogeneous. The observed heterogeneity among population pairs might be expected for sexual forms of reproductive isolation, if

different traits and different mutations are involved in different populations (Schluter & Price 1993; Parker & Partridge 1998; Rowe et al. 2003). Some specific pairs of parapatric populations exhibited significantly reduced female fecundity in between-population crosses, but most did not (Table 12.2). In contrast to lifetime fecundity, oviposition rates themselves were significantly reduced for crosses between parapatric pairs on different hosts (Fig 12.1; Table 12.1). To the extent that female fitness is reduced in these matings, reductions in female fecundity in between-host matings represent a cost to hybridization that could have facilitated the reinforcement of mating preferences that has been observed in these parapatric populations (Howard and Gregory 1993; Nosil et al. 2003).

Unlike mating preferences, cryptic isolation itself is not consistently strongest in parapatry, perhaps because behavioral processes that occur earlier in the life history are more effective at minimizing the costs associated with hybridization (Coyne & Orr 2004). Another explanation involves gene flow, which is known to occur between parapatric populations (Nosil et al. 2003). Homogenizing gene flow may have constrained divergence in cryptic reproductive barriers between parapatric populations of *T. cristinae*, as has been observed for other traits such as color-pattern, body size, body shape, host preference and mate preference (Sandoval 1994a; Nosil et al. 2003; Nosil & Crespi 2004; Nosil 2004; Nosil et al. 2005)

Reproductive isolation is expected to increase with divergence time (Coyne & Orr 2004). However, previous analyses of mitochondrial (COI) and nuclear (ITS) DNA sequence data in *T. cristinae* indicate that differences among population pairs in divergence time are unlikely to account for our results. Two lines of evidence support this claim. First, geographically-separated pairs of populations on the same versus different hosts show similar levels of sequence divergence (Nosil et al. 2002), yet only the latter show reduced female fitness in between-population crosses. Moreover, because levels of sequence differentiation are substantial and indicative of long periods of time since divergence (on average 3-4% and 1-2% divergence at COI and ITS respectively; Nosil et al. 2002, 2003 for details), different population pairs represent relatively independent evolutionary replicates. Second, substantial sequence divergence was detected only for population pairs that were not directly adjacent to one another (Nosil et al. 2003), yet such adjacent pairs on different hosts show more evidence for reduced female fitness than

do geographically-separated pairs on the same host (all the populations from Nosil et al. 2002, 2003 are represented in the current study). Thus ecological divergence in host-plant use, rather than neutral differentiation, predicts cryptic reproductive isolation.

The results of population crosses are not necessarily diagnostic of the existence of sexual conflict (Rowe et al. 2003), so we do not claim that sexual conflict does or does not occur in these populations. Rather, our results indicate that if sexual conflict occurs, it must interact with ecological divergence to drive the evolution of reproductive isolation. Some models of speciation via sexual selection include a role for natural selection, but they focus on the evolution of premating isolation (Lande & Kirkpatrick 1982; Schluter 2000). For example, the 'sensory drive' hypothesis predicts that mating signals will involve in correlation with aspects of the environment (Endler 1992). When this occurs, ecologically-divergent pairs of populations diverge in mating signals due to an interaction between natural and sexual selection, and show strong sexual (behavioural) isolation as a consequence (Boughman 2002). Sensory drive applies to premating signals, and in fact pre-mating sexual isolation is greater between *T. cristinae* populations using different host species than between populations using the same host (Nosil et al. 2002, 2003). Our current findings show that post-mating sexual interactions can also be influenced by the ecological environment.

Most generally, our results demonstrate that even inherently sexual forms of reproductive isolation can evolve as a by-product of ecological divergence. Thus cryptic reproductive isolation can involve both sexual interactions and ecological divergence (see also Knowles et al. 2004). Additional evidence for this hypothesis stems from studies of fertilization success in plants, where cross-fertilization occurs only when style lengths of different species are similar, and style length may be affected by natural selection (Williams & Rouse 1988; Diaz & McNair 1999). Collectively, these findings show that inclusion of ecology in models of speciation via sexual interactions may lead to novel insights into the evolution of reproductive isolation.

