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AN ANALYSIS OF CLIMATE INDUCED HYBRID SPECIATION IN TIGER SWALLOWTAIL BUTTERFLIES (*PAPILIO*)

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AN ANALYSIS OF CLIMATE INDUCED HYBRID SPECIATION IN TIGER SWALLOWTAIL BUTTERFLIES (*PAPILIO*)

By

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ABSTRACT

AN ANALYSIS OF CLIMATE INDUCED HYBRID SPECIATION IN TIGER SWALLOWTAIL BUTTERFLIES (*PAPILIO*)

By

Gabriel J. Ording

North American *Papilio canadensis* and *P. glaucus* (Lepidoptera: Papilionodae, these *Papilio* = *Pterourus*), have been described as having allopatric distributions separated by a narrow hybrid zone. The range of hybridization is directly correlated with a well-defined thermal landscape. In light of recent climate shifts, potentially associated with global warming, there have been increased levels of genetic introgression. This dissertation describes the morphological and genetic status of unique isolated hybrid swarm populations, on South Manitou Island in Michigan and in the Battenkill River Valley near the border of New York and Vermont, that have arisen near the range boundaries of these two closely related species of Tiger Swallowtail butterflies. Climate induced genomic mixing may be responsible for 'climatic speciation', as appears to be occurring in a case of incipient speciation near the border of southern New York and Vermont. Additionally, it is suggested that the generation of these unique genetic populations best explains the origins of a recently described new *Papilio* species (*Pterourus appalachiensis* Pavulaan & Wright, 2002) via hybrid speciation. Furthermore, the significance of an identified X-linked gene complex controlling both diapause and the expression of the lactate dehydrogenase enzyme is examined in laboratory crosses and is proposed as a primary mechanism in the historic maintenance of the hybrid zone and also is likely instrumental in the reported cases of hybrid speciation.

Again, to my family

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CHAPTER 1:

INTRODUCTION

An American Entomologist first coined the term *speciation* in 1906. In the words of Orator F. Cook, "*Speciation*, to give the process a name, is the origination or multiplication of *species* by subdivision, *usually*, if not always, as a result of environmental incidents" (Berlocher 1998). Before and since that time there has been an increasingly heated debate about the "species concept" and the best definition to apply (Hey 2006). Until recently the biological species concept (BSC) championed by Ernst Mayr (1963) has been used as the primary default in most biological investigations. The BSC indicates that species are groups of interbreeding populations that are reproductively isolated from each other (Mayr 1963, 1995). However, there are weaknesses associated with the BSC that make it difficult to apply to every biological situation. As a result there have been many attempts at redefining *species*, but so far no single definition works in every situation. Concisely defining the species concept will likely be a never-ending source of scientific controversy. A concrete definition is of great importance in certain situations, in taxonomy for example. A concise definition is also important in conservation efforts, where funding and resources are limiting. For many evolutionary biologists however, who are more concerned with evolutionary processes, the need for a single *species* definition is less crucial, and accurately identifying the mechanisms by which differentiated populations of organisms arise is of the utmost importance. Identification of the mechanisms that drive evolution will allow for explanations as to the "origins" of the vast amount of biodiversity on the planet.

As indicated by Cook, *speciation* is best described as a process rather than a singular event, and is the series of events that lead to populations of organisms becoming genetically differentiated and ultimately reproductively isolated from one another (Harrison 1993; Coyne and Orr 2004). The relationship between two distinct populations can be placed along a continuum on a sliding scale between early phases of differentiation and true species. Central to the BSC is the establishment of reproductive isolation between populations, such that the genetic differences between unique populations can become enhanced through further evolutionary processes. As first developed by Mayr, the BSC indicated that reproductive isolation is accomplished through intervening "isolating mechanisms". Many evolutionary biologists have opted for the use of the alternative phrase "isolating barriers" (Coyne and Orr 2004), to describe the combination of factors that either prevent mating altogether (prezygotic) and / or those factors that result in infertile or inviable offspring being produced if mating were to occur (postzygotic). It has been suggested that the presence of only postzygotic barriers to gene flow indicates that the differentiating populations have only recently begun down the evolutionary pathway towards complete speciation, and that the presence of prezygotic barriers is indicative of being further along the evolutionary pathway towards complete speciation (Presgraves 2002). Reinforcement has been proposed as the process by which prezygotic barriers to gene flow arise in areas where recently diverged populations overlap, such as in hybrid zones (Howard 1993). These additional prezygotic barriers would act to prevent futile attempts at mating that would only result in wasted energy expenditure.

When originally developed, the BSC relied upon geographic isolation, to allow for divergence in allopatry (Mayr 1963). The work of Cook indicates that he too meant that *speciation* occurred as a result of geographic isolation, when he referred to "environmental incidents" (Berlocher 1998). Any geographic barrier (a newly arising mountain range, river, ocean, or glacial advance) that can prevent gene flow between populations can act as a ready made isolating mechanism. This is a piece of what makes allopatric speciation so appealing and intuitive is that there is a clear barrier that prevents any gene flow, thus allowing for an accumulation of genetic changes to occur independently in both populations. Because of its elegant simplicity, allopatric speciation has been the dominating view as to how speciation has occurred.

A recognized alternative to allopatry, heavily championed by Guy Bush of Michigan State University in the early 1960's, is the concept of sympatric speciation. Sympatric speciation does not require any geographic distance or barrier to allow speciation to occur. Typically the mechanism that allows for sympatric speciation involves disruptive selection leading to a shift in the ecological niche of an organism. Two of the model examples exploring sympatric speciation have been an alteration in the gene complexes that determine host plant preferences in *Rhagoletis pomonella* (Bush 1975, 1993; Bush and Smith 1998) and sexual selection in African cichlids (Higashi et al. 1999; Mendelson and Shaw 2005).

Another potential pathway to allow for speciation has been proposed, *allochronic speciation* (Alexander and Bigelow 1960). Allochronic speciation, similar to and perhaps best described as a subcategory of sympatry (Bush 1975; Tauber and Tauber 1981), does not require any geographic boundaries to prevent gene flow, but relies upon populations

becoming temporally isolated. If populations are separated by time, during the reproductive portion of their lifecycle, this has the same effect of preventing mating opportunities, as do strong host plant preferences, mate recognition systems, or mountain chains and glaciers. Frequently cited examples of speciation, proposed to have been driven by allochronic speciation include North American crickets (Alexander and Bigelow 1960; Harrison 1979), periodic cicadas (Cooley et al. 2001), and gall-forming aphids (Abbot and Withgott 2004). The ultimate ecological effect of both sympatric and allochronic speciation is "niche packing", allowing unique populations to take advantage of slightly different and untapped ecological resources, while minimizing or eliminating niche overlap and competition.

Speciation being viewed as a process suggests that there must be a sequence of events that initiates the process of divergence, and that an accumulation of genetic changes must occur before complete reproductive isolation can be achieved. Temporal isolation may be the first step in the process of speciation, but it may also be the only necessary step to allow for complete reproductive isolation (Tauber and Tauber 1981). This being the case, if the timing of significant life-history traits is controlled by a relatively small number of genes, or even an individual locus, minimal genetic modifications could result in striking effects.

Climate Change

Biogeography is the ecological discipline devoted to investigating the interaction of environmental factors that ultimately determine the distribution and abundance of organisms. There is a unique and intricate interplay of ecological factors (abiotic and

biotic) for which every species has evolved its own range(s) of tolerance. Global climate change is providing evolutionary ecologists unique opportunities to investigate factors that are involved in the maintenance of distinct populations and also the factors that may contribute to the processes of speciation. Global warming is having a diverse combination of ecological impacts on natural populations. Climate change is leading to shifting environmental conditions and selective pressures. Increased average global temperatures have resulted in a shortening of winter like weather in many locations (Stenseth and Mysterud 2002; Parmesan and Yohe 2003). One result of this is the phenology of flowering plants and animals is being drastically altered. Earlier annual flowering times have been described for many plants (Miller-Rushing et al. 2006) and the observed flight times of many insects have been altered (Parmesan 2006). Physiological shifts in the photoperiodic induction of diapause have been reported in response to climate change (Bradshaw and Holzapfel 2001). These shifts then have resulted in the disruption of coevolved synchronized insect plant interactions (Parmesan 2006). The genetic diversity of some insect species has rapidly eroded in response to natural selection associated with climate change (Rodriguez-Trelles and Rodriguiz 1998). Ultimately, this loss of genetic variation will reduce these populations ability to cope with environmental changes. The predicted rates at which global temperatures are expected to shift will be far too rapid for even robust populations to cope with, let alone populations that are genetically challenged.

However, climate change may actually lead to increased levels of genetic diversity and rates of evolution in some insect populations for a combination of reasons. Insect population growth rates are strongly influenced by thermodynamics. Increased

temperatures associated with anthropogenic climate change are expected to lead to increased population growth rates of certain species in many locations (Frazier et al. 2006). This in itself may have tremendous ecological and evolutionary impacts. In addition, climate-speciation hypotheses have been proposed that suggest that increased metabolic rates brought about by increased temperatures may result in increased rates of mutation, and in turn increased rates of evolution (Rohde 1992; Balanya et al. 2006; Wright et al. 2006). The ranges of many insects, including butterflies, in both Europe and North America have been found to expand and shift towards higher latitudes and elevations (Karban and Strauss 2004; Parmesan and Yohe 2003). Shifting species ranges is now allowing for increased contact and increased levels of gene flow between historically isolated populations (Ording 2001). Additionally, it has been shown that mutation rates and genome diversity frequently increases in organisms that are under stressful environmental conditions (Nevo 2001), as would be the case for animals near range boundaries or during periods of climatic change. When historically stable hybrid zones between closely related species are destabilized by shifting climates, this often serves to promote increased levels of introgression. This in turn, promotes enhanced levels of genetic variability that can allow newly formed genotypes to evolutionarily shadow environmental changes. This in effect can lead to 'climatic speciation' (Masaki 1978) through the recombination of preexisting characters (Dowling and Secor 1997; Balanya et al. 2006).

Hybridization

For a long time, botanists have viewed the occurrence of hybridization in nature as an important process in the evolution of plants. Zoologists on the other hand, have historically viewed animal hybrids to be evolutionary dead ends that have little or no ecological value, with hybrids expressing reduced fitness (Mayr 1942). Recently however, more and more animal investigations are identifying potential ecological and evolutionary value in hybridization as sources of new genetic combinations (Dowling and Secor 1997). In some situations, these novel genotypes may exhibit hybrid vigor and levels of increased fitness as compared to either parental genotype (Arnold and Hodges 1995; Harrison 1993). Additionally, it has been suggested that an evolutionary value of hybrids is that they can potentially be well adapted to new and unique environmental conditions (Arnold 1997).

A hybrid zone is a geographic region where the ranges of two genetically distinct populations overlap, and in which a certain amount of interspecific mating occurs that results in some offspring of mixed ancestry (Harrison 1993). Hybrid zones have been described as unique locations in which to study the processes of evolution in that they act as "windows into the evolutionary process" or as "natural laboratories" (Barton and Hewitt 1985). Hybrid zones can be identified by the presence of clines. A cline is a geographic location between two genetically distinct populations in which there is a gradient identifiable from one genetic character state to another. Clines between unique populations allow for unique opportunities to investigate the processes and mechanisms that are driving the evolution of isolating mechanisms between distinct populations, and cline theory is of extreme significance when discussing hybrid zones.

Closely related, but distinct species, are often times recognizable based upon one or more diagnostic characters or markers (morphological or genetic). A cline would represent the geographic location that demarcates the hybrid zone, the geographic location in which one could expect to find specimens of mixed ancestry. Some authors use the terms cline and hybrid zone interchangeably. Investigations conducted in these hybrid zones can help to shed light on the various mechanisms involved in evolutionary processes, such as speciation (Harrison 1993; Arnold 1997; Coyne and Orr 2004).

The maintenance of the width and shape of a cline is largely the result of a balance between dispersal and selection. There is a direct relationship between the ability for long-range dispersal and cline width (Harrison 1993). Similarly, the strength of selective pressures on various characters will help to dictate cline width. If a cline exhibits a comparable width along its entire length, this might indicate that the strongest selective pressures acting to maintain distinct populations are the result of negative endogenous selection through genetic incompatibilities (Barton and Hewitt 1985). However, if the cline exhibits a mosaic pattern, this could be indicative of a heterogeneous environment in which exogenous selective pressures are acting more strongly. Mosaic clines and hybrid zones, transitions from one population to another, often times coincide with ecotones (distinct boundaries across an environmental gradient). The varying environmental conditions on either side of these ecotone boundaries may in fact be the primary selective pressures that initiated population divergence (Barton and Hewitt 1985).

The slope of a cline can potentially infer the strength of selection (Kirkpatrick and Barton 1997). A steep cline for any given character would indicate extremely strong

selective pressures. Different diagnostic characters may exhibit differences in the mean slope. This would indicate that the relative strength of selection on each locus is variable. If a steep slope indicates strong selection, a subtler slope would be indicative of relaxed selective pressures. This differential strength of selection allows for asymmetrical gene flow at different loci. Certain genetic alleles may have an increased ability to introgress further beyond the center of the cline than can other alleles. A steep cline for one trait can potentially be the result of strong selection on that locus directly, or perhaps could be the result of tight genetic linkage with some other loci under strong selective pressures (Harrison 1993). In this way, clines and hybrid zones can act as semipermeable barriers to gene flow.

When sampling and identifying the genotypes of specimens within a cline, hybrid zones can be categorized along a continuum, ranging from unimodal to bimodal, with unimodal hybrid zones being primarily composed of individuals that are genotypically intermediate between both parental forms, and bimodal hybrid zones being composed of individuals whose genotypes resemble one of the two parental form (Jiggins and Mallet 2000). Bimodality in a hybrid zone is thought to represent a system in which the parental forms are more fully diverged and unimodal hybrid zones represent the juncture between two populations that are less fully reproductively isolation (Kondrashov et al. 1998).

Hybrid zones and cline centers often remain highly stable over the course of time and do not shift in position. This is often the result of the cline being coincident with an ecological gradient that maintains the position of the cline center through exogenous selection (Coyne and Orr 2004). In fact, the frequency of hybrid individuals can some times be much higher than would be expected in these intermediate environmental

conditions due to bounded hybrid superiority (Woodruff 1989), a condition in which hybrid offspring express increased fitness to that of either parental population under unique environmental conditions (Collins 1984). Cline movement, the gradual shifting of the cline center farther towards one or the other parent population has however been known to occur. This is most often the case when endogenous selective pressures are acting to prevent complete mixing of the parental gene pools, but some amount of introgression and "leaking" is occurring near the population borders (Harrison 1993).

Genetic Incompatibilities

There is an ongoing quest to identify common patterns and mechanisms that can help to explain the various processes of evolution and speciation in plants and animals. Among the common patterns, identified by both botanists and zoologists, that aids to prevent hybridization is that of hybrid breakdown. The fitness of hybrid offspring is frequently greatly reduced from that of either parent population. Without knowing the molecular mechanisms by which it could arise, Darwin suggested that hybrid sterility was the result of unknown differences in the parent populations leading to incompatibilities in hybrid individuals (Darwin 1859; Presgraves 2007). There are intrinsic forces at work that help to prevent gene flow between distinct populations and thus the melding of the respective gene pools.

In the vast majority of taxa studied, hybrid offspring are frequently found to be sterile or inviable. It has been identified that the combination of diverged genetic backgrounds leads to intrinsic genetic incompatibilities (Coyne and Orr 2004). Over time, populations evolve coadapted gene complexes that have been "tested" and "sculpted" by selective forces under unique local environmental conditions. These gene

combinations have been "crafted", through natural selection, to work in concert. When hybridization occurs, incompatibilities arise due to disruptions of these coadapted gene complexes that result in negative epistatic interactions between alleles that have not coevolved. These endogenous factors resulting in a reduction of fitness, due to the crossing of diverged genetic backgrounds, is one mechanism that results in outbreeding depression.

Among the most widely accepted "rules" that can be applied to the vast majority of evolutionary systems that helps to in part offer a mechanism that explains outbreeding depression is Haldane's Rule, or the Haldane Effect (Orr 1997). Haldane's Rule suggests that when hybridization occurs the result is a distinct increase in the occurrence of sterility or inviability in the hybrid offspring of the heterogametic sex. According to Haldane's Rule, in a hybrid zone the heterogametic sex will be extremely rare or completely absent. This has been largely supported in the vast majority of animal hybrid zones investigated. This indicates that there are some seemingly universal processes driving evolution and speciation. The result of Haldane's Rule is postzygotic isolation driven by genetic incompatibilities of loci on sex chromosomes (Presgraves 2002).

There are several hypotheses that have been proposed to explain the mechanism(s) underlying Haldane's Rule, for which the Dominance Theory has received the vast majority of support. The Dominance Theory suggests that *complementary* alleles on the X chromosome can lead to sterility or inviability and are partially recessive. A hybrid individual in a hemizygous state (receiving only one X chromosome) would have no alternative allele to mask the deleterious recessive (Orr 1997).

The severity of the Haldane Effect has been found to increase as the level of genetic divergence between the hybridizing taxa increases. This process of enhanced genetic incompatibility is the result of a "snowballing effect" (Coyne and Orr 2004). Use of a model put forth by Dobzhansky (1937) and Muller (1942) is very useful in explaining how this would occur. Consider two populations with common ancestry but that have diverged over time. Before divergence had occurred, the fixed genotype for the population at two loci could have been described as *aabb*. After divergence a new allele arose through mutation in population 1 for the *a* locus and through drift and/or selection, populations 1 genotypes had become fixed as AAbb. Similarly, population 2 became fixed for a mutation at the *b* locus, leading to the population 2 genotype being aaBB. These mutations arose independently of each other but in the presence of comparable genetic backgrounds. However, the new A and B alleles had never been "tested" together. In hybrid offspring, these are the *complementary* alleles, in that they are genetically incompatible (Orr 1997).

Extend this idea to populations that have diverged and have become distinct at multiple loci. With each new allele arising in each of the populations, there are increasing opportunities for allele combinations that are incompatible. Figure 1.1 illustrates the process of incompatibilities arising in diverging populations. Time is represented as moving forward moving up the diverged lineages. In each case the lower case letters represent the ancestral allele, the upper case letters represent the derived characters. Each derived allele has not been "tested" against the genetic background of the opposite lineage. With each new derived allele there is an increasing opportunity and

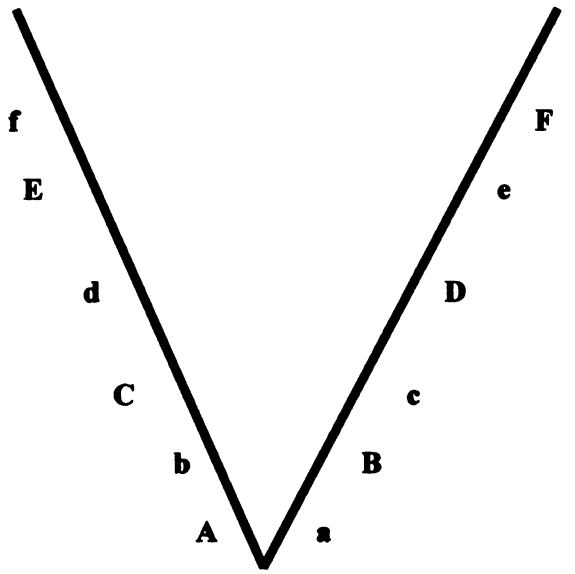


Figure 1.1. A diagrammatic illustration of the process by which incompatibilities could arise in diverging populations. Time is represented as moving forward moving up the diverging lineages. In each case the lower case letters represent the ancestral allele, the upper case letters represent the derived characters. Each derived allele has not been "tested" against the genetic background of the opposite lineage. With each newly derived allele there is an increasing opportunity and number of possible combinations in which genetic incompatibilities could occur. The end result of the "snowballing effect" would be complete reproductive isolation. (Figure modified from Coyne and Orr 2004.)

number of possible combinations in which genetic incompatibilities could occur. The end result of the "snowballing effect" would be complete isolation.

When Haldane's Rule is identified between hybridizing populations, this is an indication that complete reproductive isolation has not yet been achieved. If genetic incompatibilities, especially on the X-chromosome, are a major driving force in the process of speciation, the "snowballing" effect associated with Haldane's Rule will in theory ultimately lead to complete isolation and speciation (Coyne and Orr 2004).

Exogenous factors can also lead to outbreeding depression. Populations of organisms can become highly adapted to local environmental conditions. The alleles that form the coadapted gene complexes are suited to specific ecological conditions. Ecological constraints can prevent the movement of certain alleles across environmental gradients for which they are not adapted. Often times, this is one of the mechanisms found to delineate the range boundaries between closely related species. A widely recognized example of an ecological gradient constraining the adjacent distribution of closely related populations is temperature and the effect that it can have on certain metabolic enzymes (Powers et al. 1986; Dimichele and Powers 1991).

Distinct alleles of certain enzymes have been shown to exhibit structural variability and temperature dependent differences in thermal stability and function (Adams et al. 1973). Some alleles perform much better at higher or lower temperatures, whereas others perform poorly under certain temperature regimes (Angiletta et al. 2003). Different allelic forms of enzymes having differing thermal stabilities have resulted in a steep latitudinal cline in marine fishes directly correlating with environmental temperatures (Crawford and Powers 1989; Dimichele and Powers 1991). Evidence that

differing thermal environments impose striking selective pressures on different structural forms of enzymes has been clearly shown (Eanes 1999). A combination of these genetic and ecological incompatibilities prevents widespread hybridization and complete mixing of distinct population genomes. However, where population ranges meet, there can be opportunities for hybridization and a localized zone of genetic mixing. Hybrid zones are typically narrow geographic ranges, often times correlated with some distinct ecological boundary, in which specimens of mixed ancestry can be found (Harrison 1993).

Typically, hybrid zones are narrow geographic regions where extensive genetic exchange and introgression is prevented through some form of hybrid unfitness (Barton and Hewitt 1985). However, under conditions where two closely related populations meet and hybridize, localized populations may arise, that under localized environmental conditions, exhibit no apparent loss of fitness. These populations may be composed of individuals that exhibit a diverse array of genetically recombined forms. These populations composed of diverse recombinant types are best described as hybrid swarms (Harrison 1993). Given that hybrid zones often correlate to environmental gradients, they represent boundaries for both parental populations that are potentially imposed as a result of ecological limitations (e.g., thermal landscape). The mixing of two distinct parental genotypes can result in genetic incompatibilities and a loss of fitness, but alternatively can offer an opportunity for the production of unique genetic combinations that can actually result in offspring that have increased fitness compared to either parental form under the localized environmental conditions. This state of hybrid vigor, where offspring exhibit higher fitness than either parental form under the extreme environmental

conditions associated with an ecotone is described as "bounded hybrid superiority" (Moore 1977).

Populations near species range boundaries are often times under stress and experience relatively increased levels of mutation and recombination (Hoffmann and Hercus 2000). A common phenomenon has been described in many hybrid zones, the rare allele effect, where allozymes that are extremely rare or absent from either of the parental populations appear at surprisingly high frequencies within hybrid swarm populations (Woodruff 1989). The most frequently proposed mechanism by which these alleles are brought to high frequencies suggests that it is the result of the strong selection that can occur within hybrid zones, purging genetic incompatibilities and the alleles that are closely linked to them. Alleles that had been rare, but associated with genetically viable allelic combinations, are then brought to high frequencies through genetic "hitchhiking" (Schilthuizen et al. 1999). As a result of this rare allele phenomenon occurring in association with hybrid zones, these novel allozymes have been referred to as "hybrizymes" (Woodruff 1989). Often times, these hybrizymes are completely unique to the hybrid population, and are completely absent from either parental population. These alleles are not the result of recombination of parental genetic material, but instead are typically the result of single nucleotide substitution in a parental allele (Schilthuizen et al. 2001). These hybrizymes are established local evolutionary novelties that have been described in systems as neutral alleles, but could also potentially provide adaptive benefits under local environmental conditions.

Hybridization between distinct populations has historically been viewed as an evolutionary waste of reproductive energy. More recently however a great deal of

research is indicating that the novel genotypes that arise through the crossing of distinct parental populations, can possess great evolutionary potential. Hybrid offspring are genetically distinct from either parental population and can potentially exhibit higher fitness in a novel habitat or under extreme conditions for which neither parental population is well adapted (Gompert et al. 2006). There are an increasing number of investigations exploring hybridization as a mechanism that can be involved in the process of speciation. Hybrid speciation has been proposed in plants (Wolfe et al. 1998; Riesberg et al. 2003), fish (Salzburger and Sturmbauer 2002), and insects (Schwarz et al. 2005, 2007), including Lepidoptera (Scriber and Ording 2005; Gompert et al. 2006; Maverez et al. 2006). The intent of this dissertation is to explore the multiple components that are thought to be responsible for, in part, the current distribution of the hybrid zone between two closely related species of Tiger Swallowtail, *Papilio canadensis* and *Papilio* glaucus, but more importantly, to investigate the ecological and genetic factors that are thought to have led to unique cases of hybrid speciation.

Butterflies as Model Organisms for Ecological and Evolutionary Study

Insects in general have served as ideal model systems through which to gain a tremendous amount of scientific insight into genetics, associated with both developmental and evolutionary biology. There have been a great number of significant advances in population genetics and evolutionary theory accomplished through investigations on Lepidoptera. Classic textbook examples of genetic research conducted on Lepidoptera include natural selection and industrial melanism in the Peppered Moth (*Biston betularia*) originally reported by J.W. Tutt (1896) and later made widely known

by B. Kettlewell (1973) (see reviews by Coyne 1998; Grant 1999; Cook 2003; Majerus 2005); the migratory movements of Monarch Butterflies (*Danaus plexippus*) originally described by F.A. Urquhart (1960) and their associated population genetic structuring later described by Eanes and Koehn (1978); the idea of *coevolution* of insects and plants described by Ehrlich and Raven (1964) was heavily based upon descriptions of butterflies and their associated host plants; and widely cited examples of the variety of processes that potentially drive *speciation* have been put forth by Chris Jiggins and James Mallet (Jiggins and Mallet 2000; Jiggins et al. 2001).