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Figure 12.1 Lifetime fecundity and oviposition rate is reduced for females used in between-population versus within-population crosses, but only when crosses are between populations that use different host-plant species. Differences among the four types of crosses are highly significant ($p < 0.001$, ANOVA). Letters above the 95% confidence intervals denote which specific pairwise comparisons are statistically different from one another in post-hoc analyses (same letter = no statistical difference; different letters = statistically different at $p < 0.01$). See Table 12.1 for paired t-tests and Table 12.2 for analysis of individual population pairs. A) Lifetime fecundity. B) Oviposition rate.

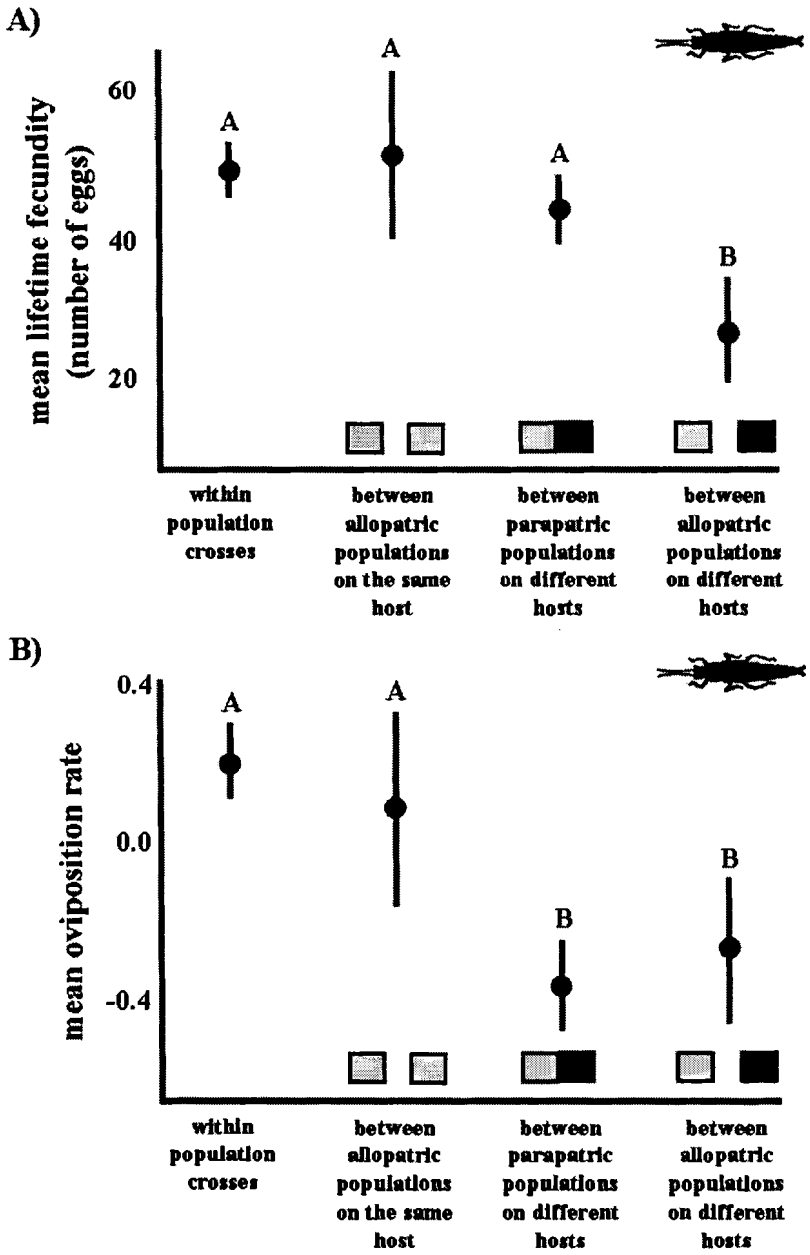

Table 12.1 Results of paired t-tests examining whether female fitness is reduced in between-population versus within population crosses (see Table 12.2 for means from individual pairs of populations).
Three different scenarios are examined; allopatric pairs of populations using the same host, parapatric pairs of populations using different hosts, and allopatric pairs of populations using different hosts. Mean difference is calculated as within-population minus between-population. Analyses confined to only females originating from *Ceanothus* yielded trends in the same direction in all cases (significance levels for these analyses are show in the extreme right-hand column).

comparison	Mean difference	Test statistic	df	p-value	p-value (C only)
Fecundity					
Allopatric same host	2.33	0.47	2	0.69	0.90
Parapatric different host	4.25	0.72	7	0.49	0.38
Allopatric different host	16.00	6.36	2	0.02	0.01
Oviposition rate					
Allopatric same host	0.00	0.00	2	1.00	0.88
Parapatric different host	0.41	2.54	7	0.04	0.02
Allopatric different host	0.60	0.60	2	0.03	0.04

Table 12.2 Results for individual pairs of populations.

Three distinct types of pairwise comparisons are considered; allopatric pairs on the same host (A), parapatric pairs on different hosts (B) and allopatric pairs on different hosts (C). Mean fecundity (fecun.), female longevity (long.) and oviposition rate (ovip. rate) is given for each pair as: mean for between-population crosses, mean for within-population crosses. F refers to the F-ratio from ANOVA analyses testing whether female fitness is dependent on an interaction between male population and female population. Combined probabilities for allopatric pairs of populations on different hosts are <0.005 and < 0.001 for fecundity and oviposition rate respectively.

pair	fecun.	n	F	p	long.	F	p	Ovip. rate	F	p
A										
1	60, 53	62	0.03	0.86	56, 54	0.13	0.72	0.0, -0.1	0.53	0.47
2	44, 54	56	1.70	0.21	39, 47	1.03	0.32	0.3, 0.3	0.48	0.49
3	23, 27	12	0.10	0.77	34, 35	0.00	0.96	-0.5, -0.4	0.40	0.54
B										
4	57, 61	85	0.32	0.57	51, 52	0.00	0.99	-0.0, 0.3	1.94	0.17
5	46, 32	32	0.70	0.41	48, 38	1.22	0.28	-0.5, -0.3	1.59	0.22
6	52, 50	16	0.01	0.94	62, 60	0.02	0.90	-0.8, -0.4	0.55	0.47
7	43, 59	52	1.60	0.21	45, 60	4.99	0.03	-0.2, -0.3	0.40	0.53
8	18, 54	70	13.73	0.01	30, 34	0.65	0.42	-0.5, 0.9	23.98	<0.001
9	50, 56	43	0.04	0.85	55, 49	0.67	0.42	-0.5, 0.2	2.64	0.11
10	44, 28	30	3.07	0.09	54, 37	3.61	0.07	-0.5, -0.4	0.25	0.62
11	44, 48	49	0.15	0.71	51, 50	0.06	0.81	-0.4, -0.1	2.79	0.10
C										
12	28, 39	53	1.97	0.17	35, 35	0.00	0.99	-0.3, 0.1	4.47	<0.05
13	25, 44	76	6.46	0.01	26, 30	0.72	0.40	-0.1, 0.6	10.70	<0.01
14	28, 46	53	6.54	0.01	40, 42	0.05	0.82	-0.5, 0.2	10.119	<0.01

Letter for each population pair refers to the codes used to denote populations in previous studies (Nosil et al. 2002, 2003; Nosil 2004). 1 = P x PR, 2 = PE x WCC, 3 = LOG x BT, 4 = HVA x HVC, 5 = MA x MC, 6 = HA x HC, 7 = OUTA x OUTC, 8 = R12A x R12C, 9 = MBOXC x MBOXA, 10 = OGC x OGA, 11 = VPA x VPAC, 12 = LA x VPC, 13 = R6C x R23A, 14 = SC x LRN.