Studies on Lepidoptera have also greatly enhanced our understanding of the dynamic genetic processes associated with hybrid zones (Barton and Hewitt 1985; Mallet and Barton 1989; Dasmahapatra et al. 2002). The emphasis of hybrid zone research has primarily been focused on making inferences from distinct populations as to the mechanisms that initially drove differentiation and speciation, and then also the mechanisms that allow for the maintenance of two distinct species across hybrid zone boundaries (Harrision 1993; Jiggins et al. 1997). Recently however, there have been an increasing number of investigations identifying the possibility of hybridization as a mechanism that can ultimately allow for speciation (Ungerer et al. 1998; Salzburger et al. 2002; Gross and Rieseberg 2005; Scriber and Ording 2005; Schwarz et al. 2005, 2007; Gompert et al. 2006). The hybrid zone between *Papilio glaucus* and *Papilio canadensis* provides an ideal system with which to investigate this possibility.

Two Species

North America is home to two closely related species of Tiger Swallowtail butterflies, *Papilio canadensis* and *Papilio glaucus*. Only recently were these two species elevated from subspecies ranking and granted distinct species status (Hagen et al. 1991). These two species are distinguishable on the basis of multiple morphometric, physiologic, behavioral, and biochemical traits (Table 1.1; Scriber 1994). The documented range of the northern of the two species, *Papilio canadensis*, extends all the way into Alaska, whereas the range of the southern species, *Papilio glaucus*, extends southward all the way to the tip of Florida (Figure 1.2; Scriber 1994).

For many species across North America, a Berengial refuge has long been proposed as an historic isolated geographic location that would have allowed for allopatric speciation to occur. After the glacial retreat, some 15,000 – 25,000 years ago, diverged populations would have been able to repopulate areas to the south and come into secondary contact with ancestral populations. It has been suggested that these two *Papilio* species arose through genetic differentiation in allopatry during the Pleistocene ice age. With the spread of the ice sheets over vast portions of North America, a small relic population, that eventually became *P. canadensis*, was isolated in the far northwestern Berengial refuge (Scriber 1988, Scriber et al. 1991). *P. glaucus* populations were forced southward by the ice sheets. Glacial retreat then allowed for the ranges of these two populations to expand and again meet.

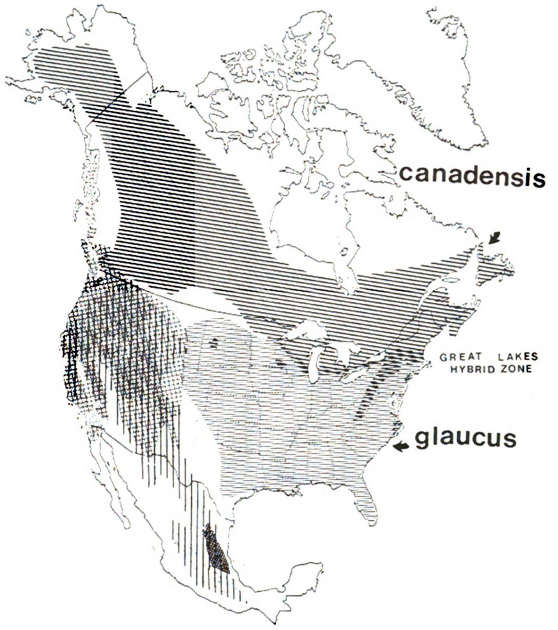


Figure 1.2. The geographic ranges of two closely related species of Tiger Swallowtail Butterflies, *Papilio canadensis* to the north and *Papilio glaucus* to the south (modified from Scriber 1996a). Indicated in the Western United States are the ranges of three largely sympatric species, *P. rutulus, P. eurymedon, and P. multicaudatus*. Represented in Mexico is the range of *P. alexiares*.

Table 1.1 – Summary of genetic differences discussed between *Papilio glaucus* and *Papilio canadensis*. (Modified from Table 1 in Scriber 1990).

P. glaucus trait	*P. canadensis* trait	Mode of inheritance	Reference
1. Pgd 100, 50	Pgd 150,125,80	x-linked allozyme	Hagen & Scriber 1991
2. Ldh 100	Ldh 80, 40	x-linked allozyme	Hagen & Scriber 1991
3. Hk 110	Hk 100	autosomal allozyme	Hagen & Scriber 1991
4. Tuliptree detoxification		2-4 loci? dominant autosomal?	Scriber 1986
5.	Quaking aspen detoxification	2-4 loci? dominant autosomal?	Scriber 1986
6. Narrow anal wing band	Broad anal wing band	?	Luebke et al. 1988
7. Large Forewing length	Small Forewing length	polygenic	Luebke et al. 1988
8. Prefers tuliptree for oviposition	Prefers aspen for oviposition	?	Scriber 1994
9. Dark Morph ♀ (b+)	(b-)	y-linked	Scriber 1994
10. Dark Morph "Enabler" (s-)	Dark Morph Suppressor (s+)	x-linked	Scriber 1994

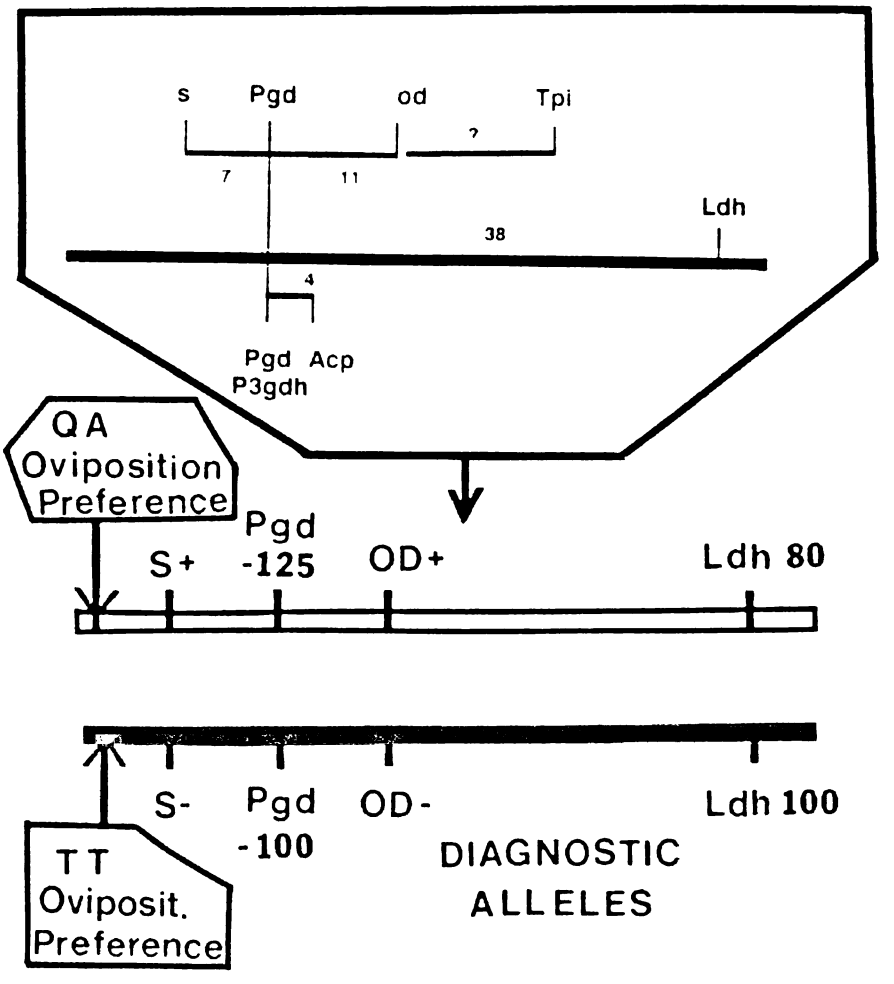


Figure 1.3. Proposed linkage map of the X-chromosome loci in Tiger Swallowtail Butterflies (Modified from Hagen and Scriber 1989). The chromosome represented in white depicts the canadensis-type chromosome, whereas that represented in black depicts the glaucus-type. Chapter 4 identifies the likely possibility that the Ldh 100 and od- loci are in fact much more closely linked that suggested by this linkage map.

Papilio Hybrid Zone

After the Pleistocene glaciers retreated, the ranges of *P. canadensis* and *P. glaucus* were able to again expand and meet in secondary contact. There is currently a narrow band that extends across the Midwest into New England at approximately 41-44 °N where the ranges of these two species overlap and hybridization is able to occur (Figure 1.4; Remington 1968; Scriber 1996a). This hybrid zone is best characterized as unimodal in nature (Jiggins and Mallet 2000). Towards the East, this range of overlap becomes more confused and sweeps southward into the Appalachian Mountains (Pavulaan and Wright 2002; Scriber 1996a). Much of this narrow region of hybridization has remained stable for more than a century (Scriber and Lederhouse 1992; Scriber et al. 1996). As is commonly reported of hybrid zones (Harrison 1993), this *P. canadensis* and *P. glaucus* hybrid zone, at first glance, corresponds to the boundary between two ecotones, the boreal coniferous forest to the north (or high altitudes in mountainous regions) and the temperate deciduous forest to the south (or lower altitudes). It has been shown that perhaps a more accurate tool, than latitude, by which to predict the location of potential hybridization between these two species would be the geographic location that corresponds to between approximately 2300 and 2700 F degree days of thermal unit accumulation. A minimum of 2800 F degree-days is the amount of thermal energy required allowing for the bivoltine physiology of *P. glaucus* in a single season (Scriber 1994).

Under laboratory conditions, *P. canadensis* and *P. glaucus* can be readily hand paired, resulting in viable offspring. The resulting F1 hybrids are intermediate in many

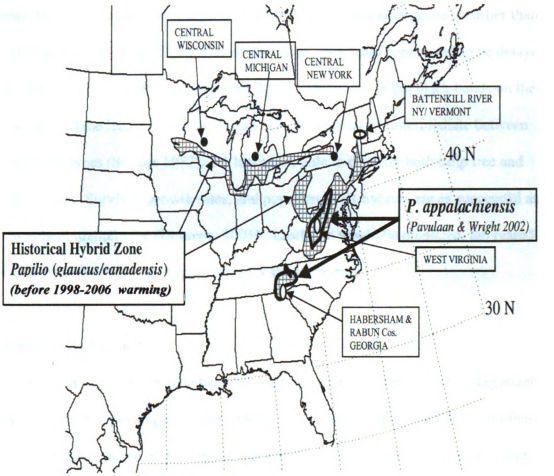


Figure 1.4. Geographic representation of the historic hybrid zone as well as the identified distribution of the recently described *Papilio appalachiensis*, and also the location of incipient speciation in the Battenkill river valley of New York and Vermont.

respects with regards to significant life-history traits and morphological characters. Under growth chamber controlled environmental conditions, it has been shown that pupal eclosion of male F1 hybrids (*P. glaucus* X *P. canadensis*) is intermediate between both parental types, being delayed compared to *P. canadensis* and emergence is earlier than that of *P. glaucus*. Female F1 hybrids of this same cross have been found to be delayed by an average of two weeks (Scriber et al. 2002). The width of the black bands on the hind wings and the length of the forewings of hybrid adults are intermediate between both parental types (Scriber 1982). F1 larvae are able to detoxify both tulip tree and quaking aspen. Survival, growth rates, and pupation of F1 hybrids are as successful as that of either parental type (Donovan 2001). Lastly, as would be predicted, lab reared offspring exhibit a mixture of species diagnostic allozymes.

Barriers to Gene Flow?

In nature, barriers to gene flow between natural populations can be categorized as either prezygotic or postzygotic. Mechanisms under either of these categories ultimately result in the maintenance of distinct populations and therefore, species reinforcement (Harrison 1993). Even in the heart of the known hybrid zone, identification of a field-captured individual exhibiting a genotype of mixed *glaucus* and *canadensis* ancestry has been rare in occurrence (<10%) (Leubke et al. 1988; Scriber et al. 2003). A great deal of research has been conducted investigating what mechanisms maintain and shape the narrow zone of hybridization between *P. glaucus* and *P. canadensis*. If hybrids are viable, what prevents these two populations from fusing into a single panmictic population?

P. canadensis and *P. glaucus* have species specific host plant preferences and detoxification abilities that correspond to some of the available plants in their respective ranges. In three choice host plant investigations, *P. canadensis* females consistently prefer ovipositing on quaking aspen (*Populus tremuloides*), whereas *P. glaucus* prefer ovipositing on tulip tree (*Liriodendron tulipifera*). Additionally, the larvae of each species are able to successfully detoxify the secondary chemicals associated with their respective choice of host plant, but an inability to detoxify the alternative host plant leads to larval death. However, there are suitable host plants, including black cherry (*Prunus serotina*) and white ash (*Fraxinus americana*) that the two species share in common, which exist throughout their combined species ranges (Scriber 1982; Scriber 2002). Host plant availability is therefore not likely a sufficient mechanism preventing range expansion of either species.

It is frequently the case that species reinforcement and reproductive isolation are accomplished through mechanisms associated with mating systems (Harrison 1993). Male mate preference has not been shown to be a strong candidate explanation. Presented with a choice of size-matched tethered virgin females of both species, wild males in both *glaucus* and *canadensis* ranges significantly prefer mating with *P. glaucus* females (Deering and Scriber 2002). It has also been shown that conspecific sperm does not exhibit sperm precedence in multiple mating situations (Stump and Scriber 2006). These investigations alone do not identify any strong barriers to bi-directional gene flow.

Impacts of Temperature

Many life history trait patterns of insects are dramatically influenced, or are directly driven, as a result of temperature. It has been hypothesized that temperature is the strongest selective force dictating the geographic range limits of many polyphagous insects, including *P. glaucus* (Tesar and Scriber 2000). A great deal of research has indicated that both *P. canadensis* and *P. glaucus* are drastically impacted by, and can exhibit strong local adaptations to cope with local thermal environments (Ayres and Scriber 1994; Scriber 1996b, 2002; Scriber and Lederhouse 1992). It is very likely that temperature, acting directly or indirectly, is the major factor preventing unchecked gene flow between populations of *P. canadensis* and *P. glaucus.*

Consider the farthest northern reaches of the range of *P. glaucus*. It consistently correlates with the geographic range that has >2800 degree day thermal unit accumulation. This makes intuitive sense given the genetically based multivoltine physiology of *P. glaucus*. Both *P. canadensis* and *P. glaucus* over winter as pupae on the ground in the leaf litter. Diapause is facultative in nature in *P. glaucus*. Environmental cues, primarily photoperiod, either induce diapause (relatively shortened daylight period) or induce direct development (relatively long daylight period). It would be suicidal for a second generation of *P. glaucus* to be attempted in locations without sufficient thermal units accumulated annually. An absolute minimum of 2800 degree-days is necessary to allow for the completion of two full generations. Additionally, field studies have been conducted that indicate that *P. glaucus* pupae are less cold tolerant than are pupae of *P. canadensis*. *Glaucus* pupae have been shown to experience increased mortality under the thermal environments frequently encountered in the *P. canadensis* home range (Kukal et

al. 1991). In these ways, temperature likely acts to prevent the advancement of *P. glaucus* northward.

Alternatively, it appears as though temperature may also play a role in preventing significant movement of *P. canadensis* southward. Diapause in *P. canadensis* is obligate in nature, requiring a cold period (winter) followed by spring like temperatures, in order to induce eclosion. Physiologically, diapause has likely evolved to help insects avoid prolonged times of environmental stress. However, while in diapause butterflies are unable to behaviorally modify their immediate environment. It has been shown that the extreme summer temperatures that frequently occur south of the hybrid zone could induce heat stress in diapausing *canadensis* pupae. In fact, experimental results identified an inability of nearly 100% of both pure *P. canadensis* and hybrid *glaucus* X *canadensis* pupae from eclosing after brief exposure to temperatures of 36°C (Scriber et al. 2002). This kind of strong selection would prevent significant amounts of extensive southward *P. canadensis* gene flow.

Recent Evidence of Introgressive Hybridization

Likely as a result of thermal constraints acting on a variety of life history traits, the geographic location of the hybrid zone between *P. canadensis* and *P. glaucus* has remained relatively stable for more than a century (Scriber and Lederhouse 1992; Scriber et al. 1996). However, recent global climate changes have led to drastic alterations in the typical thermal environment across much of North America. It appears as though this has greatly facilitated increased levels of gene flow between populations of *P. canadensis* and *P. glaucus*. Over the course of the past 8-10 years there has been both direct and

indirect evidence of northward gene flow of *glaucus* genes into various populations of historically "pure" *P. canadensis*.

There are two isolated pieces of evidence indicating the possibility for long-range gene flow of *P. glaucus* northward into populations of *P. canadensis*. The first direct observation of potential gene flow was the capture of a dark morph female in the Upper Peninsula of Northern Michigan in the summer of 1997. This is the farthest northern collection of a dark morph female on record. The location where it was captured is nearly 300 kilometers from where a dark morph female would be anticipated. This isolated incident of a pure dark morph female being captured so far from where it would be expected was hypothesized to be the result of long-range transport by a strong storm front that had passed through the day before (Scriber et al. 1998). There is no guarantee that the arrival of this dark *P. glaucus* would have resulted in genetic introgression. Potentially no male *P. canadensis* would have recognized her as a suitable mate. The second isolated source of evidence was a wild *P. canadensis* female captured in northern Michigan in June of 2000. The eggs produced by this female resulted in a brood of offspring that in every way appeared to be primary hybrids between *P. glaucus* and *P. canadensis* (Donovan and Scriber 2003). These isolated incidents should highlight that opportunities do exist for gene flow well beyond species boundaries.

A Newly Described Species

In 2002 a new species of Tiger Swallowtail, *Papilio appalachiensis* (Lepidoptera: Papilionidae), was described from various sites of the Appalachian Mountains in the Southeastern United States (Figure 1.4). It has been described as a univoltine species

sympatric to populations of *P. glaucus*. The primary differences from *P. glaucus* initially justifying it being given unique species status were based on a delayed spring emergence pattern, an apparent obligate diapause physiology, an absence of dark morph females, larger size (than typical spring form *glaucus* in that region), and multiple wing morphometrics. The wing morphometrics of this new species actually appeared to be very hybrid like. However, preliminary mtDNA analysis indicated that this unique species was more closely related to *P. glaucus* (Pavulaan and Wright 2002).

Since first being described, collaborative investigations have indicated that *P. appalachiensis* is a hybrid species between *P. glaucus* and *P. canadensis* (Scriber and Ording 2005). This hypothesis is supported by data collections associated with oviposition preferences, larval survival abilities, and morphometric analysis, each of which show this new species to be intermediate between both parental types in all categories, as is the case with lab reared hybrids. The most compelling data indicating the mixed ancestry of this new species has been the allozyme electrophoresis. Specimens collected and described as *P. appalachiensis* by the original authors (Pavulaan and Wright), that have been analyzed through allozyme gel electrophoresis, for two X-linked species diagnostic loci, exhibit a striking genetic pattern. Nearly all individuals scored have *glaucus* like Pgd and *canadensis* like Ldh (Scriber and Ording 2005). Highly noteworthy, is that this new species has been identified from geographic regions that correspond to an accumulated 2300-2700 thermal degree-days.

There is clearly an intimate link and an interaction between the thermal landscape and population genetics, that initially allowed for differentiation between *P. glaucus* and *P. canadensis*, has maintained these two groups as unique populations with distinct ranges, and now allows for differential rates of gene flow and introgression. Figure 1.5 (adapted from Scriber et al. 2008) represents the allele frequencies of two X-linked species diagnostic allozymes along the thermal landscape across the Eastern United States. Those populations represented from regions in which there are less than 2300 degree days are univoltine and essentially pure *P. canadensis*, whereas those populations from regions in which there are 2800 degree days or more are bivoltine and essentially pure *P. glaucus*. Those populations represented between 2350 and 2750 degree days are found relatively near species range boundaries or in the midst of the species hybrid zone, and are therefore prone to varying degrees of genetic introgression. This dissertation will clearly illustrate how recent shifting thermal landscapes have differentially impacted the range limits of *P. glaucus* in certain areas, allowing for variable levels of genetic introgression into once pure populations of *P. canadensis*. In those populations in which the highest levels of genetic introgression have been recorded, there is clear evidence of genetic recombination and divergence in key species diagnostic traits.

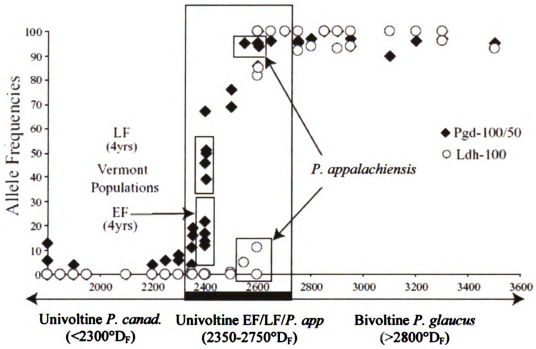


Figure 1.5. X-linked allele frequencies (*Pgd*-100 and *Ldh*-100 allozymes) along the thermal landscape (degree-days above a base of 50°F [$°D_F$]) across the eastern United States. Note the sharp decline in *Ldh*-100 at 2700$°D_F$ and the nonconcordant but steep increase in *Pgd*-100 frequencies between 2300 and 2800$°D_F$. The 70 different populations (each represented by 10-125 males) are from Vermont and northern Michigan to southern Ohio and North Carolina (data from Scriber and Ording 2005; and JMS unpublished data). The bivoltine *P. glaucus* are at the right (>2800$°D_F$), while everything else is univoltine. These other populations include a range of *P. canadensis* populations that exhibit differential degrees of *P. glaucus* introgression. (Figure modified from Scriber et al. 2008).

Chapter 2 of this dissertation investigates an isolated hybrid swarm located on South Manitou Island in Lake Michigan. This population of *P. canadensis* is unique in that it is located over 150 km north of the historic hybrid zone across a relatively broad thermal landscape, and yet levels of *P. glaucus* introgression there are as high if not higher than across the heart of the hybrid zone in the State of Michigan. It is thought that the introgression exhibited on South Manitou Island is the result of historic episodes of gene flow into the area, and given the isolated nature of this island population, significant levels of introgression are not the result of ongoing gene flow (Ording 2001). The environmental conditions of this northern location are unique in that the typically severe northern temperatures are heavily moderated by the significant lake effect of this region. This provides an opportunity to monitor the stability of an introgressed hybrid swarm population and to investigate how environmental conditions on the "cooler side of the hybrid zone" exert differential selective pressures on the variety of hybrid like trait combinations.

Chapter 3 will describe populations what were once pure *P. canadensis* near the New York and Vermont border, which have exhibited extremely high levels of genetic introgression over the course of the past 10 years. The combination of reasons for the high levels of introgression in these populations includes the recent series of unusually warm years associated with anthropogenic climate change, allowing for shifting species boundaries. Additionally, the topography of the region results in an extremely condensed thermal landscape (compared to that in the State of Michigan), bringing populations of *P. glaucus* much closer contact to populations of *P. canadensis*. The high levels of genetic

mixing that have occurred have resulted in unique combinations of hybrid traits being expressed, including changes in significant life history traits (diapause). This has resulted in rapid divergence and provided a mechanism for temporal isolation. This temporal isolation is an example of a rapidly developed mechanism that could allow for hybrid speciation (Scriber and Ording 2005) and could be identical in nature to the processes that have led to the formation of the newly described *P. appalachiensis* (Pavulaan and Wright 2002).

Chapter 4 will highlight the genetic mechanisms that allow for the recombinant genotypes that exist in the introgressed populations of *P. canadensis* to arise, through the use and analysis of laboratory reared hybrids. These laboratory investigations provide evidence as to the frequency with which recombination likely occurs in nature, and also provides support for the presence of genetic incompatibilities and a Haldane effect that has likely played a central role in the historic maintenance of the *P. canadensis* and *P. glaucus* range boundaries.

CHAPTER 2:

A STABLE HYBRID SWARM

Introduction

Ongoing research (1998-2007) conducted on and adjacent to the Manitou Islands in Lake Michigan, has identified an isolated "hybrid swarm" over 150 km. north of the historic *Papilio canadensis* and *P. glaucus* hybrid zone in Michigan (Figure 2.1). Specimens collected from this hybrid swarm (1998-2001) exhibited intermediacy and mixed ancestry for several of the species diagnostic characters (Ording 2001). The hypothesis being tested is that the levels of *P. glaucus* introgression in this hybrid swarm have remained stable and consistent in the years following the original investigation. The extent to which *P. glaucus* introgression continues to be present, and the stability of the hybrid swarm on South Manitou Island has been investigated through further analysis of those same diagnostic characters between *P. canadensis* and *P. glaucus*. A combination of morphologic, ecological and biochemical traits have been considered utilizing samples taken over an eight-year period (1998-2005).

Figure 2.1. *Papilio canadensis* and *P. glaucus* hybrid zone across the Midwest. The shaded region in Michigan roughly indicates what has historically been considered the hybrid zone between *Papilio glaucus* and *P. canadensis* (Nielsen, 1999). The shaded region across Wisconsin into Minnesota represents the 50 year average degree-day accumulation and northern limit allowing for two generations of *Papilio glaucus*. The star indicates the location of South Manitou Island.

Banding patterns on butterfly wings is a common method by which species can be distinguished, as is the case between *Papilio glaucus* and *P. canadensis* (Hagen et al. 1991), or can also be used to discern differences between populations within a species. The Monarch butterfly (*Danaus plexippus*) has a range that extends across the Western Hemisphere, from Alaska in the north to Patagonia in the south. There are differences in wing banding patterns that allow individuals coming from one region to be distinguished from an individual coming from another (Williams et al. 1942).

In *P. canadensis* and *P. glaucus* there is a distinct black band in the hind wing that partially fills the anal cell. This morphologic character is one used to distinguish between *Papilio canadensis* and *P. glaucus* (Hagen et al. 1991). This dark band is significantly wider in *P. canadensis*, on average filling 70 percent of the anal cell, whereas in *P. glaucus* this black band fills on average only 30 percent of the anal cell (Hagen et al. 1991). Lab reared hybrids and field collected specimens of mixed ancestry exhibit intermediacy for this trait (Luebke et al. 1988). As a result this black band character has been heavily relied upon as a reliable morphometric character with which to identify specimens of mixed ancestry.