CHAPTER 13.
GENERAL CONCLUSIONS AND UNRESOLVED ISSUES

13.1 Summary of Results and Future Directions for *Timema Cristinae*

Populations of *T. cristinae* living on two different host-plant species are divergently adapted to these hosts, and can be considered 'host-ecotypes'. For the host-ecotypes, multiple forms of reproductive isolation were greater between populations using different hosts than between similar-aged populations using the same host. This pattern was detected for habitat isolation, immigrant inviability, sexual isolation, and cryptic postmating isolation, indicating that divergent host-plant adaptation promoted the evolution of multiple reproductive barriers. The genetic details of this host-associated divergence are unknown. The genetics of speciation is a huge and burgeoning field, and progress is being made in understanding the genetics of speciation by natural selection in particular (reviewed in Chapter 2, Rundle and Nosil 2005). Key factors of interest are the role of pleiotropy versus linkage disequilibrium, the number of genes or gene regions involved, the types of gene involved, and the role of physical linkage and chromosomal inversions.

13.1.1 Genetic basis of adaptive divergence and reproductive isolation

The genetics of adaptive divergence and reproductive isolation in the *T. cristinae* ecotypes is currently being investigated using Amplified Fragment Length Polymorphism markers (AFLP's, Vos et al. 1995) and a 'population genomic approach' (Nosil, Egan, Funk unpublished data). The signature of divergent, host-associated selection can be detected at the genetic level by examining numerous molecular markers: loci that show unusually high levels of genetic differentiation between populations, but only between populations on different hosts, are assumed to be subject to divergent, host-associated natural selection (Bowcock et al. 1991; Beaumont and Nichols 1996; Luikart et al. 2003; Beaumont 2005; Vasemagi and Primmer 2005). We examined differentiation at hundreds of AFLP loci between populations of *Timema cristinae*. Simulations were used to establish the level of genetic divergence expected under neutrality. A small proportion of loci exhibit much greater divergence than predicted under neutrality in multiple comparisons between pairs of populations on different host-plant species, but conformed to neutral expectation in comparisons between pairs of populations using the same host

species. These loci are implicated in divergence due to divergent, host-associated natural selection (Nosil, Egan, Funk unpublished data). The results suggest that only a small proportion of loci are linked to genes involved in adaptation and reproductive isolation, and support other studies indicating that adaptive radiation can proceed via just a few key genes or genomic regions (e.g. AFLP genome scans for: snails, Wilding et al. 2001; budmoths, Emenialov et al. 2003; whitefish, Campbell and Bernatchez 2004; frogs, Bonin et al. 2006; also candidate gene examples from sticklebacks, Colosimo et al. 2005; monkeyflowers, Schemske and Bradshaw 1999; Bradshaw and Schemske 2003).

Additionally, associations between genotype and ecologically-relevant phenotypes can be examined within populations (e.g. Nachman et al. 2003; Hoekstra and Nachman 2003). This approach can identify molecular markers associated with ecologically-important phenotypic traits and thus functionally/ecologically-relevant genetic variation (such markers are likely in genes, or linked to genes, affecting selected traits; Gupta et al. 2005; Vasemagi and Primmer 2005). For example, several AFLP markers associate strongly with the presence / absence of the stripe phenotype within populations of *T. cristinae* (Nosil, Egan, Funk unpublished data). This work is preliminary and ongoing, but holds promise for unlocking the genetic basis of the divergence documented in this thesis.

13.2 Retrospective Thoughts – What Now?

Detailed studies of the role of ecology, geography and genetics on the evolution of reproductive isolation can yield a comprehensive picture of the evolution of reproductive isolation within a particular pair of ecotypes or species. The question is no longer simply 'can natural selection promote the evolution of reproductive isolation?', because there are numerous examples where clearly selection has done so (as reviewed in Chapter 2, Rundle and Nosil 2005). However, these detailed studies of one pair of ecotypes or species captures but a single point in the continuous process of speciation, in a single taxon. Replicated studies across the species boundary are now required to build up a picture of how speciation unfolds from beginning to end, within a particular group of organisms. If such studies are conducted across disparate taxa, tests for generalities can be made.

13.2.1 Transitions along the continuum of speciation

Speciation is often a continuous process whereby populations diverge from randomly mating units to reproductively isolated species. Case studies of single taxa capture a single point in the extended process of speciation. Case studies of single reproductive barriers capture a single contribution to the evolution of reproductive isolation. Such studies are absolutely critical to a detailed understanding of particular aspects of speciation, as demonstrated by the work on the *T. cristinae* ecotypes outlined in this thesis. However, gaining a more complete understanding of speciation as a process requires the comparative study of populations representing diverse stages of evolutionary divergence, and of the contributions of multiple reproductive barriers (Dres and Mallet 2001; Coyne and Orr 2004). Studies examining multiple forms of reproductive isolation even within a single stage of divergence are relatively rare (but see Craig et al. 1993; McMillan et al. 1997; Funk 1998; Mendelson 2003; Ramsey et al. 2003; Levitan et al. 2004), and studies of diverse stages of divergence almost completely lacking (but see Mallet et al. 1998).

The host-associated forms of *T. cristinae* are unlikely to have achieved species status, as indicated by only partial barriers to gene flow at the premating level and weak mtDNA differentiation between adjacent populations on different hosts due to ongoing gene flow (Nosil *et al.* 2003). The host forms represent either an ongoing speciation event or population divergence that has reached equilibrium such that we have examined the early stages of the speciation process. A similar scenario appears to occur for host-plant ecotypes of *T. podura*, a non-sister species of *T. cristinae*. *T. podura* has also formed ecotypes on *Ceanothus* and *Adenostoma,* and these ecotypes exhibit divergent host-adaptation and the evolution of partial reproductive isolation as a by-product of host adaptation (Sandoval and Nosil 2005). But again, complete speciation does not appear to have occurred. Studies of more divergent taxa are required to elucidate the factors facilitating the transition from ecotype to species, from weak to strong reproductive isolation. The degree to which the same traits and processes are involved at different stages of speciation is poorly understood (Claridge and Morgan 1993; Ryan and Rand 1993; Mallet et al. 1998; Boake et al. 1997; Bordenstein et al. 2000; Jiggins et al. 2004). Studies examining multiple selective processes and reproductive barriers, at different

stages of divergence, will likely provide the most complete picture of the how the entire process of speciation unfolds, from beginning to end.

13.2.2. Generality of ecological speciation

Examples of ecologically driven reproductive isolation in a few individual taxa have begun to accumulate, but it remains unclear whether these cases represent the exception – reflecting the non-random selection of study taxa – or the rule. Examining patterns across disparate taxa is required to determine the generality of ecological speciation; thus addressing the question of generality requires a comparative approach. A potentially powerful approach for studying ecological speciation was offered by Funk et al. (2002) who proposed adding an ecological dimension to comparative studies investigating the relationship between reproductive isolation and divergence time (where genetic distance is used as a proxy for time, reviewed in Coyne and Orr 2004). This approach was recently applied by quantifying ecological divergence for >500 species pairs from eight plant, invertebrate, and vertebrate taxa where the association between reproductive isolation and time had been previously determined, and then statistically isolating the association of ecological divergence with reproductive isolation (Funk et al. 2006). The results revealed a consistently positive association between ecological divergence and reproductive isolation across the disparate taxa (independent of time). These findings are consistent with the hypothesis that ecological adaptation plays a taxonomically general role in promoting reproductive isolation and speciation. However, further studies are required as only eight taxa were represented in the Funk et al. (2006) study, and the role of factors such as geography and genetics was not examined. Clearly though, comparative studies offer promise for uncovering evolutionary generalities.

13.3 Concluding Statement

As described in this final chapter, replicated studies of the role of ecology, geography and genetics, both within and among species and from many disparate taxa, may allow us to eventually build up a comprehensive understanding of Darwin's 'mystery of mysteries'.

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