Analysis of species diagnostic allozyme allele frequencies is extremely useful in estimating levels of genetic introgression in a population. *P. canadensis* specimens from South Manitou Island have been analyzed for levels of *P. glaucus* introgression using the Pgd (6-Phosphogluconate dehydrogenase) allozyme locus. This locus was chosen as a result of it being highly polymorphic (Hagen & Scriber 1991), relatively consistent and easy to interpret, and is among the species diagnostic characters.

MATERIALS AND METHODS

Specimen Acquisition and Transport

Papilio specimens utilized in this research were live-captured by net, from a variety of locations on South Manitou Island each year (Table 2.1). Specimen collections were primarily made during mid-day, between approximately ten o'clock a.m. and four o'clock p.m. on warm sunny days, these being the predominant hours for flight activity. Specimens were most frequently captured while puddling, feeding on available sources of nectar, and sometimes while in flight. This method of specimen collection often led to the discovery of desirable puddling locations or high concentrations of appropriate nectar sources, each often times with high densities of butterflies. These locations could then be returned to multiple times in a day and within a season for further collections. These collection locations also proved to be consistently productive in subsequent years.

Upon capture, individual specimens were placed with their wings folded back into 2 oz. Glassine envelopes, which were labeled with specimen sex, date and location of capture. Collections were transported alive to the laboratory in Tupperware® plastic containers in ice coolers, which lowered specimen body temperatures and slowed metabolism. Upon arrival to Michigan State University in East Lansing, Michigan, specimens were preserved by freezing them alive in an -80° C ultra-low biological freezer for later processing.

Table 2.1. South Manitou Island male *P. canadensis* specimen collection data.

Year	Date(s)	Sample Sizes (n)	Total (n)
1998	June 17	32	32
1999	June 18	120	120
2000	June 10	72	72
2001	June 11	100	100
2002	June 29 and July 8	6 and 3	9
2003	June 20	70	70
2004	June 18	43	43
2005	June 25	25	25

Wing Morphometrics

Two wing characteristics were chosen for analysis in order to identify the presence of *Papilio glaucus* introgression into the South Manitou Island population of *P. canadensis*. Forewing length (Fig. 2.2) and hind wing anal cell black bandwidth (Fig. 2.3) are morphometric characters of adult butterflies, which can be used in the field to help distinguish between *P. canadensis* and *P. glaucus*. On the average, *P. glaucus* forewings are significantly larger than those of *P. canadensis*. *P. glaucus* forewings have been shown to be 8-10 mm longer, from thoracic attachment to tip, than *P. canadensis* forewings (Hagen et al 1991). The more powerful diagnostic morphometric wing character is the width of the black band along the anal margin of the hind wing. For *P. glaucus* males, this band fills on average 30 percent of the width from the wing margin to the CuA2 vein; whereas for *P. canadensis* the width of the band fills 70 percent of this anal cell (Hagen et al. 1991). Hybrid individuals display a black bandwidth intermediate between the two, averaging 50 percent (Scriber et al. 2001). Intermediacy for genetically based morphometric traits is a good indicator of a hybrid individuals or populations (Harrison 1993).

After the wings were detached from adult swallowtail specimens during the allozyme electrophoresis preparatory protocol, they were assayed for the two major morphological features. Forewing length, from the distal tip of the wing to the basal thoracic attachment (Fig. 2.2), was measured using a clear plastic metric ruler to the nearest mm. On the ventral surface of the wings, the width of the anal black band was assessed as a percentage of the distance from the wing edge to the CuA2 vein. This measurement was taken at a line of intersection with the junction of vein CuA2

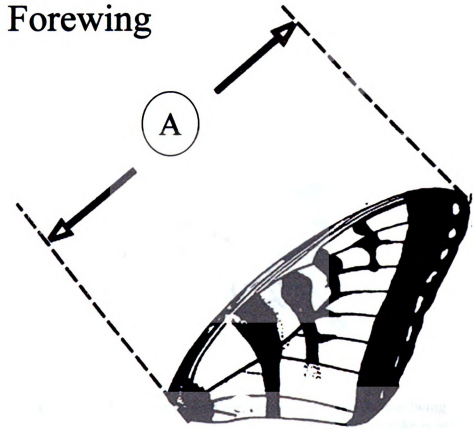


Figure 2.2. Forewing length measurements (measurement A) are the distance from the tip of the forewing to the thoracic wing base attachment (Figure modified from Leubke et al. 1988).

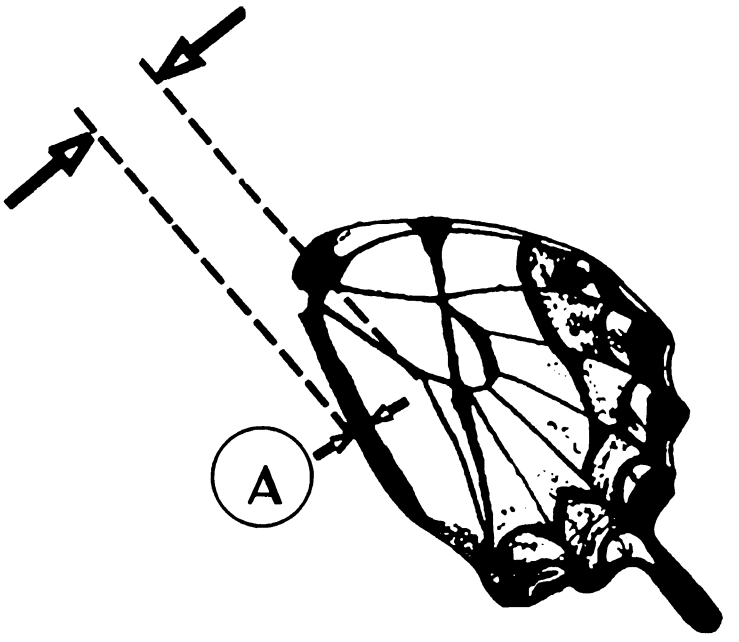


Figure 2.3 Black band width measurements are the percentage of the hindwing anal cell that is filled by the dark band labeled A. (Modified from Luebke et al. 1988).

and the discal cell (Fig. 2.3). This anal black band measurement was taken to the nearest .1mm using a dissecting microscope and a WILD glass micrometer slip. For both of these morphometric characters assayed, measurement values for both wings were taken when available, and the mean values for each individual have been utilized for analysis. In cases where wings were damaged and ripped, preventing an accurate measurement; if one wing was undamaged a single measurement has been utilized for analysis; if both wings were damaged, the specimen has not been included in the analyses.

Allozyme Electrophoresis

Allozyme electrophoresis was performed on adult male Tiger Swallowtail Butterflies in this study. Electrophoresis protocol follows that of Hagen and Scriber 1991. Adult specimens were removed from -80° C and processed in a 4° C cold room. Using a scalpel or razorblade, wings were dissected from the thorax at their place of attachment and returned to Glassine envelopes for morphometric analysis. Tissue extracts were prepared by grinding one half of the abdomen with 100 µl of grinding buffer. The lower half of the abdomen was utilized in male specimens. The remaining abdomen portion, head and thorax, were returned to the –80° C freezer for future use. The extract was centrifuged for 10 minutes at 14,000 rpm. At this point the extract could be stored at –80° C until ready to continue the electrophoresis protocol. Female specimens were not utilized in the electrophoretic portions of this study for several reasons. First, the sample sizes for females were relatively low for many of the years of sampling. More importantly however, the allozyme banding patterns produced by females are frequently not as clear as those produced by male

specimens and prove to be difficult to interpret. It is possible that the eggs contained in the abdomen somehow disrupt the normal staining process.

Samples were removed from -80° C and allowed to thaw in 4° cold room for approximately 10 minutes and were then centrifuged for 5 minutes at 14,000 rpm. 7.5 µl of extract from each sample was applied to thin layer acetate plates (Titan III [94 by 76 mm], Helena Laboratories) for electrophoresis. The allozyme locus scored for this study portion is Pgd (6-Phosphogluconate dehydrogenase). Scoring of gel banding patterns was accomplished following methods of Hagen and Scriber 1991 using original gels and photographs. Known *P.g.* and *P.c.* standards were run alongside all samples analyzed, to provide for allozyme banding pattern standardization.

Statistical Analysis

In order to determine whether the wing morphometric characters in the South Manitou Island hybrid swarm had remained stable, multiple statistical analyses of both forewing length and black bandwidths were performed using JMP statistical software version 6.0 by the SAS Institute Inc. An analysis of variance (ANOVA), a nonparametric Wilcoxon test and a Student's t-test were performed comparing both forewing lengths and hind wing black bandwidths across and between years (1998-2005). To be sure that there was no effect of forewing length on black bandwidth, a bivariate linear regression was performed to be sure that these characters are in fact independent of one another.

The stability of the hybrid swarm was also investigated through statistical analysis of the allele frequencies that were identified through gel electrophoresis.

These statistical analyses were performed using Genepop 3.6 (Raymond and Roussett 1995). Tests for both genotypic and genic differentiation were performed comparing allele frequencies from each year (1998-2005).

RESULTS

Wing Morphometrics

The bivariate linear regression indicates that the two characters are in fact independent of one another (Fig. 2.4). Plotting black bandwidth against forewing length indicates that there was no effect of forewing length on black bandwidth (R^2 = 0.004). This provides validity to the use of these two characters as being independent of one another. Both the ANOVA and the pairwise statistical analysis comparing the forewing lengths of specimens from South Manitou Island across each year indicates that the average forewing length does vary significantly between certain pairs of years (Table 2.2). There are not significant pairwise differences however between six of the eight years analyzed, and these include the earliest year sampled (1998) and the four most recent years analyzed (2002 – 2005). The same pairwise analysis of the black bandwidths of these same South Manitou Island specimens across each year indicates that there is no significant difference between any pair of years analyzed (Table 2.3).

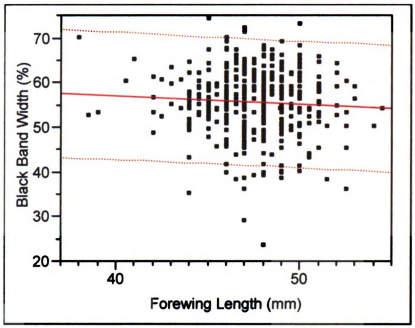


Figure 2.4. Bivariate linear regression of black band width by forewing length (R^2 = 0.004) for male *Papilio canadensis* specimens collected on South Manitou Island from 1998-2005. Analysis was performed using JMP statistical software version 6.0.

Table 2.2. Forewing length measurements for male specimens collected on South Manitou Island, Leelenau County, Michigan from 1998-2005. Values represented are the average length in mm +/- s.e. from the distal tip of the wing to the basal thoracic attachment. Values are based upon the calculated means of the left and right forewing lengths for each specimen analyzed. The years not connected by the same letter are significantly different based upon a comparison for each pair using the Tukey's HSD (adjusted P-value of significance <0.05).

Year	Similarity	Sample Size (n)	Mean Forewing Length +/- s.e.
1998	A B	32	46.01 +/- 0.48
1999	A	120	48.58 +/- 0.43
2000	B	72	47.29 +/- 0.23
2001	A B	60	47.68 +/- 0.29
2002	A B	9	46.00 +/- 1.27
2003	A B	70	47.54 +/- 0.26
2004	B	43	46.86 +/- 0.37
2005	A B	25	47.48 +/- 0.39

Table 2.3. Hind Wing Black Band widths for male specimens collected on South Manitou Island, Leelenau County, Michigan from 1998-2005. Values represented are the percent of the hind wing anal cell that is filled by a dark pigmented band. Values are based upon the calculated means of the left and right hind wing black bands for each specimen analyzed. There were no significant differences across years based upon analysis using Tukey's HSD (adjusted P-value of significance <0.05).

Year	Sample Size (n)	Mean Black Band Width +/- s.e.
1998	32	55.20 +/- 1.29
1999	120	56.27 +/- 0.62
2000	72	56.14 +/- 0.96
2001	60	54.91 +/- 0.91
2002	9	55.17 +/- 2.91
2003	70	55.43 +/- 0.75
2004	43	55.45 +/- 1.23
2005	25	53.66 +/- 1.85

Allozyme Electrophoresis

Of the nine Pgd alleles that exist in the closely related species groups of Papilionidae (Hagen and Scriber 1991), six were encountered in these analyses. The South Manitou Island population allele frequencies are listed in Table 2.4. Using Genepop Version 3.6 pairwise analysis was conducted to determine the level of Genic and Genotypic differentiation between each year for which there was allozyme data available on South Manitou Island (Table 2.4). These analyses indicate that there are no significant genic or genotypic differences between any years, other than the population in 2001 significantly differs from the population in both 1998 and 2000 (P-values of significance <0.05).

Table 2.4. Allele frequencies for the species diagnostic Pgd allozyme from male specimens collected on South Manitou Island, Leelenau County, Michigan from 1998-2005 (allozyme data for 2003 and 2004 are unavailable). Allele frequency values represented are percentages of introgressed *glaucus* alleles detected through gel electrophoresis. The years not connected by the same letter are significantly different based upon a determination of both Genic Differentiation and Genotypic Differentiation as calculated using Genepop Version 3.6 1999 (P-values of <0.05).

Year	Similarity	Sample Size (n)	Allele Frequencies
1998	A	32	.109
1999	AB	120	.079
2000	A	100	.095
2001	B	100	.035
2002	AB	9	.111
2005	AB	20	.025

DISCUSSION

There are two morphometric wing characters that have consistently been applied in distinguishing between *P. glaucus* and *P. canadensis,* forewing length and hind wing black bandwidth. Intermediacy for these two characters has been applied towards the identification and characterization of hybrid like specimens. The analysis of data collected over an extended period of time (1998-2005) indicates that these characters have remained intermediate and stable in the introgressed hybrid swarm population that exists on South Manitou Island. Additionally, the allele frequencies of introgressed allozymes in this same population have remained relatively constant over this same time period, this also indicating that the isolated island hybrid swarm population has remained relatively stable.

Given the island location and the difficulty of accessing this hybrid swarm, sampling efforts have until recently been primarily restricted to making the annual collection in a single day each year. However, other more recently identified *P. canadensis* populations that show indications of *P. glaucus* introgression exhibit the possibility that detectable levels of introgression can be dependent on the timing of collections. It appears in these populations that specimens that possess certain hybrid-like genotypes exhibit a delayed emergence.

Chapter 3 introduces a unique *Papilio* population in the midst of the *P. glaucus* and *P. canadensis* hybrid zone in the Battenkill River Valley of New York and Vermont. This population possesses unique traits that appear to be the result of hybridization. The result of these new genetic combinations appear to have resulted in the production of an incipient species, reproductively isolated from either parental population through temporal mechanisms.

CHAPTER 3:

CAN INTROGRESSION LEAD TO REPRODUCTIVE ISOLATION? : INCIPIENT HYBRID SPECIATION BETWEEN *PAPILIO GLAUCUS* AND *P. CANADENSIS*

Introduction

Hybridization can lead to the production of a vast diversity of genotypes unique from that of either parental population. It has been shown that hybrid offspring can exhibit "bounded hybrid superiority", or increased fitness compared to that of either parental population, under certain environmental conditions (Collins 1984; Woodruff 1989). Bounded hybrid superiority has been implicated in cases of hybrid speciation in extreme environments (Riesberg et al. 2003; Gompert et al. 2006) and in novel ecological situations allowing for adaptive radiation (Schwarz et al. 2005). In theory, hybrid speciation would be an unlikely evolutionary phenomenon given that viable hybrid offspring would be able to mate and exchange genetic material with parental forms. This would allow for the production of a hybrid swarm, but not speciation. What is required for hybrid speciation to occur is that the hybrid offspring be afforded some significant genetic modification, which would result in reproductive isolation. Hybridization between *Papilio glaucus* and *P. canadensis* is the proposed mechanism that explains the newly described *Papilio appalachiensis* species (Scriber and Ording 2005). There are other locations in which high levels of hybridization are occurring between these closely related *Papilio* species. Investigations in these locations are of extreme value in that it is possible to identify the mechanisms that can ultimately lead to speciation and provide an opportunity to witness the process while it is occurring.

"False Second Generation"

Populations of *P. canadensis* outside of the Great Lakes have shown evidence of high levels of *P. glaucus* introgression. Several New England populations of *P. canadensis* including the Battenkill River Valley population of New York and Vermont have recently (since 1999) exhibited a strikingly uncharacteristic life history trait phenomenon. These locations have displayed what has been described as a "False Second" generation (Hagen and Lederhouse 1985). As described earlier, *P. canadensis* has a univoltine life cycle. These locations however, have recently begun displaying what appear to be two distinct flights within a single summer. The first flight occurs as would be expected, in late May through June, and a second flight occurs in mid July. This second flight was being described as a "false second generation" due to the fact that it appears far too quickly in the season (mid July) to be a true second flight, derived from the first. This second flight is composed of individuals that appear more "*glaucus*-like" than does the first flight, being significantly larger (based upon forewing length) and possessing significantly narrower hind wing black bands (see Table 1.1). In addition, individuals from this second flight appear "*glaucus*-like" for other species-diagnostic traits, including oviposition preference and host plant detoxification abilities (Scriber and Ording 2005).

With respect to flight times in the field, a variety of morphometric characters, and host use abilities, the Early Flight (EF) and the Late Flight (LF) *Papilio* populations from the Battenkill river valley appear distinct. Many of the apparent character differences in the LF population appear to be similar to populations of *P. canadensis* that have been heavily introgressed by *P. glaucus*, or similar to lab reared hybrids between the two

species. This chapter is devoted to the analysis of data that investigates three primary questions. First, is there genetic evidence of *P. glaucus* introgression into either the EF or the LF populations? Second, are the EF and LF populations genetically unique? Lastly, are the EF and LF reproductively isolated? This study provides an example of an individual trait that is greatly modified as a result of introgressive hybridization, that can provide strong reproductive isolation between parental populations and hybrid offspring.

In order to determine the presence and degree of genetic introgression of *P. glaucus* into the Battenkill Valley population of *P. canadensis*, a combination of morphological and molecular techniques were employed, similar to those described in Chapter 2. In order to determine whether the EF and the LF are genetically unique and reproductively isolated, a combination of field observations, lab controlled emergence experiments, and additional molecular analyses have been employed.

MATERIALS AND METHODS

Specimen Acquisition

Wild captured adults from the Battenkill River Valley populations that have been used for morphological and molecular analysis were field captured by net, from a variety of locations. Specimen collections were primarily made during mid-day, between approximately ten o'clock a.m. and four o'clock p.m. on warm sunny days, these being the predominant hours for flight activity. Specimens were most frequently captured while feeding on available sources of nectar, puddling, and sometimes while in flight. Sample sizes of males captured for each of the years surveyed are as follows: Early Flight (EF) (May – June) 2002 n = 48, 2003 n = 28, 2004 n = 128; Late Flight (LF) (Mid July) 2002 n = 15, 2003 n = 14, 2004 n = 12.

Offspring from the females of both EF and LF flights were collected as pupae from field-rearing in sleeved tree branches of wild black cherry (*Prunus serotina*). The eggs and larvae obtained inside of these sleeved branches were from 25-30 EF females and 15-18 LF females in 2002, and from 15-20 EF females and 12-15 LF females in 2003. The resulting pupae were collected in mid-September and stored in darkness at 3-5 °C under controlled environmental conditions beginning in October until the commencement of the emergence experiments in both 2003 and 2004.

Wing Morphometrics

The same two quantitative polygenic wing characteristics (forewing length and hind wing black band width) that were applied as a component towards the classification of the South Manitou Island population as a hybrid swarm (see details in Chapter 2), were

applied in order to compare and contrast the EF and LF flight of the Battenkill River Valley. Genetic differentiation between two populations is possible through phenotypic analysis because shared genes would be reflected in similar phenotypes (Boag and van Noordwijk 1987). The characteristics under investigation, forewing length and hind wing anal cell black bandwidth, again were chosen to apply to this investigation for the same reasons that they were applied towards the South Manitou Island investigation. Both of the traits are heritable and polygenic (Luebke et al. 1988), thus conceivably impacted by mutation, genetic drift, and natural selection under differing niche specific environmental conditions. In addition, these two traits have been consistently applied as highly informative measurements to apply when identifying interspecific hybridization. (Scriber et al. 2001; 2003). Female specimens were placed in sleeves on trees in the field to allow for oviposition. In this process, wings became tattered and were not readily usable. For this reason only male specimens were used for wing morphometric analyses. Sample sizes for each of the years analyzed are as follows: 2002 EF n = 48, LF n = 15; 2003 EF n = 28, LF n = 14; 2004 EF n = 128, LF n = 12).

Pupal Weights

There is a direct correlation between pupal weight and overall size of adult *Papilio*. *P. glaucus* consistently have larger pupae than do *P. canadensis*. This correlates directly with *P. glaucus* being a significantly larger butterfly than *P. canadensis* (Hagen et al. 1991). Pupal weight, like the wing morphometric characters, is a polygenic trait that can prove valuable in comparing genetic differences between these two species. Pupae were removed from winter diapause chambers and a random subsample were

weighed to the nearest milligram using a Mettler ® macroanalytical balance. Sample sizes for each of the years analyzed were as follows: 2003 EF n = 40♂ and 39♀, LF n = 44♂ and 42♀; 2004 EF n = 73♂ and 77♀, LF n = 78♂ and 62♀. Sex of pupae was determined post emergence for guaranteed accuracy. Only the pupal weights of those specimens that successfully emerged were used in analysis. Pupal weight data from those pupae that did not successfully emerge were omitted.

Allozyme Electrophoresis

In order to determine and compare the level of *P. glaucus* introgression into both the EF and LF populations, and to determine whether the EF and LF populations were reproductively isolated, allozyme electrophoresis was employed. In this investigation the two X-linked species diagnostic allozymes were analyzed, Pgd (6-Phosphogluconate dehydrogenase) and Ldh (lactate dehydrogenase). The same techniques as were described in Chapter 2 were applied. Of the nine Pgd alleles that exist in the closely related species groups of Papilionidae (Hagen and Scriber 1991), five were encountered in this analysis. For the purposes of determining the degree of *glaucus* introgression, the frequency of Pgd alleles that have been described as diagnostic characters associated with *glaucus* (Pgd 100 and 50) have been grouped together, and the *canadensis* diagnostic alleles (Pgd 150, 125, and 80) have also been grouped together for interpretation. Analysis was performed only on wild male individuals captured in the field. The specimens that were used for the emergence investigations that resulted from field rearing were not used due to the fact that these were many individuals from relatively few families and would not represent independent samples. Allele frequencies

for Pgd are presented for 2000-2004, whereas allele frequencies for Ldh are only presented for 2002-2004. The reason that Ldh allele frequencies were not included for 2000 and 2001 is due to the fact that before 2002 all specimens scored had indicated zero *glaucus* introgression and 2002 was the first year that a novel hybrizyme allele was recognized. Sample sizes for the number of male specimens from each year are as follows: 2000 EF n = 116, LF n = 33; 2001 EF n = 0, LF n = 51; 2002 EF n = 117, LF n = 13; 2003 EF n = 29, LF n = 14; 2004 EF n = 158, LF n = 12.

Mitochondrial DNA Analysis

Another technique employed in order to identify the extent of *P. glaucus* introgression into both the EF and LF populations was an analysis of mtDNA. Fourteen EF and the fourteen LF male specimens that were field captured during the summer of 2003 were analyzed for mtDNA characters that are known to be diagnostic between *P. glaucus* and *P. canadensis*. DNA extraction and PCR-RFLP analysis techniques follow those used by Stump et al. (2003), which were modified from Sperling & Hickey (1995). For each specimen, two legs were plucked and macerated in 800 µl of Lifton buffer (0.2M sucrose, 50mM EDTA, 100mM Tris, and 0.5% SDS). Samples were vortexed and left at room temperature for 30 minutes. Then 100 µl 8M KOAc was added and each sample was inverted and put on ice for 60 minutes. Samples were centrifuged for 20 minutes and the supernatant was transferred to a new tube. Samples were extracted once with phenol and once with chloroform/isoamyl alcohol (24:1). DNA was then precipitated with isopropanol. Samples were centrifuged at 14,000 rpm for 10 minutes.

The resulting pellet was washed with 70% ethanol, then dried and resuspended in 200 µl 1X TE buffer.

The PCR primers that were used had sequences 5' ATA ATT GGA GGA TTT GGA AAT TG 3' and 5' ATT GTA GTA ATA AAA TTA ATT GCT CC 3'. These primers were produced as a result of sequencing work on *canadensis* and *glaucus* mitochondrial COI and COII genes (Caterino and Sperling 1999), and were expected to produce a DNA fragment 294 base pairs long. Within this fragment were five potentially diagnostic restriction sites. A *Taq1* restriction site was expected to be present in specimens carrying *glaucus* mtDNA and absent in those carrying *canadensis* mtDNA. An *Ssp1* restriction site was expected to be present in specimens carrying *canadensis* mtDNA and absent in those carrying *glaucus* mtDNA.

PCR was carried out using the above primers in a total reaction volume of 100 µl using AmpliTaq Gold DNA polymerase in a Perkin Elmer GeneAmp 9600 Cycler. PCR products were verified by running them out on a 2% agarose gel along with a 100bp DNA ladder and visualized with ethidium bromide (EtBr) and ultraviolet light. All PCR products were then digested independently by both *Taq1*and *Ssp1* restriction enzymes incubated at 65 °C for 120 minutes. The two alternative diagnostic restriction enzymes were used on every specimen to provide corroboration. Digested DNA was run on a 1.5% EtBr Agarose gel with a 100bp DNA ladder for comparison.

Emergence Investigations

After pupal weights were determined, specimens were evenly distributed and randomly assigned to controlled environmental chambers (18 °C, 22 °C, and 26 °C in 2003; 14 °C, 18 °C, 22 °C, and 26 °C in 2004; all under a L18:D6 photoperiod) to

determine the length of time until adult emergence. Emergence investigations began on April 15, 2003 and on April 8, 2004. Pupae were either placed alone in a screened Petri dish or in a screened petri dish in a group of up to 20 pupae. Petri dish placement within each chamber was split, with EF and LF specimens on each shelf level, so as to avoid the possibility that the shelf height or the distance to the light sources would act as confounding variables. Temperatures of each environmental chamber were kept constant throughout the emergence investigations and were monitored for stability on a daily basis. Checks were made twice daily (early morning and late afternoon) to collect freshly eclosed specimens.

Statistical Analysis

In order to identify whether the EF and LF populations were in fact morphologically distinct, multiple statistical analyses of data associated with forewing length, black bandwidths, pupal weights, and emergence timing were performed using JMP statistical software version 6.0 by the SAS Institute Inc. Both an analysis of variance (ANOVA) and a nonparametric 2-sample Wilcoxon test was performed comparing both forewing length and hind wing black bandwidths between wild captured EF and LF for each individual year (2002-2004). These same analyses were performed comparing the pupal weights of the field-reared pupae and also their respective days to emergence from EF and LF populations from each individual year (2003 and 2004).

Analysis of the allozyme data allowed for a determination and a comparison of the amount of *P. glaucus* introgression in both the EF and the LF populations. These temporal and genetic data were also valuable in determining whether the EF and the LF

were reproductively isolated. These analyses were performed using Genepop 3.6 (Raymond and Roussett 1995). Tests for both genotypic and genic differentiation were performed comparing allele frequencies from both populations for each year (2000-2004).

Additionally, a classic method by which evolutionary biologists estimate gene flow between populations is the use of Wright's Fst (Weir and Cockerham1984), which is a comparison of the allele frequencies between populations. Fst is on a scale from 0 to 1, with low values indicating that there is a great deal of gene flow, and high values inferring that there is very little. Fst values were calculated for the EF and LF for each year (2000-2004) for both individual loci investigated using Genepop 3.6.

RESULTS

Wing Morphometrics

For both the ANOVA and the nonparametric 2-sample Wilcoxon analyses that were conducted, comparing the forewing lengths for each year (2002-2004) between EF and LF (Table 3.1), the EF forewing lengths were consistently identified as being significantly smaller than those of the LF (p-values <0.0001). The same analyses applied towards a comparison of the EF and LF black bandwidths for each year analyzed (2002-2004) also resulted in significant differences between the two populations (Table 3.2), with the black bands of the EF being significantly wider than those in the LF (p-values <0.0001).

Pupal Weights

Both an analysis of variance (ANOVA) and a nonparametric 2-sample Wilcoxon analysis were performed comparing the pupal weights of specimens field reared for each year (2003-2004) between EF and LF (Table 3.3). The EF pupal weights were consistently identified as being significantly smaller than those of the LF (p-values <0.0001). It should be noted that pupal weights are not independent observations in that the pupae are derived from only a limited number of mothers for each year and flight population.

Table 3.1. Forewing length measurements for male specimens collected in the Early Flight and the Late Flights of the Battenkill River Valley of Vermont and New York from 2002-2004. Values represented are the mean length in mm +/- s.e. from the distal tip of the wing to the basal thoracic attachment. Values are based upon the calculated means of the left and right forewing lengths for each specimen analyzed. Sample sizes are presented in parentheses. Probability values are derived from a nonparametric 2-sample Wilcoxon test performed using JMP 6.0. Probabilities for comparisons that are significantly different are indicated with an *. All comparisons are significantly different with p-values <0.0001.

	Mean Forewing Length +/- s.e.		
Year	EF (n)	LF (n)	Prob>[Z]
2002	45.42 +/- 0.34 (48)	50.60 +/- 0.51 (15)	<0.0001*
2003	45.18 +/- 0.34 (28)	51.00 +/- 0.49 (14)	<0.0001*
2004	47.05 +/- 0.15 (128)	51.42 +/- 0.65 (12)	<0.0001*

Table 3.2. Hind wing black bandwidth measurements for male specimens collected in the Early Flight and the Late Flights of the Battenkill River Valley of Vermont and New York from 2002-2004. Values represented are the mean percent +/- s.e. of the hind wing anal cell that is filled by a dark pigmented band. Values are based upon the calculated means of the left and right hind wing black bands for each specimen analyzed. Sample sizes are presented in parentheses. Probability values are derived from a nonparametric 2-sample Wilcoxon test performed using JMP 6.0. Probabilities for comparisons that are significantly different are indicated with an *. All comparisons are significantly different with P-values <0.0001.

	Mean Black Bandwidth +/- s.e.		
Year	EF (n)	LF (n)	Prob>[Z]
2002	67.46 +/- 1.23 (48)	47.97 +/- 2.23 (15)	<0.0001*
2003	69.22 +/- 1.60 (29)	50.29 +/- 1.98 (14)	<0.0001*
2004	71.02 +/- 0.66 (128)	47.46 +/- 2.09 (12)	<0.0001*

Table 3.3. Pupal weight measurements for field reared specimens from both the Early Flight and the Late Flights of the Battenkill River Valley of Vermont and New York from 2003-2004. Values represented are the mean pupal weights in milligrams +/- s.e. Sample sizes are presented in parentheses. Probability values are derived from a nonparametric 2-sample Wilcoxon test performed using JMP 6.0. Probabilities for comparisons that are significantly different are indicated with an *. All comparisons significantly different with p-values <0.0001.

	Mean Male Pupal Weight +/- s.e.		
Year	Early Flight (n)	Late Flight (n)	Prob>[Z]
2003	0.77 +/- 0.01 (40)	0.97 +/- 0.02 (44)	<0.0001*
2004	0.74 +/- 0.01 (73)	1.03 +/- 0.10 (78)	<0.0001*

	Mean Female Pupal Weight +/- s.e.		
Year	Early Flight (n)	Late Flight (n)	Prob>[Z]
2003	0.87 +/- 0.08 (39)	1.04 +/- 0.02 (42)	<0.0001*
2004	0.79 +/- 0.01 (77)	1.10 +/- 0.01 (62)	<0.0001*

Allozyme Electrophoresis

Of the nine Pgd alleles that exist in the closely related species groups of Papilionidae (Hagen and Scriber 1991), five were encountered in this analysis. For the purposes of determining the degree of *glaucus* introgression, the Pgd alleles that have been described as diagnostic characters associated with *glaucus* (Pgd 100 and 50) have been lumped together, and the *canadensis* diagnostic alleles (Pgd 150, 125, and 80) have been lumped together for interpretation (Table 3.4; Figure 3.1).

Of the four Ldh alleles that have been described in this species group, three were encountered in this analysis (Ldh 40 and 80 which are diagnostic for *P. canadensis* and Ldh 100 which is diagnostic for *P. glaucus*). In addition, a novel allele that has never been described before (here described as Ldh 20 and will be referred to as a hybrizyme) was encountered and found at high frequencies (25-50%) in the late flight. This novel hybrizyme was not detected in the EF until 2004, but was only present at an extremely low allele frequency from a very large population sampling (1.3% out of 158 specimens analyzed). Table 3.4 and Figure 3.2 represent the frequencies of Ldh diagnostic alleles, and also the allele frequencies of the novel Ldh 20, in both the EF and LF for 2000-2004.

Fst values were also calculated for the combination of the two loci (Table 3.5). Typically an Fst value of greater than 0.15 is considered to be an indication that populations are significantly differentiated (Frankham et al. 2002). Based upon this standard, the EF and the LF populations exhibit low levels of gene flow and significant genetic differentiation. This is even true in 2000 when the novel Ldh 20 hybrizyme may have been present in the LF population at frequencies comparable to that of the years since its discovery.

Table 3.4. Species diagnostic allele frequencies for two X-linked loci (Pgd and Ldh) in wild captured male specimens from the Early Flight and the Late Flight populations of the Battenkill River Valley for 2000-2004. Allele frequencies represented are the combinations of all of the diagnostic alleles for each species lumped together in order to represent the degree of genetic introgression. The species diagnostic Pgd alleles for *P. canadensis* are 150, 125 and 80, and for *P. glaucus* are 100 and 50. The species diagnostic Ldh alleles for *P. canadensis* are 80 and 40, and for *P. glaucus* is 100. The Ldh hybrizyme is the novel Ldh 20 allele that was consistently detected in the Late Flight. The *'s for this allele in the 2000 and 2001 Late Flight populations indicate that the allele was in fact present at high frequencies, but as a result of being novel, failed to be identified and had been misinterpreted as faulty gel results. An estimation of the likely allele frequency of the hybrizyme for those two years is shown in parentheses.

Loci	**Pgd**		**Ldh**	
Population	**EF**	**LF**	**EF**	**LF**
	2000		**2000**	
	n=116	n=33	n=116	n=33
canadensis	0.927	0.621	1.000	1.000 *(0.850)*
glaucus	0.073	0.379	0.000	0.000
hybrizyme			***	*** *(0.150)*
	2001		**2001**	
	n=0	n=51	n=0	n=51
canadensis		0.529		1.000 *(0.745)*
glaucus		0.471		0.000
hybrizyme				*** *(0.255)*
	2002		**2002**	
	n=117	n=13	n=117	n=13
canadensis	0.927	0.539	1.000	0.539
glaucus	0.073	0.462	0.000	0.000
hybrizyme			0.000	0.462
	2003		**2003**	
	n=29	n=14	n=29	n=14
canadensis	0.828	0.500	1.000	0.500
glaucus	0.172	0.500	0.000	0.000
hybrizyme			0.000	0.500
	2004		**2004**	
	n=158	n=12	n=158	n=12
canadensis	0.930	0.542	0.984	0.750
glaucus	0.070	0.458	0.003	0.000
hybrizyme			0.013	0.250

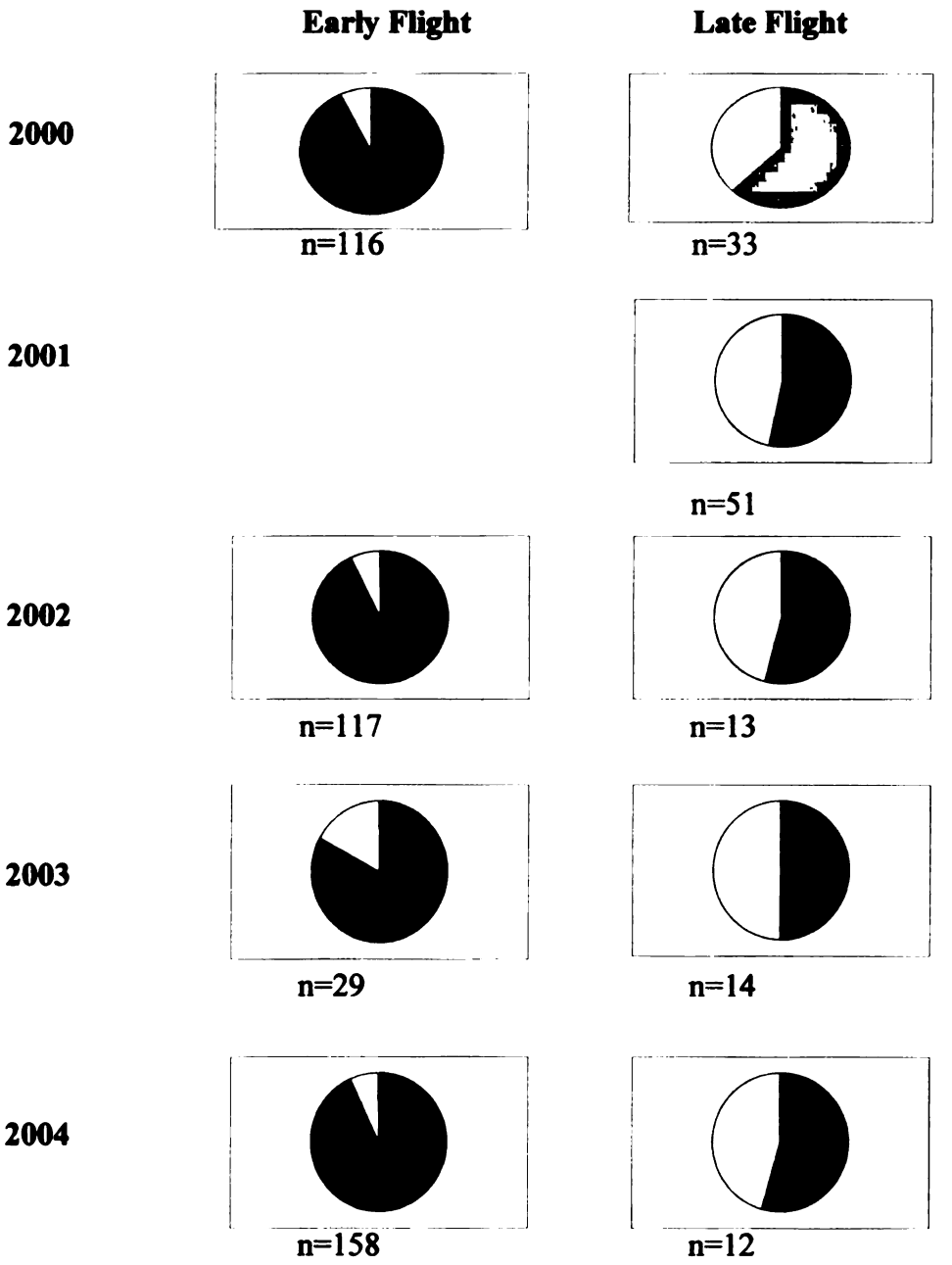


Figure 3.1. Degree of *P. glaucus* introgression into both the Early Flight and the Late Flight populations of the Battenkill River Valley, for the diagnostic X-linked Pgd locus. The portion of each graph that is filled in with white represents the proportion of introgressed *glaucus* Pgd alleles in that population for 2000-2004.

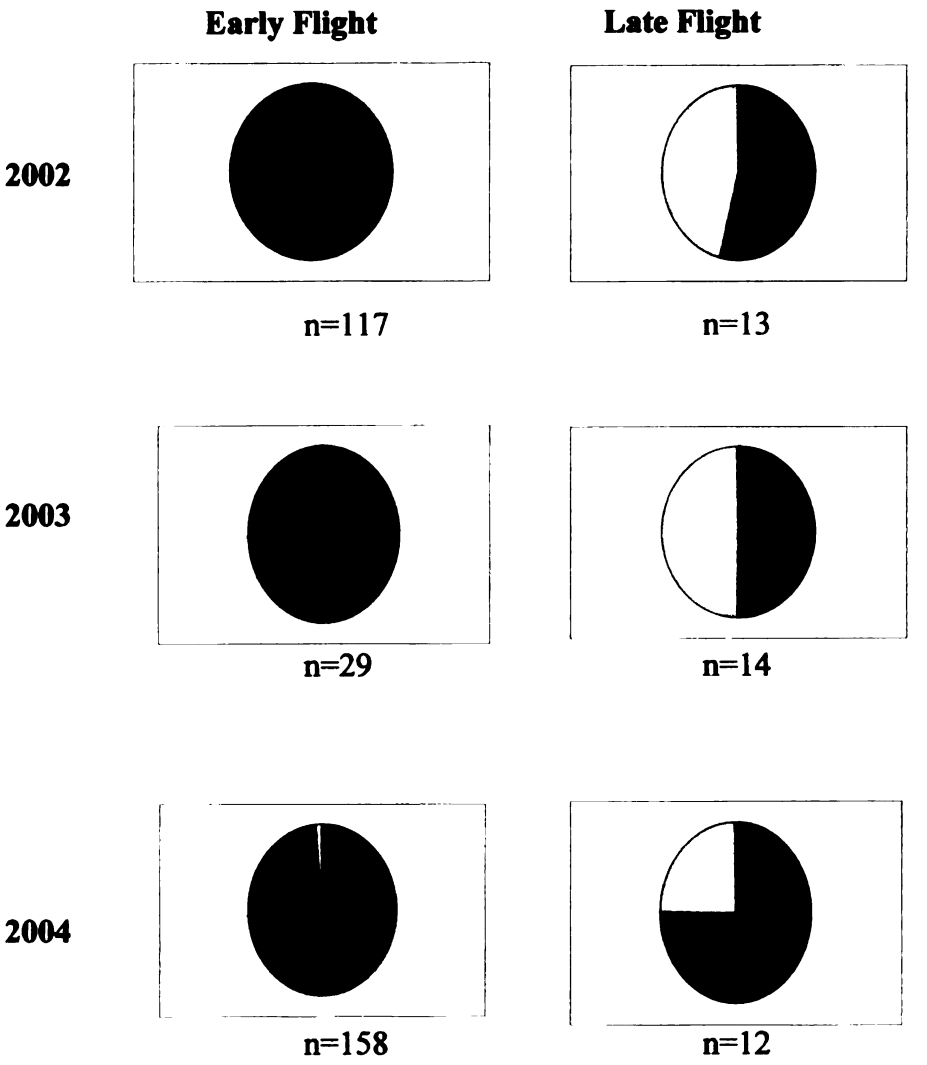


Figure 3.2. Representation of the presence and proportion of the allele frequencies of the novel X-linked Ldh 20 hybrizyme in both the Early Flight and the Late Flight populations of the Battenkill River Valley. The portion of each graph that is filled in with gray represents the proportion of the hybrizyme Ldh allele in those populations for 2002-2004.

Table 3.5. Wright's Fst values for the Early and Late Flight populations of the Battenkill River Valley (2000-2004) based upon allozyme data collected from wild captured male specimens. Pairwise values were calculated using Genepop Version 3.6. Values of greater than 0.15 indicate significant population differentiation and suggest little gene flow (Frankam et al. 2002). Fst values are presented for both of the individual X-linked loci analyzed (Pgd and Ldh). An * indicates that year for which the novel Ldh 20 hybrizyme was potentially present but unidentified and therefore not appropriately accounted for. Fst values that are significant are underlined.

Year	Pgd	Ldh
2000*	<u>0.253</u>	0.013*
2002	<u>0.284</u>	<u>0.567</u>
2003	0.148	<u>0.487</u>
2004	<u>0.381</u>	0.086

Mitochondrial DNA

All of the specimens analyzed from both the EF and the LF populations for PCR-RFLP were successfully amplified by PCR, except one specimen (LF ♂#8-03). The PCR products for all specimens were slightly shorter than 300bp long, as expected based on previous work (Sperling & Hickey 1995; Stump et al. 2003). No specimens produced two PCR fragments. All of the specimens analyzed from both the EF and the LF had PCR fragments that were both cut by *Ssp1*, and were not cut by *Taq1*, except one individual specimen from the EF (EF ♂#1-03). The PCR product from this EF specimen was both cut by *Taq1*, and was not cut by *Ssp1* (Table 3.6). In addition, for unknown reasons, one of the LF specimens (LF ♂#14-03) did not produce any banding pattern whatsoever after the restriction enzyme digestion.

The corroborated data for all specimens that were successfully analyzed indicates that the mtDNA carried by all of the LF specimens and all of the EF specimens except one (EF ♂#1), is *canadensis* like. *P. glaucus* mtDNA was only found in the single individual from the EF. Table 3.6 provides both the mtDNA haplotypes of all individuals analyzed as well as the associated genotypes for the two diagnostic X-linked loci (Pgd and Ldh). EF ♂#1 possess *glaucus* mtDNA and also exhibits some X-linked introgression. That specimen scored as heterozygous for the Pgd locus (one *canadensis* allele and one *glaucus* allele) but scored as carrying two *canadensis* alleles for Ldh. This indicates that that specimen was not an F1 hybrid, but is rather a recombinant type.

Table 3.6. mtDNA haplotypes and diagnostic X-linked loci (Pgd and Ldh) allozyme genotypes for fourteen wild captured males from 2003 from both the Early and Late flights of the Battenkill River Valley populations of *Papilio*. Introgressed *glaucus* alleles are presented in bold print.

Specimen	mtDNA	Pgd	Ldh
Early Flight ♂#1	**(+)**	125/**100**	80/80
Early Flight ♂#4	(–)	125/**100**	80/40
Early Flight ♂#6	(–)	125/125	80/80
Early Flight ♂#7	(–)	125/80	80/80
Early Flight ♂#8	(–)	125/125	80/80
Early Flight ♂#9	(–)	125/125	80/80
Early Flight ♂#10	(–)	**100**/80	80/80
Early Flight ♂#11	(–)	125/125	80/80
Early Flight ♂#12	(–)	125/80	80/80
Early Flight ♂#13	(–)	125/125	80/80
Early Flight ♂#14	(–)	125/80	80/40
Early Flight ♂#15	(–)	125/80	80/40
Early Flight ♂#16	(–)	125/125	80/80
Early Flight ♂#17	(–)	125/125	80/80
Early Flight ♂#18	(–)	125/**100**	80/80
Late Flight ♂#1	(–)	125/125	80/20
Late Flight ♂#2	(–)	125/**100**	80/20
Late Flight ♂#3	(–)	125/**100**	80/20
Late Flight ♂#4	(–)	125/**100**	80/20
Late Flight ♂#5	(–)	**100**/80	80/20
Late Flight ♂#6	(–)	**100/100**	40/20
Late Flight ♂#7	(–)	**100**/80	80/20
Late Flight ♂#8	(?)	**100/100**	40/20
Late Flight ♂#9	(–)	125/125	80/20
Late Flight ♂#10	(–)	**100/100**	80/20
Late Flight ♂#11	(–)	150/**100**	80/20
Late Flight ♂#12	(–)	125/125	40/20
Late Flight ♂#13	(–)	125/**100**	80/20
Late Flight ♂#14	(?)	125/**100**	80/20

Key

Species	mtDNA	Pgd	Ldh
canadensis	(–)	150, 125, 80	80, 40
glaucus	(+)	100	100

Emergence Investigations

The timing of pupal eclosion and adult emergence of the LF broods, that were derived from wild captured LF females, was consistently delayed compared to that of the EF broods for both years assayed under every temperature regime (2003 at 18 °C, 22 °C, 26 °C and 2004 at 14 °C, 18 °C, 22 °C, 26 °C). The mean days to emergence was significantly less for the EF broods than it was for the LF broods in all categories (Table 3.7). And in fact there was no overlap of the timing of any of the emerging broods under any of the individual temperature conditions, meaning that the last individual had emerged from the EF brood before the very first individual emerged from the LF brood with only three individual exceptions to this (see Figs. 3.3 and 3.4). In the 18 °C chamber in 2003, and in the 14 °C and 26 °C chambers in 2004, a single specimen each that were among the LF brood specimens (LF 18 °C ♂#1-03, LF 14° ♀ #1-04, and LF 26° ♂ #1-04) emerged well ahead of their conspecifics and in fact emerged directly in the midst of the EF emergence flush.

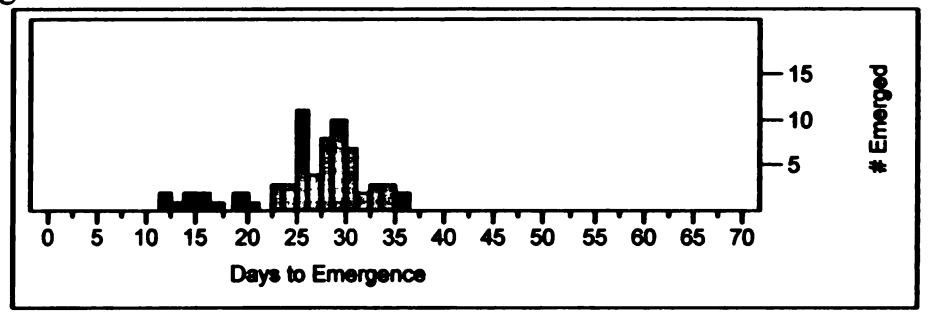
In addition to determining that the timing of emergence was distinct between the EF and LF populations, analysis was conducted to compare the timing of emergence between males and females under each temperature regime (Table 3.8). For both years that analysis was performed (2003-2004), and under every temperature regime, the mean days to emergence was slightly less for males than for females. In all categories except two, these differences in emergence timing were significantly different. The average emergence timing of females being slightly delayed from that of male specimens is highly adaptive considering how important protandry has been found to be as a component of reproductive strategies in many animals including *Papilio* (Wiklund 2003).

Table 3.7. Comparisons of the days to emergence between Early Flight and Late Flight field reared pupae. These comparisons were conducted in two sequential years across multiple temperatures (2003 at 18 °C, 22 °C, 26 °C and 2004 at 14 °C, 18 °C, 22 °C, 26 °C). Values represented are the mean number of days +/- s.e. from the time that pupae were removed from winter like conditions to the day of pupal eclosion Sample sizes are presented in parentheses. Probability values are derived from a nonparametric 2-sample Wilcoxon test performed using JMP 6.0. Probabilities for comparisons that are significantly different are indicated with an *. All comparisons are significantly different with p-values <0.0001.

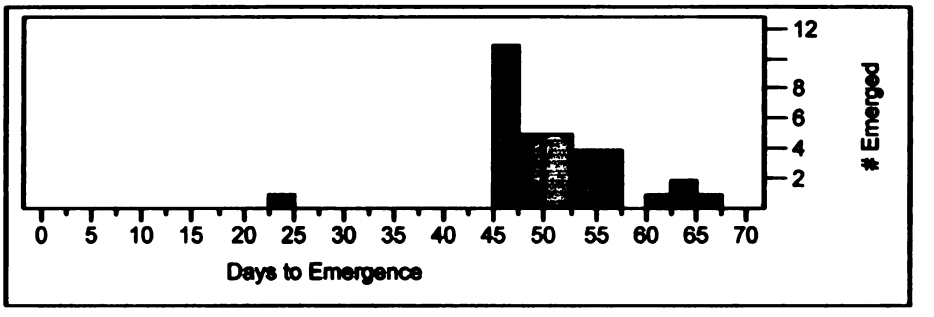
Year	Temp.	Mean Days to Emergence +/- s.e. EF (n)	LF (n)	Prob>[Z]
2003	18 °C	26.37 +/- 0.70 (67)	50.29 +/- 1.27 (34)	<0.0001*
	22 °C	15.78 +/- 0.34 (64)	32.94 +/- 1.31 (36)	<0.0001*
	26 °C	12.32 +/- 0.27 (65)	25.92 +/- 0.68 (36)	<0.0001*
2004	14 °C	50.40 +/- 0.82 (42)	93.97 +/- 2.97 (29)	<0.0001*
	18 °C	24.71 +/- 0.30 (48)	46.29 +/- 0.83 (35)	<0.0001*
	22 °C	17.02 +/- 0.28 (47)	32.78 +/- 0.82 (37)	<0.0001*
	26 °C	11.69 +/- 0.16 (48)	25.28 +/- 0.64 (39)	<0.0001*

2003 Emergence Experiments

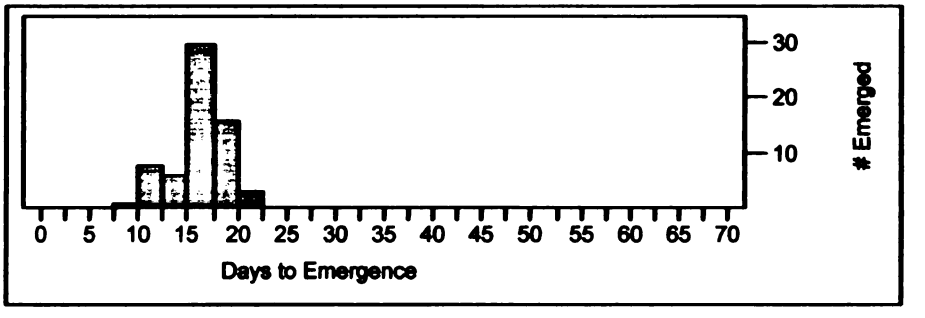
Early Flight 18 °C



Late Flight 18 °C



Early Flight 22 °C



Late Flight 22 °C

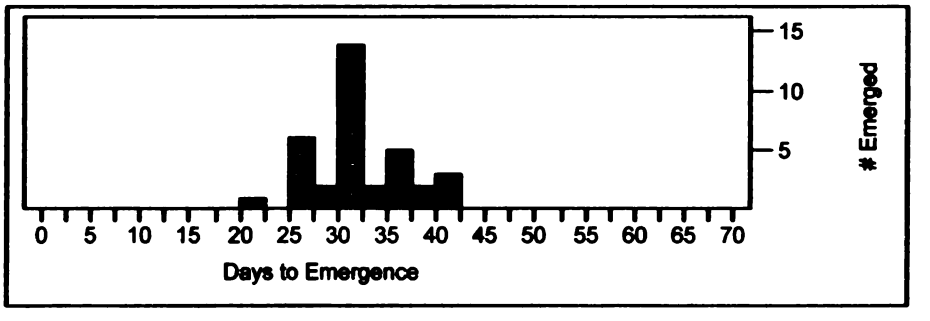
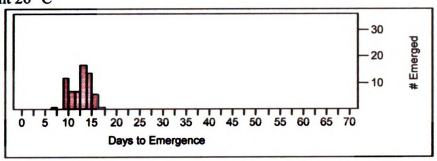


Figure 3.5. Histogram comparison of the days to emergence between Early Flight and Late Flight field reared pupae in 2003 at 18 °C, 22 °C, and 26 °C.

Early Flight 26 °C



Late Flight 26 °C

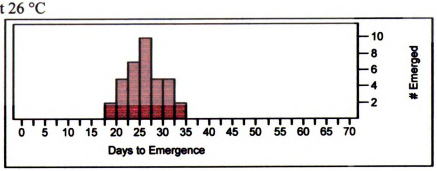


Figure 3.5 continued.

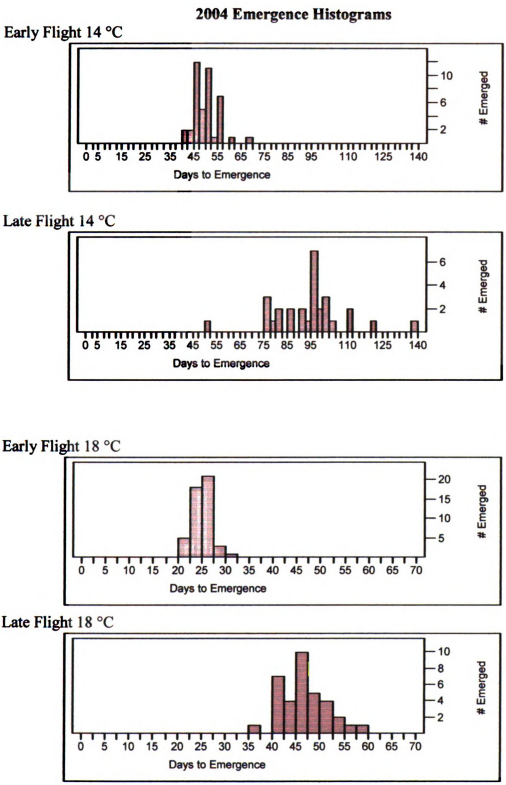
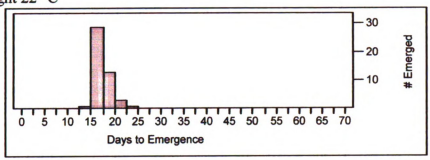
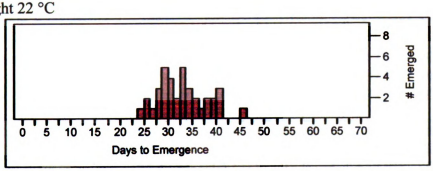


Figure 3.6. Histogram comparison of the days to emergence between Early Flight and Late Flight field reared pupae in 2004 at 14 °C, 18 °C, 22 °C, and 26 °C.

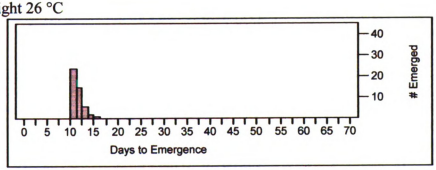
Early Flight 22 °C



Late Flight 22 °C



Early Flight 26 °C



Late Flight 26 °C

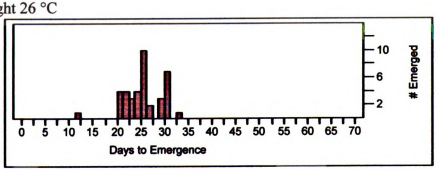


Figure 3.6 continued.

Table 3.8. Comparisons of the days to emergence between males and females in both the Early Flight and Late Flight field reared pupae. These comparisons were conducted in two sequential years across multiple temperatures (2003 at 18 °C, 22 °C, 26 °C and 2004 at 14 °C, 18 °C, 22 °C, 26 °C). Values represented are the mean number of days +/- s.e. from the time that pupae were removed from winter like conditions to the day of pupal eclosion Sample sizes are presented in parentheses. Probability values are derived from a nonparametric 2-sampleWilcoxon test performed using JMP 6.0. Probabilities for comparisons that are significantly different are indicated with an *.

		Mean Days to Emergence +/- s.e.		
Year	Temp.	Males (n)	Females (n)	Prob>[Z]
Early Flight 2003	18 °C	24.74 +/- 0.88 (38)	28.52 +/- 1.01 (29)	0.0004*
	22 °C	15.54 +/- 0.45 (28)	15.97 +/- 0.50 (36)	0.3560
	26 °C	12.03 +/- 0.31 (30)	12.57 +/- 0.42 (35)	0.1440
Late Flight 2003	18 °C	46.41 +/- 1.59 (17)	54.18 +/- 1.50 (17)	0.0008*
	22 °C	29.93 +/- 1.00 (15)	35.10 +/- 2.03 (21)	0.0250*
	26 °C	24.43 +/- 0.95 (21)	28.00 +/- 0.65 (15)	0.0025*
Early Flight 2004	14 °C	48.15 +/- 0.77 (26)	54.06 +/- 1.33 (16)	0.0002*
	18 °C	23.24 +/- 0.28 (21)	25.85 +/- 0.36 (27)	<0.0001*
	22 °C	15.80 +/- 0.19 (25)	18.41 +/- 0.38 (22)	<0.0001*
	26 °C	11.12 +/- 0.14 (26)	12.36 +/- 0.22 (22)	<0.0001*
Late Flight 2004	14 °C	89.41 +/- 2.56 (17)	100.42 +/- 5.85 (12)	0.0237*
	18 °C	44.15 +/- 0.92 (20)	49.13 +/- 1.17 (15)	0.0036*
	22 °C	30.18 +/- 0.99 (17)	35.00 +/- 1.03 (20)	0.0029*
	26 °C	23.67 +/- 0.76 (24)	27.87 +/- 0.74 (15)	0.0007*

DISCUSSION

Based upon the morphometric and molecular evidence, both the Early and Late Flight populations of the Battenkill River Valley can be characterized as hybrid swarms. Both of these flights exhibit higher levels of *P. glaucus* introgression than does the stable South Manitou Island hybrid swarm (see Chapter 2). The level of *P. glaucus* introgression exhibited in the Late Flight however, is far greater than that of the Early Flight.

The LF is likely the product of relatively high rates of *glaucus* introgression into the EF. This is made possible as a result of recent warming due to climate change in a highly condensed thermal landscape of the region near the hybrid zone (refer to Figure 1.4). Certain recombined backcross genotypes apparently result in an alteration of developmental rates leading to a significantly delayed spring emergence. It can be argued however that the LF is not merely a genetic "sink", annually being replenished by backcross offspring. The timing of the emergence of pupae derived from LF adults consistently results in their emergence being appropriately delayed, compared to that of the EF. This indicates that the LF is primarily the result of recurring annual reproductive activities within the LF. Additionally, the complete absence of the novel Ldh 20 hybrizyme in the EF through 2003 suggests that this novel allele arose as a mutation in the LF and that there has been little opportunity for genetic mixing between these two populations.

Based upon field observations and sampling success, it is clear that the LF is not nearly as numerous as is the EF. As a result, the Ldh 20 hybrizyme may have become so prevalent in the LF due to genetic drift in a small population. Alternatively, there may be

some metabolic fitness advantage associated with this hybrizyme in the novel thermal niche of the LF, favored through natural selection. It is important to note that the range of this Late Flight occurs in the geographic area that correlates with an accumulated 2300-2700 thermal degree-days. This is nearly the same thermal niche being exploited by *P. appalachiensis* (2600-2850 degree days) in the mountains of West Virginia and Georgia (Scriber et al. 2007).

An important element to the production of a stable, self-sustaining, reproductively isolated population is that the emergence of males and females is appropriately timed (i.e. synchronous), so as to maximize reproductive success. Protandry is a key element to this in many animals, and widely observed in butterflies (Wiklund 2003). The degree to which the timing of LF male emergence is appropriately just prior to the emergence of LF females (see Table 3.8) would strongly promote successful annual regeneration of a late flight derived from the LF of the previous year.

Both field observations and laboratory investigations suggest that the Late Flight population may in fact be temporally isolated enough to constrain gene flow between these two populations. Field observations over the course of the past five years in the Battenkill River Valley report the early *canadensis* like flight occurring primarily in late May and early June. The Late Flight adults are observed flying in mid to late July (Romack *personal communication*). Additionally, field reared pupae from both flights held in cold storage, have been simultaneously placed side by side under different temperature regimes (18, 22, and 26° Celsius) in climate controlled growth chambers, and monitored for emergence times (see Figures 3.5 and 3.6 and Table 3.7). Two years worth of collected data indicates that both the males and female from pupae from the

Late Flight exhibit a delayed emergence from that of the Early Flight adults. In every temperature regime, the last adult had emerged from the Early Flight before the first adult had emerged from the Late Flight, with only 3 individual exceptions. This time lag between the two broods was enhanced in the colder temperature regimes.

The comparatively premature emergence of the three individual LF specimens could potentially be explained by an error in labeling, or were in fact the result of unnoticed larvae that were already on the branch of the tree that had been oviposited by a free flying female. Either of these scenarios could potentially result in EF specimens being artificially grouped with LF specimens. This possibility was investigated by looking at the pupal weights of these individuals. One of the three specimens (LF 26° ♂ #1-04) did in fact have a very low pupal weight (0.683 grams) that is much more typical of those pupae from the EF. The other two specimens however had relatively large pupal weights (.965 and 1.116 grams), the second of which is larger than any EF pupae measured. Based upon pupal weight, these individuals would be categorized as LF specimens. An alternative possibility to explain unusually premature emergence in these specimens is that they possessed a genotypic backcross combination that resulted in this.

The emergence data is an indication of expected relative flight times but is highly temperature dependent. It should be noted that these emergence investigations were conducted at constant temperatures for both years in each temperature regime. This is not an ideal replication of field conditions in that there would be naturally occurring fluctuations in temperatures. Early spring would be somewhat cooler, and the temperatures would gradually increase as spring progressed. Additionally, fluctuations would be experienced between day and nighttime temperatures.

Under all of the laboratory maintained temperature regimes, the mean emergence times did appear to be distinct between these two populations but did not provide for a significant window of time, if any at all, between the flights. For instance in 2003 under the 26 °C temperature regime, the last EF specimen to emerge in the chamber is a female, and the very next day, the first LF specimen, a male emerges. The EF female would only have needed to survive a single day to have potentially encountered and mated with this LF male. This emergence pattern certainly would not afford sufficient temporal isolation to prevent gene flow. However, the laboratory temperature regime that is more indicative of what these populations would likely encounter in the wild is the 18 °C chamber. Considering the 2003 emergence data, there is a 10-day window between the last EF emergence and the first LF emergence under those conditions. Under these conditions, which more closely resemble field conditions, there is increased temporal isolation, which could greatly limit gene flow. In fact, it is important to remember that based upon field observations in the Battenkill River Valley, the EF in the field occurs from early to mid June and the LF emerges in mid July (Romack personal observations).

This pupal eclosion time lag does not in itself guarantee complete reproductive isolation however. It is still plausible that a late emerging Early Flight female could remain active for two weeks in the field and cross paths with one of the first to emerge protandrous males from the Late Flight. There is however, additional evidence, which suggests that this is infrequent at most. Allozyme electrophoresis has been employed to monitor the X-linked *canadensis* and *glaucus* like allele frequencies of both Pgd and Ldh enzymes. As previously stated, the frequency of *glaucus* like Pgd is much higher in the Late Flight, and there is zero *glaucus* like Ldh introgression in either flight. However, a

startling number of the Late Flight generation specimens exhibit the novel Ldh 20 hybrizyme allele that had never before been reported. Ldh 20 allele frequencies have been identified to be as high as 50% in the Late Flight (Fig. 3.2). This rare allele was identified in the Early Flight, but in only the most recent year (2004) and at a very low frequency (.003%).

In the field, those rare LF individuals (1.3% of those LF specimens reared in the sleeve rearing experiment) that were seen to have emerged well in advance of the rest of the LF brood, and in the midst of the EF emergence pattern, would serve to allow for low levels of genetic introgression from the LF population into the EF population. In this way the Ldh 20 hybrizyme could have been successfully transmitted. This would explain why it appeared in the last year of the specimens analyzed. Overall, the striking contrast of the allele frequencies for both of the X-linked loci, especially the newly identified "hybrizyme" suggests that there is very little genetic exchange between these two populations.

Incipient Speciation?

Based upon the analyzed data presented, a snapshot in time indicates that EF and LF are distinct subpopulations that appear to be largely reproductively isolated. This would indicate that this localized "Late Flight" phenomenon exhibits a combination of circumstances and mechanisms that could result in allochronic isolation that has resulted from introgressive hybridization. The timing of diapause and pupal eclosion has been reported to be a likely mechanism by which to provide an isolating mechanism other systems. Recent investigations done on the well studied *Rhagoletis* group has indicated

that in fact the host race differences associated with diapause may have been the key trait that allowed for the initiation of differentiation (Feder et al. 2003a). It has been found that this character trait actually arose in allopatry Feder et al. 2003b).

If the newly described species, *P. appalachiensis*, is in fact the result of hybrid speciation, and the Battenkill River Valley is another location where there are significantly increased levels of genetic introgression due to recent climate shifts, is it possible to identify and capture climatic speciation in the act? The Late Flight could be described as an incipient species. The morphological characteristics (forewing length and hind wing black band width) and the timing of emergence of specimens collected from this population indicate that they are virtually identical to *P. appalachiensis*. The major difference between *P. appalachiensis* and the Late Flight is that *P. appalachiensis* populations have nearly fixed allozyme frequencies (*glaucus* like Pgd and *canadensis* like Ldh), while the Late Flight still exhibits a higher retention of *canadensis* like Pgd alleles.

A combination of mechanisms could eventually allow for the Late Flight allele frequencies to become fixed for the *glaucus*-like Pgd alleles, thus making the Late Flight virtually indistinguishable from *P. appalachiensis*. First, the Late Flight would need to be sufficiently temporally, and thus reproductively isolated from the first flight of *canadensis* to prevent the continued influx of *canadensis*-like genes. Second, there would need to be either sufficient selection (intrinsic or extrinsic) against the *canadensis*-like Pgd allele to eliminate it (or something closely linked on the X-chromosome), or the elimination of the *canadensis* Pgd alleles could be accomplished through random genetic drift over time. These possibilities are examined in Chapter 4.

CHAPTER 4:

TRAIT LINKAGE ON THE X-CHROMOSOME OF *PAPILIO CANADENSIS* AND *P. GLAUCUS,* REVEALED BY A HIGHLY INFORMATIVE BACKCROSS

Introduction

In all of the *P. canadensis* populations that have begun to exhibit *P. glaucus* introgression, including the populations of *P. appalachiensis*, there appears to be a consistent pattern of "*glaucus*-like" traits, both present and absent. These populations possess individuals, who score either intermediate or *glaucus*-like for most of the species diagnostic traits (forewing length, hind wing black-band width, oviposition preference, tulip tree detoxification ability, and Pgd and Hk allozymes (Scriber and Ording 2005)). However, none of the several hundred individuals analyzed from these populations has possessed the *glaucus* X-linked Ldh allele (100). Nor have there been any individuals captured that have demonstrated the ability for direct development, which is dictated by the X-linked od- gene allowing for facultative diapause.

The latter of these two traits not being present is not so surprising. As stated previously, the offspring of any individual possessing the od- gene, that underwent direct development, would stand no chance of completing development to pupation given the limited thermal environment. As earlier stated, completion of two full generations within a single year requires 2800 degree-days, even on the highest quality host plant species for rapid larval growth (Scriber and Lederhouse 1992;

Scriber 1996b). On average, there are simply insufficient annual thermal units in the locations where these hybrid swarm populations exist. This thermal constraint would likely act to strongly select against the od- gene.

As described in Table 1.1, studies of diagnostic traits in these two *Papilio* species have determined that five known species-specific genetic differences exist on the X-chromosome (Scriber 1994, Hagen and Scriber 1995). The sequence in which these loci exist along the length of the X-chromosome, as well as an estimated relative map distance between each locus, is depicted in Figure 1.3.

Individual specimens have been field collected from these introgressed populations that have exhibited traits that suggest that they possess recombinant genotypes of X-linked diagnostic traits. There are two potential methods by which an individual could express an apparently non-concordant genotype. One method by which apparently recombined genotypes could arise is through segments of the X-chromosome being translocated to an autosome, and in this way passed on to offspring. Sex chromosome segment translocation has been described in the Mediterranean flour moth (Marec et al. 2001). A more commonly described method by which recombinant types could arise in this *Papilio* group is through chromosomal crossovers during meiosis in males.

Why is there zero introgression of "*glaucus*-like" Ldh (100)?

Given the apparent frequency with which chromosomal crossovers can occur in these hybridizing butterflies, why is it then that the "*glaucus*-like" Ldh 100 allele is never expressed in individuals sampled from the introgressed populations of *P.*

canadensis? One possible explanation is that the Ldh locus is far more closely linked to the diapause locus than has been previously suggested (Hagen and Scriber 1984). A close linkage between the "*glaucus*-like" facultative diapause and Ldh 100 would explain why neither trait occurred in these populations. As earlier stated, individuals possessing the facultative diapause gene would be rapidly eliminated from populations anywhere with fewer than 2750 °F degree days in the colder more northern regions of North America.

An alternative hypothesis explaining the absence of Ldh 100 in introgressed populations of *P. canadensis* is that there is direct selection on the Ldh gene. Allozymes are often utilized as genetic markers in population investigations and are assumed to be neutral genetic markers. However, lactate dehydrogenase is an important enzyme in the metabolism of carbohydrates. Distinct alleles of this enzyme have been shown to exhibit structural variability and temperature dependent differences in thermal stability and function (Adams et al. 1973). Some alleles perform much better at higher or lower temperatures, whereas others perform poorly under certain temperature regimes (Angiletta et al. 2003). Possession of different allelic forms of the Ldh enzyme having differing thermal stabilities has resulted in a steep latitudinal cline in marine fishes directly correlating with environmental temperatures (Crawford and Powers 1989; Dimichele and Powers 1991). There is clear evidence that differing thermal environments impose striking differential selective pressures on different structural forms of enzymes (Eanes 1999). This has been shown in several studies to be true specifically for Ldh (Crawford and Powers 1989; Johns and Somero 2004; Schulte et al. 2000).

Furthermore, it has been shown that minor modifications to the gene that codes for Ldh can result in significant structural changes that in turn result in differences in thermal stability. Minimal amino acid substitutions have been identified in the Ldh enzyme in six different species of barracuda living in different thermal environments (Holland et al. 1997). It has been shown that there is a single amino acid substitution in the Ldh enzyme between two distinct species of damselfishes, one native to cold-temperate habitats, while the other is native to tropical waters (Johns and Somero 2004). This too has been identified to be the case in the Ldh enzyme between *P. canadensis* and *P. glaucus*. Preliminary results indicate that a single base pair substitution is responsible for the structural differences between Ldh 100 (*glaucus*) and Ldh 80 (*canadensis*) (Andolfatto 2004 *personal communication*). Collaborative work is now underway to identify DNA sequence differences between the various Ldh alleles.

Lab rearing organisms under environmentally controlled conditions can be of great value in that it can help to eliminate the uncertainties and confounding variables that can arise under field conditions. Additionally, there is great value to being able to produce lab reared crosses in that you can better determine and manipulate the genetic background of the parents and resulting offspring. When investigating the mechanisms that prevent widespread gene flow across a hybrid zone, lab reared crosses between individuals of known genetic material allows controlled investigations to distinguish between intrinsic and extrinsic barriers to gene flow. Recent investigations associated with newly described hybrid species have allowed

the "recreation" of hybrid species genotypes under laboratory conditions (Riesberg et al. 2003; Maverez et al. 2006).

A great deal of work has been done with laboratory breeding investigations conducted with *Papilio glaucus* and *P. canadensis*. Most of this work has been applied towards identifying the mechanisms that are involved in the maintenance of their narrow hybrid zone and also those factors that were involved in the processes of their speciation (Luebke et al. 1988; Hagen et al 1991; Hagen and Scriber 1995; Scriber et al. 1999). It was determined through genetic analysis of laboratory-reared crosses and backcrosses between these two species, that the Haldane effect plays a significant role as a postzygotic isolating mechanism. It was found that an incompatibility between *canadensis* X-chromosome genes and the *glaucus* Y-chromosome or cytoplasm results in the disruption of female development (Hagen and Scriber 1995). A laboratory hybridization study conducted by Pavulaan and Wright (2002), who originally described the new *P. appalachiensis,* illustrates that the Haldane effect was also exhibited in a brood produced through hand pairing of an *appalachiensis* male and a dark morph *P. glaucus* female. This cross resulted in 24 eggs and 1st instar larvae but only resulted in 10 successful pupae. Of those 10 pupae, only 7 males successfully emerged. The remaining 3 were females that never successfully eclosed. (Pavulaan and Wright 2002)

There is a good deal of evidence that suggests that chromosomal crossovers frequently occur in these *Papilio* species. This is the abundance of individuals field captured from introgressed *P. canadensis* populations (South Manitou and Vermont), that when analyzed through allozyme electrophoresis, exhibit the "*glaucus*-like" Pgd

allele (100) but one of the "*canadensis*-like" Ldh alleles (40 or 80), as is the fixed condition of *P. appalachiensis* (Scriber and Ording 2005). In addition, many lab reared crosses have produced offspring expressing X-linked trait combinations that can most readily be explained through chromosomal crossovers (Scriber 1994).

Data gathered through laboratory crosses between *P. glaucus* and *P. canadensis* have also been applied toward the development of the linkage map of the X-linked loci (Fig. 1.3) for which there are species diagnostic alleles (Hagen and Scriber 1989). This linkage map provides an indication as to the sequence and an estimate of the relative distances between major landmark loci along the length of the X chromosome in this *Papilio* group. This provides a mechanism by which to predict the likelihood of certain genetic recombinations occurring through genetic crossovers.

The rate at which genetic recombinations occur in *P. glaucus* and *P. canadensis* has been found to be higher than in other insect groups including *Drosophila*, relative to mutation rates (Putnam et al. 2007). Fixed states of genetic recombination between two parental species has been an element in characterizing and a mechanism in helping to explain some cases of hybrid speciation (Riesberg et al. 2003; Schwarz et al. 2005; Gompert et al. 2006) including the new *Papilio appalachiensis* (Scriber and Ording 2005). Comparable genetic recombination between *P. glaucus* and *P. canadensis* appears to explain the origin of the Late Flight in the Battenkill River Valley of Vermont and New York. Lab-reared backcrosses could help to "recreate" the genotypes that result in a delayed emergence mimicking that of the Late Flight. Analysis of individuals of this type would then help to identify the genetic combinations that result in this shift in such a major life history

trait, as is the timing of diapause. This chapter is devoted to the analysis of a unique lab reared backcross that helps to illustrate the striking frequency with which recombination can occur, the variety of genetic combinations that can be produced, and partially determine whether it is intrinsic (genetic incompatibilities) or extrinsic (environmental) factors that have acted as a mechanism to prevent gene flow between *P. glaucus* and *P. canadensis*.

MATERIALS AND METHODS

Laboratory Cross

The most informative type of cross in this *Papilio* system is to produce a backcross between a dark morph female and a hybrid male. This provides the most information regarding X-linked loci. In this investigation, a wild captured dark morph female *P. glaucus* produced a series of offspring in the lab. One of the lab-reared offspring was a virgin dark morph female (♀#18006). A lab-reared male was produced from a hand-pairing between a dark morph *P.g.* ♀ from Pennsylvania and a wild caught *P.c.* ♂ from Oscoda County, Michigan. ♀#18006 was then hand paired to this lab-reared F1 hybrid. After the pairing, ♀#18006 was placed in an oviposition arena with suitable host plants upon which to deposit eggs. These eggs were collected and the resulting larvae were reared through to pupation on appropriate host plants under early to mid summer like photoperiod conditions (L18:D6) with the intent of inducing direct development in those offspring that possessed the X-linked facultative diapause gene (Rockey et al. 1987).

The resulting pupae were placed in screened cages and monitored for direct development. Those specimens that direct-developed (n=16 dark morph ♀, n=4 yellow ♀, and n=39♂) were placed in –80 °C freezer for storage until allozyme electrophoresis was conducted. The resulting pupae that did not direct develop were collected in mid-September and stored in darkness at 3-5 °C under controlled environmental diapause conditions until the recommencement of emergence investigations. Those pupae were brought out of cold-storage in early May and placed in screened petri dishes in a 26 °C growth chamber under a L18:D6

photoperiod. Specimens were monitored and as they emerged, they were placed in –80 °C storage to await allozyme electrophoresis. Pupae that did not emerge after this first emergence opportunity were again placed in refrigerated cold storage for 12 weeks to mimic a second winter period. They were then again removed from cold storage and monitored through another emergence period.

Allozyme Electrophoresis

Allozyme electrophoresis was conducted on ♀#18006, the parental F1 hybrid to which she was hand paired, and all of the offspring that successfully pupated and direct developed, or emerged the next year. Every specimen was assayed for both of the X-linked diagnostic loci (Pgd and Ldh). Allozyme electrophoresis was accomplished following those methods described in chapters 2 and 3.

X-Chromosome Mapping

Based upon the suite of known X-linked diagnostic characters, analysis of the sex, color, diapause behavior, and allozyme electrophoresis data, it was possible to map and determine the parental source of each portion of the X-chromosome(s) carried by each of the offspring produced in the 18006 brood. In this way it was possible to detect which individuals carried X-chromosomes that were the result of a genetic crossover.

Some assumptions have been made in the process of determining some of the specimen allelic identities. The identity of the diapause locus has been based purely on whether or not the specimen entered diapause. If they went into diapause they were assumed to be carrying the *canadensis* diapause allele od+. Also, in the case of

the X-linked dark morph suppressor gene (s+) it is impossible to say for certain in the case of male specimens, as they do not carry a Y-chromosome and therefore never express the dark morph phenotype (b+; Scriber et al. 1996). In these male specimens the allelic identity was assumed based upon the identity of the Pgd locus.

Analysis for Haldane Effect

A determination as to whether a Haldane effect is exhibited in hybrid backcrosses was accomplished by determining the relative survival probabilities for female offspring (heterogametic sex in *Papilio*) from the 18006 brood. Survival probabilities were determined relative to those of male offspring from the 18006 brood with the same paternally inherited Ldh and Pgd alleles. These probabilities were obtained by dividing the number of females with each genotype by the number of males with the corresponding paternal haplotype.

RESULTS

Emergences

Of the pupae that were produced from the 18006 brood, 39 male and 20 female specimens (4 yellow and 16 dark morph) direct developed. Four male and four dark morph female specimens emerged during the first post diapause emergence period. One additional male and three additional dark morph female specimens emerged after the second post diapause emergence period.

Allozyme Electrophoresis and X-chromosome Mapping

Table 4.1 illustrates the determined character state for each of the four X-linked loci for ♀#18006, her F1 mate, all of the direct developing offspring (n=16 dark morph ♀, n=4 yellow ♀, and n=39♂), the diapausing specimens that emerged the following year (n= 4 dark morph ♀ and n=4♂), and the diapausing specimens that emerged after two winter like diapause periods (n=3 dark morph ♀ and n=1♂). Images in this dissertation are presented in color. The degree of certainty of the mapping of the individual X-chromosomes for every individual was possible only due to the fact that the male specimen to which ♀#18006 was paired carried a relatively rare Pgd allele. From his dark morph Pennsylvania mother he received an X-chromosome that carried an extremely rare Pgd 50 allele. This allele exists in relatively low frequencies in *glaucus* populations (Hagen and Scriber 1991). Had he carried the far more commonly observed *glaucus* Pgd 100 allele, this would have served to mask and prevent a true detection of some chromosomal crossovers.

Table 4.1. X-chromosome linkage maps for each of the offspring produced in the18006 brood. The linkage maps represented indicated the following loci in sequential order: dark morph suppressor / Pgd / diapause / Ldh. Images in this dissertation are presented in color. Color-coding allows for determination of the parental origin of each chromosome or chromosomal segment. Portions of chromosomes that could not be determined with confidence are indicated with ???.

Mother – P.g. ♀ # 18006 Penn. (Dark)	s- Y	100	od-	100
Father – P.g. ♀ Dark Penn. X P.c. ♂ # 17047 Oscoda	s+	125	od+	40
	s-	50	od-	100

F1 – Direct Developing ♀s

1.	s- Y	50	od-	100	**11.**	s+ Y	125	od-	100
2.	s- Y	50	od-	100	**12.**	s- Y	50	od-	100
3.	s- Y	50	od-	100	**13.**	s- Y	50	od-	100
4.	s- Y	50	od-	100	**14.**	s+ Y	125	od-	100
5.	s- Y	50	od-	100	**15.**	s- Y	50	od-	100
6.	s- Y	50	od-	100	**16.**	s- Y	50	od-	100
7.	s- Y	50	od-	100	**17.**	s+ Y	125	od-	100
8.	s- Y	50	od-	100	**18.**	s- Y	50	od-	100
9.	s- Y	50	od-	40	**19.**	s- Y	50	od-	100
10.	s+ Y	125	od-	100	**20.**	s- Y	50	od-	100

F1 – Direct Developing ♂s

1.	s-	50	od-	100	**16.**	s-	50	od+	40
	s-	100	od-	100		s-	100	od-	100
2.	s-	50	od-	100	**17.**	s-	50	od-	100
	s-	100	od-	100		s-	100	od-	100
3.	s-	50	od-	100	**18.**	s+	125	od-	100
	s-	100	od-	100		s-	100	od-	100
4.	s+	125	od-	100	**19.**	s-	50	od-	100
	s-	100	od-	100		s-	100	od-	100
5.	s-	50	od-	100	**20.**	s+	125	od+	40
	s-	100	od-	100		s-	100	od-	100
6.	s-	50	od-	100	**21.**	s-	50	od-	100
	s-	100	od-	100		s-	100	od-	100
7.	s+	125	od-	100	**22.**	s-	50	od+	40
	s-	100	od-	100		s-	100	od-	100
8.	s-	50	???	**???**	**23.**	s-	50	od+	40
	s-	100	od-	100		s-	100	od-	100
9.	s-	50	od-	100	**24.**	s+	125	od-	100
	s-	100	od-	100		s-	100	od-	100
10.	s+	125	???	**???**	**25.**	s+	125	od-	100
	s-	100	od-	100		s-	100	od-	100
11.	s+	125	???	???	**26.**	s+	125	od+	40
	s-	100	od-	100		s-	100	od-	100
12.	s-	50	od-	100	**27.**	s-	50	od+	40
	s-	100	od-	100		s-	100	od-	100
13.	s-	50	od-	100	**28.**	s+	125	od+	40
	s-	100	od-	100		s-	100	od-	100
14.	s-	50	**???**	**???**	**29.**	s-	50	od-	100
	s-	100	od-	100		s-	100	od-	100
15.	s+	125	od-	100	**30.**	s+	125	od-	100
	s-	100	od-	100		s-	100	od-	100

31.	s+	125	od+	40
	s-	100	od-	100
32.	s-	50	od-	100
	s-	100	od-	100
33.	s-	50	od+	40
	s-	100	od-	100
34.	s-	50	od-	100
	s-	100	od-	100
35.	s+	125	od+	40
	s-	100	od-	100
36.	s+	125	od-	100
	s-	100	od-	100
37.	s+	125	od-	100
	s-	100	od-	100
38.	??	???	???	???
	s-	100	od-	100
39.	s+	125	od+	40
	s-	100	od-	100

First Emergence F1 Diapausing ♀s

21.	s-	50	od+	40
	Y			
22.	s-	50	od-	100
	Y			
23.	s-	50	od-	100
	Y			
24.	s-	50	od+	40
	Y			

First Emergence F1 Diapausing ♂s

40.	s-	50	od+	40
	s-	100	od-	100
41.	s+	125	od-	100
	s-	100	od-	100
42.	s+	125	od+	40
	s-	100	od-	100
43.	s+	125	od-	100
	s-	100	od-	100

Second Emergence F1 Diapausing ♀s

25.	s-	125	???	100
	Y			
26.	s-	125	od+	40
	Y			
27.	s-	50	???	100
	Y			

Second Emergence F1 Diapausing ♂

44.	s+	125	???	100
	s-	100	od-	100

All of the X-linked alleles that were detected in both parents were observed throughout the offspring. Of the total number of offspring analyzed, 28 out of 71 specimens (39.4%) were determined to possess a recombined X-chromosome. Of the 20 direct developing females, five of them (25%) carried recombined X-chromosomes. Of the 39 direct developing males, 15 of them (38.5%) carried recombined X-chromosomes. Four out of seven diapausing females and four out of five diapausing males carried recombined X-chromosomes. Of those crossovers that were detectable, 90% of them (26 out of 29) were the result of a cross between the Pgd and diapause loci. Two specimens (diapausing ♀#26 and ♀#27) exhibited a crossover that was determined to be between the dark morph suppressor (s) and the Pgd loci. Also, there was only a single specimen (direct developing ♀#9) that exhibited a crossover that was determined to be between the diapause and Ldh loci.

Evidence for multiple crossovers on the same individual X-chromosome exists in only one female specimen (♀#25). This is a yellow female carrying a Pgd 125 allele and an Ldh 100 allele. This indicates that a crossover must have occurred between the dark morph suppressor and Pgd loci and also somewhere between the Pgd and Ldh loci. This observation indicates that it is possible that there were other specimens in which there were multiple crossovers, some of which were undetectable based upon the markers available.

Analysis for Haldane Effect

Of the 65 total offspring that were successfully reared to adulthood from the 18006 brood, there were a total of 39 males and only 26 females. The ratios of female to male specimens that were produced in each of four genotypic categories,

these based on the species diagnostic X-linked alleles possessed for both the Pgd and Ldh loci are contained in Table 4.2. The results indicate that there is a Haldane Effect represented in this brood. The haplotype for which there is the greatest female survival are those specimens carrying an apparently in tact *glaucus* X-chromosome, and in this category survival probability is actually greater than for males. Females in each of the alternative haplotype categories, either an X-chromosome that exhibits mixed *canadensis* and *glaucus* elements, but especially those that carry an apparently pure *canadensis* X-chromosome, exhibit reduced survival probabilities compared to their male counterparts.

Table 4.2. Relative survival probabilities for 18006 backcross females. Survival probabilities are relative to those of males with the same paternally inherited Ldh and Pgd alleles. Probabilities were obtained by dividing the number of females with each genotype by the number of males with the corresponding paternal haplotype. These numbers are given in parentheses as (#females / #males).

	Pgd 50 (*glaucus*)	Pgd 125 (*canadensis*)
Ldh 100 (*glaucus*)	**1.29** (18/14)	**.33** (4/12)
Ldh 40 (*canadensis*)	**.50** (3/6)	**.14** (1/7)

DISCUSSION

The X-chromosome has been found to play a disproportionately large role in the evolution of isolating mechanisms and the process of speciation in Lepidoptera (Prowell 1998; Jiggins et al. 2001). Butterflies (and birds) are somewhat unusual in that the male sex is homogametic. It has been found that traits carried on the X-chromosome are primarily responsible for the maintenance of reproductive isolation between *Papilio glaucus* and *P. canadensis* and that there is a detectable Haldane effect (Hagen and Scriber 1995). The Haldane effect results in decreased viability of the heterogametic sex due to genetic incompatibilities. Analysis of hybrid backcrosses can help to differentiate between which X-linked loci combinations are involved in hybrid genetic incompatibilities and those that are not.

This 18006 backcross, and other backcrosses of this nature, provide valuable information as to the hybrid recombinant genotypes that can be produced in introgressed hybrid swarms. The ability to identify the relative locations of multiple loci, both those of value as species diagnostic tools and also those that impact significant life history traits, provides a clear environmental mechanism by which to explain the sharply contrasting clines that are exhibited across the hybrid zone.

The analysis of the 18006 brood, derived from a laboratory hand-pairing, has proven extremely valuable. Given that male *Papilio* are the homogametic sex, any X-chromosome crossovers that might occur could only have taken place during the process of sperm production. One thing that is obvious and striking about the genetic analysis of the 18006 brood, is the high frequency with which recombination can

occur. In this single brood, over 37% of the offspring produced were recombinant types, possessing individual X-chromosomes that carry alleles that are diagnostic for two different species.

The next observation that can be made is the location at which the crossovers have been determined to occur, and with what frequencies. Out of the high number of individuals possessing a recombinant X-chromosome, 93% of them exhibit a crossover that is between the Pgd and diapause loci, and only a single specimen exhibits a crossover between the diapause and Ldh loci. This is striking considering the relative distances between these loci as represented on the linkage map previously developed (see Fig. 1.3) by Hagen and Scriber (1989). That linkage map suggests that the distance between the Pgd and diapause loci is relatively small, and in comparison the distance between the diapause and Ldh loci is large. The results from the 18006 analysis indicates that the likelihood of a crossover occurring between the Pgd and diapause loci is extremely high, and that the likelihood of a crossover between the diapause and Ldh loci is surprisingly low. This suggests that there must be a great deal more distance between the Pgd and diapause loci than between the diapause and the Ldh loci.

The *glaucus* Ldh 100 allele has previously never been reported in field captured specimens collected in the hybrid zone or in any identified hybrid swarm population. This could be explained through extrinsic (ecological) selection. If in fact the diapause and Ldh loci are linked much more closely than has historically been suggested, environmental factors would act strongly to prevent such combinations from surviving in the field. *P. glaucus* carry a facultative diapause gene (od-)

allowing for a bivoltine life cycle in thermally appropriate conditions. Diapause in *P. glaucus* is triggered by the shortening photoperiods associated with late summer. If this od- allele, tightly linked to the *glaucus* Ldh 100 allele, were donated to a hybrid brood, the offspring would likely direct develop in preparation to produce a second flight. The photoperiod at the locations at which such a cross would occur would typically induce direct development in the offspring. However, in these northward-extended latitudes, the continuing warmth of summer would be relatively short, providing insufficient thermal energy to allow for the second brood to successfully achieve pupation. These hybrid individuals would die in the field, and the Ldh 100 allele would be strongly selected against.

The Haldane effect has been reported as a mechanism that results in postzygotic isolation between *P. glaucus* and *P. canadensis* (Hagen and Scriber 1995). The explanation has been that there is some form of genetic incompatibility between the *canadensis* X-chromosome and either the *glaucus* Y-chromosome or cytoplasm. Often times, backcross females remain in "extended" or permanent diapause. The genotypes of these female backcrosses that die as pupae would be reflected as a deficit in the sex ratio of adults carrying each allelic combination. Analysis of the offspring of the 18006 brood lends support to this. Of the 71 offspring produced only 38% are female. The 18006 brood contains male specimens, both direct developing and diapausing, that carry what appear to be completely intact X-chromosomes from both parental types. However, among the female specimens, there is not a single individual that possesses a completely intact *canadensis* X-chromosome, and a strong

deficit of females carrying either of the *canadensis* allozyme alleles (Table 4.2). This deficit is most obvious in the category in which both allozyme alleles are the *canadensis* type. The ratios derived in each of the four allelic "classes" are similar to the ratios derived in similar crosses performed when the original X-chromosome linkage map was developed (Hagen and Scriber 1995).

Closer scrutiny of the allelic identities contained in the recombined 18006 females illustrates that there are a diverse combination of partial *canadensis* X-chromosomes associated with the *glaucus* Y-chromosome. Assuming that there were no crossovers that were undetected, it would appear as though every single portion of the X-chromosome has successfully complemented the *glaucus* Y-chromosome and cytoplasm. This might suggest that any genetic incompatibilities might be the result of a combination of loci along the length of the X-chromosome or that the incompatibilities are not 100% lethal. Another possibility that needs to be considered is that X-chromosome crossovers occurred in this brood that went undetected.

This 18006 investigation clearly indicates that the frequency with which chromosomal crossovers occur within this *Papilio* system is high. Analysis of the offspring from this brood also provides additional support, identifying the genetic components that result in the Haldane effect being exhibited in hybrid backcrosses between *P. glaucus* and *P. canadensis*. It also provides evidence for the functional mechanisms by which genetic crossovers can result in the genetically compatible backcross genotypes that are exhibited in the Late Flight hybrid swarm of Vermont and the newly described species, *P. appalachiensis*. A

revised linkage map that accurately depicts the sequence and relative distances between the species diagnostic loci on the X-chromosome would be extremely valuable, but alternative techniques might be more efficiently applied to this task. An improved linkage map and alternative techniques of analyzing the X-chromosome would be invaluable in determining which portions of the *glaucus* and *canadensis* genomes were incompatible. Additional backcross investigations need to be conducted in an attempt to consistently "recreate" the genotypes that result in an obligate diapausing brood, which exhibits the delayed emergence, as is the case for the Late Flight and *P. appalachiensis*.

CHAPTER 5:

SUMMARY AND CONCLUSIONS

Speciation is typically viewed as a process through which populations gradually evolve mechanisms that confer reproductive isolation (Harrison 1993; Coyne and Orr 2004). Populations that have recently diverged in allopatry may be lacking strong reinforcement mechanisms (Howard 1993). If these populations are brought back together in secondary contact, weak isolating mechanisms allow for introgressive hybridization. Historically hybridization has been viewed as an evolutionary dead end. More recently however, it has been discovered that hybridization can allow for rapid recombination and can ultimately act to enhance genetic diversity (Lewontin and Birch 1966; Seehausen 2004). Levels of enhanced genetic diversity can allow for rapid adaptation to unique environmental conditions. Furthermore increased rates of mutation near species borders can help to enhance the rate at which these populations can become adapted to a unique ecological niche (Nevo 2001).

Hybridization between closely related populations with distinct life history traits, such as timing of diapause and / or patterns of voltinism can potentially lead to offspring whose expression of these traits are truly distinct from either parental population (Gross and Riesberg 2005). These distinct patterns can act as a mechanism by which to rapidly isolate these offspring populations from either parental population (Thomas et al. 2003). This is especially true when the genetic determination of these life history traits is based relatively few genes (Henrich and Denlinger 1983) or even a single gene.

When comparing closely related species it is often difficult to disentangle whether traits that differ between the two species were actually involved in the process of speciation or whether they arose after the two species were already reproductively isolated. Most species groups are evolutionarily old enough so that it is difficult to identify the order of genetic changes. For example, the differences that exist between the host-races of *Rhagoletis,* for host plant preference, diagnostic enzymes, and differences in the timing of eclosion (Feder et al. 2003). However, it is uncertain as to the sequence in which these genetic differences arose and whether each of these had an initial role in initiating reproductive isolation.

Global climate change has resulted in dramatic changes in the thermal environment in various regions. Most ecological predictions associated with the impacts of climate change and global warming disturbingly warn of disastrous events (ecosystem simplification and collapse, decline of genetic diversity within populations, loss of biodiversity) (Parmesan and Yohe 2003; Thomas et al. 2004). However, a great deal of recent research is indicating that shifting climate patterns inducing range shifts of closely related species can provide opportunities for the shuffling of co-adapted gene complexes resulting in climate induced hybrid speciation (Dowling and Secor 1997). This genetic reshuffling would allow evolutionary changes to shadow environmental shifts, adapting organisms to new and unique niches.

It has been thought that *Papilio glaucus* and *Papilio canadensis* became initially differentiated in allopatry and have recently met in secondary contact along an ecological transition zone, which directly correlates with their range boundaries. The geographic distribution of *P. canadensis* and *P. glaucus* seem to be primarily dictated as a result of an interaction between genetics and the thermal environment. The univoltine *P. canadensis* is limited to the north perhaps due to stress induced mortality resulting from exposure to high temperatures common to the south of its range. The multivoltine *P. glaucus* is likely prevented from moving farther north, due to cold induced pupal mortality but more significantly insufficient degree-days to allow for a second generation to succeed in a single generation. An attempted *glaucus* second generation would be suicidal in any geographic range that accumulated <2800 thermal degree-days. In the absence of strong reinforcement mechanisms a narrow hybrid zone has existed along the length of this ecological ecotone.

Unique thermal landscapes exist along the hybrid zone between *Papilio canadensis* and *Papilio glaucus* into which climate change has facilitated high levels of introgressive hybridization. Eight of the ten warmest years on record coincide with the dramatic increase of introgression of *Papilio glaucus* alleles into populations of *P. canadensis*. Significant cline shifts have occurred for many of the species diagnostic characters, resulting in hybrid swarms at locations near and distant from the historic hybrid zone (Ording 2001; Scriber and Ording 2005). The newly identified species, *P. appalachiensis* (Pavulaan and Wright 2002), appears to be the result of hybrid speciation that was possibly due to climate change (Scriber and Ording 2005; Scriber et al. 2008).

This research has analyzed a variety of wild populations that have been differentially impacted by introgressive hybridization, and has also analyzed a highly informative lab reared hybrid backcross. The findings of this research help to elucidate those factors that can contribute to the process of climate induced hybrid speciation in this *Papilio* system.

South Manitou Island maintains a unique hybrid swarm population at a location over 150 km north of the hybrid zone across a wide thermal landscape. Climate change resulting in unusually warm years has likely allowed for *P. glaucus* relatively historic introgression into the region (Ording 2001). Analysis of a variety of traits in this population over the course of 10 years indicates that the relative levels of genetic introgression in this population have been retained and have remained relatively stable. Out of over 1000 samples taken from this location, no pure *glaucus* or F1 hybrids have ever been collected from this location. However, there are a suite of *glaucus*-like hybrid traits present (longer forewing length, narrow hind wing black band width, X-linked Pgd 100 allele). Specimens possessing these hybrid traits have been present across the span of this research indicating that there doesn't appear to be any strong selection against these genetically recombined individuals.

Climate change is also very likely the major contributing factor that has allowed for increased levels of introgression into the Battenkill River Valley at the Vermont / New York border. This location, in the Appalachian Mountains of the Eastern United States, is very different from South Manitou Island in that there is more significant

topography, which results in a much more condensed thermal landscape. This has allowed for significantly increased levels of introgressive hybridization. The result has been the newly described Late Flight (LF). These highly introgressed LF populations are also best described as hybrid swarms, but exhibit even higher levels of introgression compared to South Manitou Island. This Late Flight population exhibits unique life history traits, including flight times and voltinism patterns, from either parental *Papilio* species.

It appears as though the LF population reported in the Battenkill River Valley may represent an ongoing process that over time could lead to incipient species. Each of these introgressed populations shares the unique feature of retaining the *canadensis* Ldh enzyme that is closely associated with the diapause gene on the X-chromosome. It has been shown that thermal selection is strong on this enzyme in other systems (Crawford and Powers 1989; Johns and Somero 2004; Schulte et al. 2000) and that only minor changes to this gene can result in functionally different forms of the enzyme (Holland et al. 1997; Johns and Somero 2004), potentially conferring increased fitness in light of shifting thermal environments.

The Late Flight of the Battenkill River Valley provides a unique opportunity in that it is clear which trait has arisen to initiate isolation. The delayed emergence of the recently reported Late Flight is a physiological shift that appears to be the result of hybridization. This new trait provides for a significant level of prezygotic isolation. The LF is now either geographically isolated or temporally isolated from both of the parental populations. Being temporally isolated could then potentially allow for the accumulation of further genetic differences.

The analysis of the lab-reared backcross (18006) provides strong evidence that this *Papilio* system can experience high rates of genetic recombination and X-chromosome crossovers. The analysis of survival probabilities for the variety of potential genotypes indicates that there is a Haldane effect, intrinsic genetic incompatibilities that reduce the frequency with which certain female recombined genotypes survive. Additionally, the trait analysis and the mapping of the X-chromosome indicate that the diapause regulatory locus and the Ldh locus are tightly linked. This provides for an extrinsic explanation as to why the *glaucus* Ldh 100 allele would never be found in introgressed populations. Chromosomal crossovers would rarely occur between such tightly linked loci. Any individual possessing the facultative diapause regulatory gene (od- allele) would direct develop in a thermal landscape with insufficient time to complete another round of reproduction, thus eliminating Ldh 100 with the od- allele. Most importantly, the rapid rates of recombination in nature between these hybridizing taxa is the mechanism that can result in unique genotypes that are well adapted to unique thermal niches.

As a result of shifting thermal landscapes, likely caused by climate change, populations in this *Papilio* system have exhibited high levels introgression and genetic recombination. This has resulted in novel trait combinations with delayed post diapause emergences, and provides an example of hybridization leading to the formation of populations allochronically isolated from either parental population. The temporal isolation in the Late Flight of the Battenkill River Valley has been strong enough to allow

for identifiable genetic differentiation via strong divergent selection on the X-chromosome. This is a potential first step down the avenue towards complete reproductive isolation and speciation.

APPENDICES

APPENDIX 1:

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

Appendix 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2008-01

Title of thesis or dissertation (or other research projects):

AN ANALYSIS OF CLIMATE INDUCED HYBRID SPECIATION IN TIGER SWALLOWTAIL BUTTERFLIES (*PAPILIO*)

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)

Gabriel J. Ording

Date 1/10/08

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America.
Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:
Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

APPENDIX 1.1:

VOUCHER SPECIMEN DATA

Appendix 1.1

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Papilio canadensis	VERMONT Bennington co. 17-Jun-04						5		MSU
	VERMONT Sandgate co. 15-Jul-04						5		MSU
	MICHIGAN South Manitou Island Leelenau co. 25-Jun-05						5		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Gabriel J. Ording

Date 9-Jan-08

Voucher No. 2008-01

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator Date 1/10/2008

APPENDIX 2:

POPULATION WING MEASUREMENTS AND ALLOZYME DATA

APPENDIX

Table 1. South Manitou island specimen information for each year (1998-2005). Forewing length, hind wing black bandwidth, and/or allozyme data are given for all specimens collected. Each specimen allozyme data presented represents two alleles. If only one allele is indicated, the individual is homozygous for that allele. If two alleles are indicated the individual is heterozygous for those two alleles.

South Manitou Island Data

1998

	Hind Wing Black Band Width	Fore Wing Length
♂ #1	61.5	49.5
♂ #2	55	49.5
♂ #3	52.5	38.5
♂ #4	54	47
♂ #5	53	39
♂ #6	60.5	46.5
♂ #7	57	44.5
♂ #8	53	49
♂ #9	50	49
♂ #10	61	48.5
♂ #11	73	50
♂ #12	38.5	47
♂ #13	60.5	48.5
♂ #14	62.5	46.5
♂ #15	54	49.5
♂ #16	49	49.5
♂ #17	52	45
♂ #18	43	44
♂ #19	38	46.5
♂ #20	62.5	45
♂ #21	53.5	45.5
♂ #22	52	48
♂ #23	60	48
♂ #24	57	48.5
♂ #25	57	46.5
♂ #26	53	46.5
♂ #27	56.5	48
♂ #28	53.5	45
♂ #29	62	47.5
♂ #30	64	49
♂ #31	60.5	42.5
♂ #32	47	46

1999

	Hind Wing Black Band Width	Fore Wing Length
♂ #1	51	48
♂ #2	65	46.5
♂ #3	55	51
♂ #4	55	46
♂ #5	67	46
♂ #6	55	44.5
♂ #7	60	40.5
♂ #8	56	45
♂ #9	36	52.5
♂ #10	53	45
♂ #11	64	46
♂ #12	52	42.5
♂ #13	62	46
♂ #14	60	50
♂ #15	51	47
♂ #16	57	46
♂ #17	57	
♂ #18	66	48
♂ #19	52	49.5
♂ #20	66	49
♂ #21	56	46
♂ #22	56	44.5
♂ #23	52	49.5
♂ #24	50	51.5
♂ #25	60	52.5
♂ #26	64	47.5
♂ #27	57	49.5
♂ #28	58	48
♂ #29	58	48
♂ #30	72	47
♂ #31	66	48.5
♂ #32	60	50
♂ #33	55	47

♂ #34	53	50
♂ #35	54	54.5
♂ #36	54	43.5
♂ #37	65	50
♂ #38	52	51
♂ #39	57	48.5
♂ #40	59	47.5
♂ #41	58	47.5
♂ #42	62	52
♂ #43	61	46
♂ #44	59	50
♂ #45	66	46.5
♂ #46	68	48
♂ #47	60	52.5
♂ #48	45	47
♂ #49	54	49.5
♂ #50	62	49.5
♂ #51	52	50.5
♂ #52	58	46
♂ #53	66	50
♂ #54	54	50
♂ #55	61	48
♂ #56	52	48
♂ #57	53	52.5
♂ #58	61	49
♂ #59	49	44
♂ #60	45	49
♂ #61	58	49.5
♂ #62	51	51
♂ #63	48	50
♂ #64	59	50
♂ #65	57	49.5
♂ #66	54	48.9
♂ #67	60	46
♂ #68	74	45
♂ #69	63	47
♂ #70	48	49
♂ #71	62	47.5
♂ #72	67	46.5
♂ #73	64	46.5
♂ #74	55	49.5
♂ #75	61	51
♂ #76	59	48.5
♂ #77	44	50.5
♂ #78	55	46.5
♂ #79	47	48
♂ #80	62	48.5
♂ #81	45	52.5

♂ #82	63	50
♂ #83	50	52.5
♂ #84	56	49
♂ #85	54	47
♂ #86	49	46
♂ #87	59	45.5
♂ #88	54	46
♂ #89	50	50
♂ #90	47	48
♂ #91	50	49
♂ #92	55	45
♂ #93	59	51
♂ #94	56	47.5
♂ #95	43	50.5
♂ #96	58	46
♂ #97	65	47.5
♂ #98	56	49
♂ #99	62	47
♂ #100	58	48.5
♂ #101	53	49
♂ #102	52	49.5
♂ #103	46	50
♂ #104	52	45
♂ #105	50	49.5
♂ #106	63	46
♂ #107	58	51
♂ #108	58	51
♂ #109	53	44.5
♂ #110	59	48
♂ #111	50	54
♂ #112	48	47
♂ #113	46	46
♂ #114	54	49
♂ #115	53	51
♂ #116	49	47.5
♂ #117	56	48
♂ #118	73	50
♂ #119	39	47.5
♂ #120	59	47

2000

	Hind Wing Black Band Width	Fore Wing Length
♂ #1	56	50
♂ #2	61.5	47.5
♂ #3	53.5	48

2001

	Hind Wing Black Band Width	Fore Wing Length	Allozymes Pgd
♂ #1			125
♂ #2			125
♂ #3			125/80

♂ #4	50.5	50.5	♂ #4			125
♂ #5	57.5	50	♂ #5			125/80
♂ #6	55.5	48	♂ #6			125
♂ #7	43.5	46.5	♂ #7			125
♂ #8	45.5	47	♂ #8			125
♂ #9	58	45	♂ #9			125
♂ #10	56	50	♂ #10			125
♂ #11	63	47	♂ #11			125
♂ #12	55.5	46.5	♂ #12			125
♂ #13	60	47	♂ #13			125
♂ #14	58.5	49	♂ #14			125
♂ #15	46	47	♂ #15			125
♂ #16	61	48	♂ #16			125/100
♂ #17	58.5	44.5	♂ #17			125/100
♂ #18	67	48	♂ #18			125
♂ #19	41.5	46	♂ #19			125
♂ #20	55.5	48	♂ #20			125/100
♂ #21	54.5	49	♂ #21			125
♂ #22	64.5	49	♂ #22			125/80
♂ #23	67	45	♂ #23			125
♂ #24	52	46	♂ #24			125/80
♂ #25	51.5	45	♂ #25			125/150
♂ #26	56	47	♂ #26			125
♂ #27	60.5	49	♂ #27			125
♂ #28	54	50.5	♂ #28			125
♂ #29	53.5	50.5	♂ #29			125
♂ #30	64	46.5	♂ #30			125
♂ #31	56	46.5	♂ #31			125
♂ #32	63.5	48.5	♂ #32			125
♂ #33	53	47.5	♂ #33			125/100
♂ #34	54	46	♂ #34			125
♂ #35	60	44.5	♂ #35			125
♂ #36	65.5	51	♂ #36			125/100
♂ #37	57	45.5	♂ #37			150/125
♂ #38	63.5	48	♂ #38			125
♂ #39	65	48.5	♂ #39			150/125
♂ #40	60.5	43.5	♂ #40			125
♂ #41	48	49	♂ #41	50.5	46	125
♂ #42	65.5	48	♂ #42	53.5	46	125/80
♂ #43	64	46	♂ #43	53	50	125
♂ #44	51.5	46	♂ #44	47.5	47	125
♂ #45	45.5	46	♂ #45	64	52	125
♂ #46	62	48.5	♂ #46	54.5	49	125
♂ #47	56	45	♂ #47	55	48	125
♂ #48	67	48.5	♂ #48	47	48	125
♂ #49	60.5	46.5	♂ #49	58.5	47.5	125
♂ #50	57	48	♂ #50	59.5	51	125
♂ #51	45.5	45.5	♂ #51	49.5	47	125

♂ #52	46.5	48
♂ #53	61	50
♂ #54	59	46.5
♂ #55	51	45.5
♂ #56	55.5	48
♂ #57	60.5	46.5
♂ #58	53.5	47
♂ #59	53.5	49
♂ #60	62.5	47.5
♂ #61	71	47
♂ #62	51	45.5
♂ #63	51.5	44
♂ #64	54	45.5
♂ #65	23.5	48
♂ #66	59	47
♂ #67	47.5	48
♂ #68	64.5	47
♂ #69	51.5	49
♂ #70	57	50
♂ #71	52.5	44.5
♂ #72	54	44

2002

	Hind Wing Black Band Width	Fore Wing Length	Allozyme Pgd
♂ #1	55	43	125
♂ #2	64	50	125
♂ #3	55	46	125
♂ #4	70	38	125
♂ #5	57.5	47	125/80
♂ #6	50.5	49	125/80
♂ #7	49	48	100
♂ #8	39.5	49	125
♂ #9	56	44	125
♂ #52	54.5	47	125
♂ #53	69	49	150/125
♂ #54	41.5	48	150/125
♂ #55	58	48	125
♂ #56	58	51	125
♂ #57	59	48	80
♂ #58	63	46	125
♂ #59	51	51	125
♂ #60	60.5	47.5	125
♂ #61	54.5	52	125
♂ #62	41	50	125/80
♂ #63	56.5	42	125
♂ #64	47	47.5	125
♂ #65	67.5	48	125
♂ #66	38	51.5	125
♂ #67	56	48	125
♂ #68	56	46	125
♂ #69	54	44	125/80
♂ #70	64	45	125/80
♂ #71	59	44.5	125/80
♂ #72	59	48.5	125
♂ #73	48.5	47.5	125/80
♂ #74	60	47.5	125
♂ #75	62	50	125
♂ #76	62	44.5	125
♂ #77	56.5	50	125
♂ #78	43.5	50.5	125/80
♂ #79	54	45	125
♂ #80	59	46	125
♂ #81	63.5	47	125
♂ #82	43.5	48.5	125/100
♂ #83	49.5	45	125
♂ #84	59	48	125
♂ #85	59.5	45.5	125
♂ #86	52	51	125
♂ #87	61	47.5	125
♂ #88	56	44	125/80
♂ #89	50	49	125
♂ #90	50	49	125
♂ #91	58	47	125/80
♂ #92	53	45	125/100
♂ #93	39	48	125
♂ #94	63.5	46	125
♂ #95	62	47	125
♂ #96	49	48	125
♂ #97	61	46.5	125
♂ #98	57	44	125
♂ #99	59	49.5	125

♂ #100	44	50.5	125

2003

	Hind Wing Black Band Width	Fore Wing Length
♂ #1	57.5	46
♂ #2	51.5	48
♂ #3	48	49
♂ #4	48.5	47
♂ #5	46	46
♂ #6	40	49
♂ #7	50.5	44
♂ #8	53	48
♂ #9	49	47
♂ #10	54	47
♂ #11	67	48
♂ #12	54	47
♂ #13	62	48
♂ #14	59	53
♂ #15	64.5	51
♂ #16	53.5	45
♂ #18	57.5	48
♂ #19	62.5	47
♂ #20	64	49
♂ #21	57	47
♂ #22	58.5	47
♂ #23	62	48
♂ #24	58.5	51
♂ #25	64.5	44
♂ #26	61.5	47
♂ #27	52.5	52
♂ #28	47.5	45
♂ #29	59	52
♂ #30	60	49
♂ #31	55.5	45
♂ #32	62.5	47
♂ #33	55.5	46
♂ #34	57.5	51
♂ #35	64	47
♂ #36	47.5	50
♂ #37	57	49
♂ #38	59.5	45
♂ #39	56	53
♂ #40	49	48
♂ #41	58.5	45
♂ #42	55	47

2004

	Hind Wing Black Band Width	Fore Wing Length
♂ #1	61	49
♂ #2	54.5	44
♂ #3	46.5	49
♂ #4	60	50
♂ #5	52.5	46
♂ #6	65	41
♂ #7	60	48
♂ #8	58.5	49
♂ #9	69	49
♂ #10	59.5	43
♂ #11	65	47
♂ #12	39.5	46
♂ #13	51.5	46
♂ #14	69	46
♂ #15	61.5	47
♂ #16	57.5	46
♂ #17	60	46
♂ #18	62	44
♂ #19	55	43
♂ #20	52	47
♂ #21	53	42
♂ #22	50	50
♂ #23	35	44
♂ #24	56.5	45
♂ #25	47.5	47
♂ #26	55.5	51
♂ #27	58	49
♂ #28	63.5	47
♂ #29	62.5	49
♂ #30	41	49
♂ #31	51	48
♂ #32	46.5	47
♂ #33	59	45
♂ #34	44	47
♂ #35	56	47
♂ #36	68	50
♂ #37	60	48
♂ #38	45	51
♂ #39	63	43
♂ #40	50	47
♂ #41	60	47

♂ #43	59.5	48
♂ #44	48.5	48
♂ #45	54.5	48
♂ #46	60	48
♂ #47	56.5	46
♂ #48	54.5	45
♂ #49	70	46
♂ #50	48.5	47
♂ #51	57.5	44
♂ #52	51.5	45
♂ #53	58	49
♂ #54	62	50
♂ #55	49	49
♂ #56	54	48
♂ #57	48	48
♂ #58	50.5	50
♂ #59	60.5	48
♂ #60	50	44
♂ #61	54.5	48
♂ #62	52.5	45
♂ #63	48.5	42
♂ #64	36	47
♂ #65	55.5	46
♂ #66	63	49
♂ #67	60	48
♂ #68	55.5	48
♂ #69	51.5	48
♂ #70	54.5	46
♂ #42	56	48
♂ #43	44	48

2005

	Hind Wing Black Band Width	Fore Wing Length	Allozyme Pgd
♂ #1	39.5	48	125
♂ #2	53.5	49	125
♂ #3	46.5	47	125
♂ #4	57.5	48	125/80
♂ #5	57.5	46	125
♂ #6	40	50	125
♂ #7	60.5	49	125
♂ #8	56	50	125
♂ #9	63.5	46	125
♂ #10	62	46	125
♂ #11	58.5	49	125/100
♂ #12	59	49	125
♂ #13	55.5	44	125
♂ #14	57.5	49	125
♂ #15	63.5	47	125
♂ #16	58.5	48	125
♂ #17	67.5	46	125/80
♂ #18	56.5	50	125
♂ #19	53.5	46	125
♂ #20	29	47	125
♂ #21	49	48	
♂ #22	61	42	
♂ #23	38.5	49	
♂ #24	47	46	
♂ #25	50.5	48	

Table 2. Wild collected Vermont specimen information for each year (2002-2004). Forewing length, hind wing black bandwidth, and/or allozyme data are given for specimens collected. Each specimen allozyme data presented represents two alleles. If only one allele is indicated, the individual is homozygous for that allele. If two alleles are indicated the individual is heterozygous for those two alleles.

	Vermont 2002			
	Forewing	Hindwing		
Wild Early Flight	Length	Blackband	Pgd	Ldh
♂ #1			*150/80	80
♂ #2			125	80
♂ #3			125	80
♂ #4			125/80	80
♂ #5			150/125	40
♂ #6			*125	80
♂ #7			*	80
♂ #8			*150	80
♂ #9			*125	80
♂ #10			*125	80
♂ #11			125/80	80
♂ #12			125/80	80
♂ #13			125/80	80
♂ #14			125	80
♂ #15			100	80
♂ #16			100/50	80
♂ #17	46	60	100	80
♂ #18	48	58	100/50	80
♂ #19	49	56	125	80
♂ #20	44	54.5	80	80
♂ #21	44	73	125	80
♂ #22	49	76.5	*125	80
♂ #23	48	52.5	*	80
♂ #24	45	63	*	80
♂ #25	47	65	*	80
♂ #26	41	68	*	80
♂ #27	42	77.5	125	80
♂ #28	49	61	125	80
♂ #29	44	69.5	125	80
♂ #30	47	70.5	125	80
♂ #31	48	58	125	80
♂ #32	45	60	125	80
♂ #33	44	76	125	80
♂ #34	44	83.5	125	80
♂ #35	45	82	125/80*	*
♂ #36	39	62	125	*
♂ #37	40	69.5	125	80
♂ #38	46	73	125	80

♂ #39	45	65.5	125	80
♂ #40	44	75	125	80
♂ #41	44	76	125	80
♂ #42	43	70	*	80
♂ #43	45	83	*	80
♂ #44	45	74.5	125	80
♂ #45	46	73.5	125	80
♂ #46	45	59.5	125/80	80
♂ #47	45	69.5	125	80
♂ #48	47	56	125	80
♂ #49	43	67.5	125	80
♂ #50	47	79.5	**	80
♂ #51	45	74.5	**	80
♂ #52	48	73.5	125	80
♂ #53	45	68.5	125	80
♂ #54	47	70	125	80
♂ #55	46	58.5	125	80
♂ #56	50	72.5	125	80
♂ #57	43	67.5	150/125	*
♂ #58	46	50	125/80	**
♂ #59	46	52	125	80
♂ #60	48	72.5	125	80
♂ #61	48	71	125	*
♂ #62	46	65	125	40
♂ #63	42	66	125/100	80
♂ #64	47	58	**125/80	80
♂ #65			125/80	80
♂ #66			125/100	80
♂ #67			100	80
♂ #68			**125	*
♂ #69			**125	80/40
♂ #70			125/100	80
♂ #71			**125/80	*
♂ #72			125	80
♂ #73			125	80
♂ #74			125	80
♂ #75			125/80	80
♂ #76			125/100	80
♂ #77			*100	**
♂ #78			125/80	80
♂ #79			125	80
♂ #80			125	80
♂ #81			125/80	80
♂ #82			125	80
♂ #83			125	40
♂ #84			125	80
♂ #85			125	80
♂ #86			125	80

♂ #87	125	80
♂ #88	125	80
♂ #89	125	80
♂ #90	125	80
♂ #91	125/80	80
♂ #92	125/80	80
♂ #93	150/100	*40 or 20
♂ #94	125	80
♂ #95	125/80	80
♂ #96	125/80	*80
♂ #97	125	80
♂ #98	125	40
♂ #99	125	80
♂ #100	125	80
♂ #101	125	*80/40
♂ #102	125	40
♂ #103	125	80
♂ #104	150/125	*
♂ #105	125	80
♂ #106	125	*
♂ #107	125	*80/40
♂ #108	125	80
♂ #109	125/80	80
♂ #110	125	80
♂ #111	125	40
♂ #112	125	80
♂ #113	125	40
♂ #114	150/125	*
♂ #115	125	80
♂ #116	125	80
♂ #117	125/100	80
♂ #118	125	80
♂ #119	125	80
♂ #120	150/125	80
♂ #121	125	40
♂ #122	125	80
♂ #123	125	80
♂ #124	125	80
♂ #125	150/125	80
♂ #126	125/80	80
♂ #127	125/80	80
♂ #128	125	80
♂ #129	125	80
♂ #130	125	80
♂ #131	125/100	80
♂ #132	125	80
♂ #133	125	80
♂ #134	125	80

♂ #135	125	80
♂ #136	**	80
♂ #137	**	40
♂ #138	125/100	80
♂ #139	*	80
♂ #140	125	80
♂ #141	*	80
♂ #142	125	80
♂ #143	125	80

Vermont 2002

Wild Late Flight	Forewing Length	Hindwing Blackband	Pgd	Ldh
♂ #1	50	48	100/80	80/20
♂ #2	49	52	125	40/20
♂ #3	51	39	100	80/20
♂ #4	54	39.5	100	40
♂ #5	49	63.5	125	80/20
♂ #6	52	58.5	125/100	80/20
♂ #7	51	43.5	125/100	80/20
♂ #8	47	57.5	125	80/20
♂ #9	54	33.5	125	80/20
♂ #10	48	37.5	80	80
♂ #11	50	49	100	80/20
♂ #12	50	48.5	125/100	20
♂ #13	51	57	100	80/20
♂ #14	51	47.5	**	**
♂ #6B	52	45		

Vermont 2003

Wild Early Flight	Forewing Length	Hindwing Blackband	Pgd	Ldh
♂ #1	45	72	125/100	80
♂ #2	45	74	125/100	80
♂ #3	49	73.5	125	80/40
♂ #4	43	70	125/100	80/40
♂ #5	45	63	125	80
♂ #6	46	59	125	80
♂ #7	45	77	125/80	80
♂ #8	47	66.5	125	80
♂ #9	48	66.5	125	80
♂ #10	47	68	100/80	80
♂ #11	44	70	125	80
♂ #12	46	65.5	125/80	80

♂ #13	44	54.5	125	80
♂ #14	44	69	125/80	80/40
♂ #15	45	62	125/80	80/40
♂ #16	45	75.5	125	80
♂ #17	46	72	125	80
♂ #18	44	69.5	125/100	80
♂ #19	46	74	125/100	80
♂ #20	43	72.5	125/80	80
♂ #21		95	125	80
♂ #22	46	74.5	125	80
♂ #23	43	63.5	125	80
♂ #24	43	72.5	100	80
♂ #25	47	77	125	80
♂ #26	48	73	125/100	80
♂ #27	41	44.5	125	80
♂ #28	44	70	125	80
♂ #29	46	63.5	150/100	80

Vermont 2003

Wild Late Flight	Forewing Length	Hindwing Blackband	Pgd	Ldh
♂ #1	49	53	125	80/20
♂ #2	52	44.5	125/100	*80/20
♂ #3	53	53	125/100	*80/20
♂ #4	51	46.5	125/100	80/20
♂ #5	51	54	100/80	80/20
♂ #6	51	51	100	40/20
♂ #7	53	41.5	100/80	80/20
♂ #8	49	41	100	40/20
♂ #9	50	54.5	125	80/20
♂ #10	49	59.5	100	*80/20
♂ #11	48	53.5	150/100	80/20
♂ #12	51	61	125	40/20
♂ #13	53	35.5	125/100	80/20
♂ #14	54	55.5	125/100	80/20

Vermont 2004

Wild Early Flight	Forewing Length	Hindwing Blackband	Pgd	Ldh
♂ #1			150/125	80
♂ #2			125	80
♂ #3			125	80

♂ #4	125	80
♂ #5	125	80
♂ #6	125	80
♂ #7	125	80
♂ #8	125	80
♂ #9	125	80
♂ #10	125/100	40
♂ #11	125	80
♂ #12	125	40
♂ #13	125	80
♂ #14	125	40
♂ #15	125	80
♂ #16	125	80
♂ #17	125	40
♂ #18	125	80
♂ #19	125	80
♂ #20	125	80
♂ #21	125/100	80
♂ #22	125/100	80
♂ #23	125	80
♂ #24	125	*
♂ #25	125/80	*
♂ #26	125	80
♂ #27	125	80
♂ #28	125	40
♂ #29	125	80
♂ #30	125	80
♂ #31	125/80	80
♂ #32	150/125	80
♂ #33	125	80
♂ #34	125	80
♂ #35	125/100	80
♂ #36	125	80
♂ #37	125	80
♂ #38	125	80
♂ #39	125	80
♂ #40	125	80
♂ #200	125	80
♂ #201	125	80
♂ #202	125/80	80
♂ #203	125	80
♂ #204	125/80	80
♂ #205	125/80	80
♂ #206	125/100	*
♂ #207	125	40
♂ #208	125	40
♂ #209	125	80

♂ #210			125	80
♂ #211			125	80
♂ #212			125/80	80
♂ #213			125	80
♂ #214			125	80
♂ #215			150/125	80
♂ #216			125/100	40
♂ #217			125/80	80
♂ #218			125	*
♂ #219			125	80
♂ #220			125	80
♂ #221			125	80
♂ #222			125/100	80
♂ #223			150/125	80
♂ #224			150/125	80
♂ #225			125	80
♂ #226			125	80
♂ #227			125/100	80
♂ #228			100	40
♂ #229			150/125	80
♂ #230			125	80
♂ #231			125/80	80
♂ #232			125	80
♂ #233			125	80
♂ #234			125/100	80
♂ #235			125	80
♂ #236			125	80
♂ #237			125	80
♂ #238			125	80
♂ #239			125	80
♂ #240			125/80	40
♂ #241	48	72	125	80
♂ #242	47	76.5	125	80
♂ #243	47	67.5	125/80	80
♂ #244	48	78.5	125	40
♂ #245	48	68	125	80
♂ #246	53	75.5	125	80
♂ #247	48	80	125/80	80
♂ #248	47	72.5	125	20
♂ #249	49	51.5	125	80
♂ #250	48	68.5	125	80
♂ #251	48	72.5	150/125	80
♂ #252	46	68.5	125	80
♂ #253	48	85.5	125	40
♂ #254	47	68	125	80
♂ #255	45	69	125	80
♂ #256	48	73.5	125	80
♂ #257	48	62.5	125	80

♂ #258	49	62.5	80	80
♂ #259	50	49.5	125	80
♂ #260	44	71.5	125/50	80
♂ #261	48	67.5	125/100	80
♂ #262	42	65.5	125/80	40
♂ #263	48	69.5	125	80
♂ #264	49	79	125	80
♂ #265	48	74.5	125	80
♂ #266	49	64	125/80	80
♂ #267	47	81	125	80
♂ #268	45	72	125/100	40
♂ #269	48	62	125	80
♂ #270	48	66.5	125	40
♂ #271	45	73.5	125/80	80
♂ #272	47	75	125/50	80
♂ #273	46	68	125	100/80
♂ #274	48	68	125	80
♂ #275	49	76	125	80
♂ #276	44	87	80	80
♂ #277	50	72	125	40
♂ #278	46	71	125	80
♂ #279	47	70	125	80
♂ #280	47	82	125/80	80
♂ #281	47	79	150/125	80
♂ #282	48	76	125	80
♂ #283	44	65.5	125	40
♂ #284	48	74	125	80
♂ #285	46	65.5	125	80
♂ #286	47	60.5	125	40
♂ #287	47	80.5	125	80
♂ #288	45	63.5	125	80
♂ #289	47	76	125	80
♂ #290	48	70	125	80
♂ #291	46	73.5	125/100	80
♂ #292	45	76	125	80
♂ #293	48	69.5	125	40
♂ #294	48	63	125	40
♂ #295	45	74	125	80
♂ #296	49	63.5	125	80
♂ #297	48	77	100	80
♂ #298	48	76	125	80
♂ #299	49	64.5	125	80
♂ #300	47	79.5	125/100	80
♂ #301	48	53	125/100	80
♂ #302	49	61.5	125	80
♂ #303	49	76	125	80
♂ #304	47	75.5	125	40
♂ #305	49	76	125	40

♂ #306	45	85.5	125/100	20
♂ #307	42	69	125	40
♂ #308	47	70.5	125	80
♂ #309	47	72	125/100	40
♂ #310	47	77.5	125	80
♂ #311	45	76.5	150/125	80
♂ #312	48	65	125	80
♂ #313	48	67	80	80
♂ #314	43	83.5	125	80
♂ #315	48	69	125/80	80
♂ #316	48	77.5	125	80
♂ #317	47	72	125	80
♂ #318	46	70.5	150/125	40
♂ #319	50	74	125	80
♂ #320	46	57	125	40

♂ #344		
♂ #345		
♂ #346		
♂ #347		
♂ #348		
♂ #349		
♂ #350		
♂ #351		
♂ #352		
♂ #353	48	64
♂ #354	47	74.5
♂ #355	50	76.5
♂ #356	47	79.5
♂ #357	45	71
♂ #358	47	76.5
♂ #359	45	57.5
♂ #360	47	78
♂ #361	45	71
♂ #362	48	76.5
♂ #363	47	77.5
♂ #364	48	66
♂ #365	46	75.5
♂ #366	44	82.5
♂ #367	49	79
♂ #368	49	85.5
♂ #369	46	63.5
♂ #370	47	74.5
♂ #371	46	57
♂ #372	50	44.5
♂ #373	49	72
♂ #374	47	66
♂ #375	48	72.5

♂ #376	47	82.5
♂ #377	48	65.5
♂ #378	48	67
♂ #379	47	67
♂ #380	47	74
♂ #381	45	68.5
♂ #382	46	76
♂ #383	46	80
♂ #384	47	66.5
♂ #385	46	66.5
♂ #386	44	74.5
♂ #387	47	70
♂ #388	44	63
♂ #389	47	63.5
♂ #390	48	65.5
♂ #391	47	58
♂ #392	43	75
♂ #393	47	69.5
♂ #394	45	68
♂ #395	49	82
♂ #396	45	64.5
♂ #397	46	70.5
♂ #398	47	77
♂ #399	48	74
♂ #400	47	68.5

Vermont 2004

Wild Late Flight	Forewing Length	Hindwing Blackband	Pgd	Ldh
♂ #1	50	45	125/80	80/20
♂ #2	54	48	125/100	80
♂ #3	52	44.5	100/80	80
♂ #4	52	39	125/100	80/20
♂ #10	52	44	125/100	80
♂ #11	54	60.5	125	40
♂ #12	51	46.5	100	80/20
♂ #13	48	55	100/80	80
♂ #14	55	46.5	100	80/20
♂ #15	48	53.5	150/100	80
♂ #16	51	34	125	80/20
♂ #17	50	53	100/80	80/20

APPENDIX 3:

POPULATION EMERGENCE DATA

APPENDIX

Table 3. Specimen emergence data for Early and Late Flight Vermont pupae reared in sleeved branches of black cherry (*Prunus serotina*). Data represented are the number of days from the beginning emergence investigation under controlled laboratory conditions.

Vermont Early Flight 2003 Emergence Data

Pupae placed in emergence chambers 4/15/03

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
18° ♂ #1	0.866	12	60	43
18° ♂ #3	0.724	13	80	
18° ♂ #4	0.599	14	55	40
18° ♂ #6	0.73	15	80	43
18° ♂ #7	0.852	16	80	43
18° ♂ #8	0.758	19	80	46
18° ♂ #10	0.623	17	60	42
18° ♂ #11	0.7	21	85	45
18° ♂ #12		23	80	41
18° ♂ #14	0.729	23	70	43
18° ♂ #15	0.756	24	75	41
18° ♂ #17	0.691	24	75	44
18° ♂ #18		25	75	48
18° ♂ #19		25	60	40
18° ♂ #21		26	65	41
18° ♂ #22		26	75	47
18° ♂ #23		26	70	45
18° ♂ #24		26	60	40
18° ♂ #25	0.888	26	75	42
18° ♂ #26	0.654	26	75	42
18° ♂ #27	0.967	26	55	48
18° ♂ #28	0.743	26	55	42
18° ♂ #29		27	65	44
18° ♂ #30	0.822	27	65	45
18° ♂ #32		27	65	42
18° ♂ #33		28	70	46
18° ♂ #37	0.806	28	75	44
18° ♂ #38	0.832	28	70	45
18° ♂ #39		28	65	47
18° ♂ #40		28	75	42
18° ♂ #42		29	65	42
18° ♂ #43		29	70	45

18° ♂ #44		29	60	43
18° ♂ #46		29	70	46
18° ♂ #47		29	70	44
18° ♂ #52		30	60	45
18° ♂ #53		30	50	43
18° ♂ #66	0.872	35	60	42

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
18° ♀ #2	0.913	12	70	46
18° ♀ #5	0.897	14	80	45
18° ♀ #9	0.702	19	90	32
18° ♀ #13	0.923	23	75	47
18° ♀ #16	0.803	24	75	45
18° ♀ #20		25	85	48
18° ♀ #31	0.916	27	80	46
18° ♀ #34		28	90	44
18° ♀ #35	0.844	28	75	45
18° ♀ #36	1.004	28	85	46
18° ♀ #41		29	85	45
18° ♀ #45		29	65	47
18° ♀ #48		29	70	43
18° ♀ #49		29	75	43
18° ♀ #50	0.861	29	70	46
18° ♀ #51		30	60	45
18° ♀ #54	0.796	31	75	42
18° ♀ #55		31	75	44
18° ♀ #56		31	70	43
18° ♀ #57		31	60	45
18° ♀ #58		32	60	45
18° ♀ #59		32	60	44
18° ♀ #60		33	75	48
18° ♀ #61		33	70	43
18° ♀ #62		33	65	45
18° ♀ #63		34	65	46
18° ♀ #64		34	75	40
18° ♀ #65		34	75	41
18° ♀ #67		35	75	45

Vermont Late Flight 2003 Emergence Data

Pupae placed in emergence chambers 4/15/03

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
18° ♂ #1	1.116	23	50	47
18° ♂ #2	1.092	45	50	51
18° ♂ #4	0.981	45	75	47
18° ♂ #5		45	60	47
18° ♂ #6	0.841	46	60	45
18° ♂ #8	0.986	46	65	48
18° ♂ #9	0.934	46	55	49
18° ♂ #10	1.11	46	40	53
18° ♂ #11		47	50	45
18° ♂ #12	1.087	47	55	50
18° ♂ #14	1.109	48	50	50
18° ♂ #16		49	45	48
18° ♂ #17	0.873	49	75	45
18° ♂ #20	1.229	51	65	52
18° ♂ #21	0.914	51	70	49
18° ♂ #22	0.829	52	60	43
18° ♂ #24	1.089	53	70	48

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
18° ♀ #3	1.072	45	65	50
18° ♀ #7	1.085	46	70	47
18° ♀ #13	0.888	48		
18° ♀ #15	0.841	48	70	46
18° ♀ #18	1.139	50	70	48
18° ♀ #19	1.099	50	60	49
18° ♀ #23		53	65	44
18° ♀ #25	1.035	53	70	46
18° ♀ #26	1.133	54	70	48
18° ♀ #27	1.291	55	75	49
18° ♀ #28	1.168	55	70	50
18° ♀ #29		55	65	48
18° ♀ #30	0.905	56	65	46
18° ♀ #31		61	75	46
18° ♀ #32	1.15	63	55	48
18° ♀ #33	1.041	64	50	48
18° ♀ #34		65	55	49

Vermont Early Flight 2003 Emergence Data

Pupae placed in emergence chambers 4/15/03

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
22° ♂ #1	0.679	9	65	40
22° ♂ #2	0.645	10	60	42
22° ♂ #6	0.695	11	80	41
22° ♂ #12		13	70	43
22° ♂ #13	0.645	14	65	40
22° ♂ #14	0.904	14	60	47
22° ♂ #15		14	60	46
22° ♂ #18		15	60	42
22° ♂ #20		15	50	45
22° ♂ #21		15	65	46
22° ♂ #26	0.785	16	60	45
22° ♂ #28	0.805	16	70	45
22° ♂ #30	0.742	16	60	44
22° ♂ #32		16	65	46
22° ♂ #36		16	50	46
22° ♂ #37	0.772	17	55	48
22° ♂ #38		17	60	45
22° ♂ #39		17	70	47
22° ♂ #40		17	60	40
22° ♂ #41		17	55	46
22° ♂ #42		17	60	41
22° ♂ #43		17	45	43
22° ♂ #44		17	50	46
22° ♂ #46		17	60	45
22° ♂ #49		18	70	45
22° ♂ #50		18	70	41
22° ♂ #51		18	80	43
22° ♂ #59		18	60	47

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
22° ♀ #3	0.753	10	75	
22° ♀ #4	0.903	10	60	49
22° ♀ #5	0.747	10	90	43
22° ♀ #7	0.843	12	60	47
22° ♀ #8	0.754	11	70	43
22° ♀ #9	0.834	11	75	45
22° ♀ #10	0.822	13	70	43
22° ♀ #11	0.839	13	60	46

22° ♀ #16	0.829	15	60	42
22° ♀ #17		15	70	48
22° ♀ #19	0.996	15	85	47
22° ♀ #22		15	45	48
22° ♀ #23		16	55	42
22° ♀ #24		16	75	47
22° ♀ #25		16	75	47
22° ♀ #27	1.044	16	60	49
22° ♀ #29	0.842	16	75	47
22° ♀ #31	0.898	16	70	48
22° ♀ #33		16	75	49
22° ♀ #34		16	60	49
22° ♀ #35		16	70	48
22° ♀ #45	1.0007	18	65	50
22° ♀ #47	0.856	18	70	46
22° ♀ #48		18	75	49
22° ♀ #52		19	50	48
22° ♀ #53		19	65	45
22° ♀ #54		19	65	44
22° ♀ #55		18	70	44
22° ♀ #56		18	50	45
22° ♀ #57		18	65	49
22° ♀ #58		18	70	46
22° ♀ #60		19	75	47
22° ♀ #61		19	75	50
22° ♀ #62		20	80	47
22° ♀ #63		20	90	46
22° ♀ #64		20	50	49

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
22° ♀ #1	0.581	21	50	40
22° ♀ #8		29	60	50
22° ♀ #9	1.081	29	55	52
22° ♀ #12	1.236	30	50	52
22° ♀ #13	0.901	30	60	48
22° ♀ #14	1.033	31	70	50
22° ♀ #16		31	60	51
22° ♀ #18	1.168	32	60	50
22° ♀ #19	1.008	32	50	46
22° ♀ #20	1.177	32	70	51
22° ♀ #22	0.916	32		43
22° ♀ #25	1.19	34	45	54
22° ♀ #26		35	55	49
22° ♀ #27		35	60	47
22° ♀ #28	0.979	36	40	48

22° ♀ #31	1.149	38	50	50
22° ♀ #32	1.232	39	50	53
22° ♀ #33	1.286	40	45	50
22° ♀ #34	0.929	40	45	47
22° ♀ #35	1.021	41	45	50
22° ♀ #36	1.103	70	65	

Vermont Late Flight 2003 Emergence Data

Pupae placed in emergence chambers 4/15/03

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
22° ♂ #2		25	35	49
22° ♂ #3	1.073	25	50	50
22° ♂ #4	0.779	25	55	47
22° ♂ #5	0.833	27	75	46
22° ♂ #6	1.051	27	50	48
22° ♂ #7	0.805	27	60	47
22° ♂ #10	0.951	30	40	47
22° ♂ #11	0.756	30	50	43
22° ♂ #15	0.972	31	50	46
22° ♂ #17		32	40	50
22° ♂ #21	0.939	32	50	48
22° ♂ #23	0.945	32	40	43
22° ♂ #24		33	55	45
22° ♂ #29	1.027	36	40	49
22° ♂ #30	0.973	37	50	48

Vermont Early Flight 2003 Emergence Data

Pupae placed in emergence chambers 4/15/03

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
26° ♂ #1	0.835	9	70	42
26° ♂ #2	0.804	9	65	44
26° ♂ #3	0.704	9	70	45
26° ♂ #10	0.73	9	55	44

26° ♂ #13	0.718	9	70	39
26° ♂ #14		10	50	48
26° ♂ #16	0.785	11	50	44
26° ♂ #18	0.737	12	60	45
26° ♂ #19	0.857	12	75	47
26° ♂ #20		12	55	43
26° ♂ #21		12	60	42
26° ♂ #22		12	70	44
26° ♂ #23		12	60	45
26° ♂ #27	0.847	11	60	47
26° ♂ #29		13	55	45
26° ♂ #30		13	50	43
26° ♂ #31		13	60	44
26° ♂ #32		13	55	45
26° ♂ #34		13	65	43
26° ♂ #36		13	60	43
26° ♂ #37		13	55	42
26° ♂ #38		13	60	48
26° ♂ #42		11	60	44
26° ♂ #44	0.893	13	60	45
26° ♂ #50		14	45	43
26° ♂ #51		14	50	44
26° ♂ #52	0.801	14	60	39
26° ♂ #53	0.837	14	65	44
26° ♂ #55		14	60	45
26° ♂ #57		14	65	44

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
26° ♀ #4	0.878	9	70	43
26° ♀ #5	0.833	9	70	42
26° ♀ #6	0.807	9	65	43
26° ♀ #7	0.836	9	60	43
26° ♀ #8	0.754	7		
26° ♀ #9	0.891	9	70	46
26° ♀ #11	0.948	9	65	43
26° ♀ #12	0.771	9	75	42
26° ♀ #15		11	55	50
26° ♀ #17	0.921	12	50	50
26° ♀ #24		13	60	47
26° ♀ #25		13	60	45
26° ♀ #26	0.885	13	65	47
26° ♀ #28	0.9	11	75	46
26° ♀ #33		13	65	51
26° ♀ #35		13	50	47
26° ♀ #39		13	65	44
26° ♀ #40		13	55	48
26° ♀ #41		11	45	45

26° ♀ #43	0.844	13	55	46
26° ♀ #45	0.83	14	50	47
26° ♀ #46	1.026	14	50	49
26° ♀ #47		14	60	50
26° ♀ #48		14	70	46
26° ♀ #49		14	55	49
26° ♀ #54		14	60	49
26° ♀ #56		14	60	46
26° ♀ #58		14	50	48
26° ♀ #59		15	65	43
26° ♀ #60		15	55	47
26° ♀ #61		15	60	46
26° ♀ #62		15	65	48
26° ♀ #63		16	70	42
26° ♀ #64		16	80	47
26° ♀ #65		17	60	47

Vermont Late Flight 2003 Emergence Data

Pupae placed in emergence chambers 4/15/03

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
26° ♂ #1	1.16	18	60	51
26° ♂ #2	0.985	18	45	50
26° ♂ #3	0.99	20	45	50
26° ♂ #4	0.968	21	60	48
26° ♂ #5	1.086	21	50	51
26° ♂ #6	0.925	22	55	48
26° ♂ #8	1.09	23	50	50
26° ♂ #9	0.826	23	45	44
26° ♂ #10	0.855	23	45	47
26° ♂ #11		24	50	51
26° ♂ #12	0.628	24	50	44
26° ♂ #13	1.046	24	35	48
26° ♂ #14	0.896	24	45	48
26° ♂ #16	0.891	25	45	50
26° ♂ #17	0.932	25	60	47
26° ♂ #18		26	55	50
26° ♂ #19		26	60	45
26° ♂ #24	1.076	27	50	49
26° ♂ #32	0.949	31	60	47
26° ♂ #35	0.955	34	50	49
26° ♂ #36	1.056	34	50	42

ID	Pupal Wt.	Days to Emerge	Black Band Width (Eye)	Fore Wing Length
26° ♀ #7	0.884	22	55	47
26° ♀ #15	0.926	25	75	46
26° ♀ #20	1.131	26	50	50
26° ♀ #21		27		
26° ♀ #22	1.072	27	75	50
26° ♀ #23	1.18	27	40	54
26° ♀ #25		28	50	52
26° ♀ #26	0.997	28	60	50
26° ♀ #27		29	50	50
26° ♀ #28	0.846	29	65	48
26° ♀ #29	0.896	29	60	50
26° ♀ #30	0.86	30	55	45
26° ♀ #31	1.072	30	55	50
26° ♀ #33	1.222	31	75	48
26° ♀ #34	0.848	32	70	45

Vermont Early Flight 2004 Emergence Data

Pupae placed in emergence chambers 4/8/04

ID	Pupal Wt.	Days to Emerge	Fore Wing Length	ID	Pupal Wt.	Days to Emerge	Fore Wing Length
14° ♂ #1	0.7082	42	43	14° ♀ #1		49	41
14° ♂ #2	0.6633	42	42	14° ♀ #2	0.8209	49	49
14° ♂ #3	0.6288	44	40	14° ♀ #3	0.7219	49	44
14° ♂ #4	0.7929	44	45	14° ♀ #4	0.733	49	46
14° ♂ #5	0.7194	45	44	14° ♀ #5	0.6102	51	41
14° ♂ #6	0.6078	46	39	14° ♀ #6	0.6859	52	38
14° ♂ #7	0.7098	46	42	14° ♀ #7	0.8296	52	45
14° ♂ #8	0.7755	46	43	14° ♀ #8	0.7352	52	44
14° ♂ #9	0.7081	46		14° ♀ #9	0.8312	52	43
14° ♂ #10	0.7658	46	43	14° ♀ #10	0.7451	54	45
14° ♂ #11	0.7835	47	44	14° ♀ #11		56	43
14° ♂ #12	0.6802	47	42	14° ♀ #12		56	
14° ♂ #13	0.7922	47	44	14° ♀ #13	0.7556	57	47
14° ♂ #14	0.7397	47	44	14° ♀ #14	0.7377	57	44
14° ♂ #15	0.8514	47	46	14° ♀ #15	0.9398	61	47
14° ♂ #16	0.7219	47	42	14° ♀ #16	0.8187	69	44
14° ♂ #17	0.7468	49	44				
14° ♂ #18		50	43				
14° ♂ #19		50	38				
14° ♂ #20		52	43				
14° ♂ #21	0.8341	51	44				
14° ♂ #22	0.7631	51					
14° ♂ #23	0.8016	52	43				
14° ♂ #24		56	42				
14° ♂ #25	0.6972	56					
14° ♂ #26	0.6519	56	40				

Vermont Early Flight 2004 Emergence Data

Pupae placed in emergence chambers 4/8/04

ID	Pupal Wt.	Days to Emerge	Fore Wing Length	ID	Pupal Wt.	Days to Emerge	Fore Wing Length
18° ♂ #1		21	41	18° ♀ #1	0.8936	21	47
18° ♂ #2	0.6597	22	41	18° ♀ #2	0.7848	23	46
18° ♂ #3	0.7469	21	44	18° ♀ #3	0.6932	24	44
18° ♂ #4	0.8441	21	46	18° ♀ #4	0.6873	24	45

18° ♂ #5		23	44	18° ♀ #5	0.6269	24	41
18° ♂ #6		23	42	18° ♀ #6	0.7958	24	46
18° ♂ #7		23	41	18° ♀ #7	0.7936	25	48
18° ♂ #8	0.6866	23	42	18° ♀ #8	0.8618	25	45
18° ♂ #9	0.8687	23	48	18° ♀ #9	0.7166	25	45
18° ♂ #10	0.6463	23	40	18° ♀ #10	0.8091	25	45
18° ♂ #11	0.8251	23	44	18° ♀ #11	0.8182	25	46
18° ♂ #12	0.8173	23	44	18° ♀ #12	0.8194	26	47
18° ♂ #13	0.697	23	43	18° ♀ #13	0.9585	26	48
18° ♂ #14		24	43	18° ♀ #14	0.6287	26	43
18° ♂ #15	0.7388	24	44	18° ♀ #15	0.678	26	43
18° ♂ #16	0.8116	24	46	18° ♀ #16	0.9076	26	46
18° ♂ #17	0.7578	24	45	18° ♀ #17		26	45
18° ♂ #18		25	41	18° ♀ #18	0.8164	27	45
18° ♂ #19		25	43	18° ♀ #19	0.8177	27	46
18° ♂ #20	0.7274	25	44	18° ♀ #20	0.7426	27	47
18° ♂ #21	0.7979	25	45	18° ♀ #21	0.7704	27	45
				18° ♀ #22	0.8132	27	46
				18° ♀ #23	0.8105	27	45
				18° ♀ #24		28	41
				18° ♀ #25	0.8531	28	46
				18° ♀ #26	0.7448	29	45
				18° ♀ #27	0.7773	30	

Vermont Early Flight 2004 Emergence Data

Pupae placed in emergence chambers 4/8/04

ID	Pupal Wt.	Days to Emerge	Fore Wing Length	ID	Pupal Wt.	Days to Emerge	Fore Wing Length
22° ♂ #1	0.7952	14	45	22° ♀ #1	0.8315	15	45
22° ♂ #2	0.7089	15	45	22° ♀ #2	0.8502	16	48
22° ♂ #3	0.7122	15	42	22° ♀ #3	0.6795	17	44
22° ♂ #4		15	44	22° ♀ #4	0.7478	17	42
22° ♂ #5	0.6756	15	41	22° ♀ #5	0.7169	17	46
22° ♂ #6	0.808	15	45	22° ♀ #6	0.7821	17	45
22° ♂ #7	0.7166	15	44	22° ♀ #7	0.7664	17	
22° ♂ #8	0.7361	15	44	22° ♀ #8	0.8242	18	45
22° ♂ #9	0.658	15	41	22° ♀ #9	0.8933	18	47
22° ♂ #10	0.5528	16	42	22° ♀ #10	0.6964	18	42
22° ♂ #11	0.7653	16	46	22° ♀ #11	0.7329	18	42
22° ♂ #12		15	42	22° ♀ #12	0.8497	18	47
22° ♂ #13		16	42	22° ♀ #13	0.7485	19	46
22° ♂ #14		16	43	22° ♀ #14	0.7402	19	45
22° ♂ #15		16	43	22° ♀ #15	0.8601	19	48
22° ♂ #16		16	44	22° ♀ #16	0.79	19	45
22° ♂ #17		16		22° ♀ #17	1.0246	19	51

22° ♂ #18	0.7038	16	42	22° ♀ #18	0.6982	19	44
22° ♂ #19	0.7124	16	43	22° ♀ #19		20	46
22° ♂ #20	0.6738	16	43	22° ♀ #20	0.7567	21	44
22° ♂ #21	0.7676	16	45	22° ♀ #21	0.7766	21	45
22° ♂ #22		17		22° ♀ #22	0.8079	23	45
22° ♂ #23	0.7249	17	45				
22° ♂ #24	0.8256	18	44				
22° ♂ #25	0.6613	18	43				

Vermont Early Flight 2004 Emergence Data

Pupae placed in emergence chambers 4/8/04

ID	Pupal Wt.	Days to Emerge	Fore Wing Length	ID	Pupal Wt.	Days to Emerge	Fore Wing Length
26° ♂ #1		10	45	26° ♀ #1		11	45
26° ♂ #2	0.6623	10	42	26° ♀ #2	0.8187	11	47
26° ♂ #3	0.6991	10	42	26° ♀ #3	0.9599	11	48
26° ♂ #4	0.706	10	44	26° ♀ #4	0.9187	11	48
26° ♂ #5		11	46	26° ♀ #5		12	45
26° ♂ #6		11	46	26° ♀ #6		12	45
26° ♂ #7		11	47	26° ♀ #7	0.7358	12	44
26° ♂ #8		11	47	26° ♀ #8	0.7573	12	46
26° ♂ #9	0.7802	11	45	26° ♀ #9	0.8493	12	47
26° ♂ #10	0.7873	11	46	26° ♀ #10	0.7318	12	44
26° ♂ #11	0.8738	11	47	26° ♀ #11	0.7133	12	45
26° ♂ #12	0.6173	11	40	26° ♀ #12	0.6901	12	44
26° ♂ #13	0.7047	11	42	26° ♀ #13	0.8221	12	45
26° ♂ #14	0.7451	11	44	26° ♀ #14	0.8363	12	46
26° ♂ #15	0.6825	11	43	26° ♀ #15	0.838	13	46
26° ♂ #16	0.6704	11	45	26° ♀ #16	0.7049	13	44
26° ♂ #17	0.829	11	47	26° ♀ #17	0.8784	13	48
26° ♂ #18	0.8224	11	46	26° ♀ #18	0.8546	13	47
26° ♂ #19	0.7084	11	44	26° ♀ #19	0.8063	13	43
26° ♂ #20	0.786	11	44	26° ♀ #20	0.8508	14	47
26° ♂ #21		12	45	26° ♀ #21		14	45
26° ♂ #22	0.8002	12	45	26° ♀ #22	0.8335	15	45
26° ♂ #23	0.7381	12	45				
26° ♂ #24	0.6328	12	42				
26° ♂ #25	0.7469	12	45				
26° ♂ #26	0.8703	13					

Vermont Late Flight 2004 Emergence Data

Pupae placed in emergence chambers 4/8/04

ID	Pupal Wt.	Days to	Fore Wing	ID	Pupal Wt.	Days to	Fore Wing

		Emerge	Length			Emerge	Length
14° ♂ #1	0.8936	75	50	14° ♀ #1	0.9654	52	47
14° ♂ #2	1.0219	75	49	14° ♀ #2	1.0707	91	49
14° ♂ #3	1.0303	75	46	14° ♀ #3	0.9808	95	49
14° ♂ #4	1.0224	78	46	14° ♀ #4	1.1026	96	49
14° ♂ #5	0.9916	81	46	14° ♀ #5	1.0659	96	
14° ♂ #6	0.9891	82	47	14° ♀ #6	1.1122	98	48
14° ♂ #7	0.9538	86	49	14° ♀ #7	0.9525	100	48
14° ♂ #8	0.9083	87	45	14° ♀ #8	1.1722	102	48
14° ♂ #9	1.1649	91	48	14° ♀ #9	1.1144	104	47
14° ♂ #10	1.0353	94	47	14° ♀ #10	1.0167	111	48
14° ♂ #11	1.1038	95	48	14° ♀ #11	0.9724	122	45
14° ♂ #12	1.1506	96	50	14° ♀ #12	1.1149	138	47
14° ♂ #13	1.0923	97	47				
14° ♂ #14	1.0086	97	47				
14° ♂ #15	1.1023	99	48				
14° ♂ #16	1.2209	102	52				
14° ♂ #17	1.108	110	46				

Vermont Late Flight 2004 Emergence Data

Pupae placed in emergence chambers 4/8/04

ID	Pupal Wt.	Days to Emerge	Fore Wing Length	ID	Pupal Wt.	Days to Emerge	Fore Wing Length
18° ♂ #1	1.1192	35	50	18° ♀ #1	0.936	43	50
18° ♂ #2	1.1706	41	52	18° ♀ #2	1.223	45	51
18° ♂ #3	1.0548	41	46	18° ♀ #3	0.8084	45	41
18° ♂ #4	1.1164	41	48	18° ♀ #4	1.0102	45	49
18° ♂ #5	1.0317	40	48	18° ♀ #5	0.985	46	48
18° ♂ #6	1.0502	40	51	18° ♀ #6	1.2856	47	51
18° ♂ #7	1.1943	41	52	18° ♀ #7	1.0223	47	49
18° ♂ #8	1.0163	42	50	18° ♀ #8	1.2892	48	52
18° ♂ #9	1.0607	44	50	18° ♀ #9	1.0409	49	47
18° ♂ #10	0.8168	44	46	18° ♀ #10	1.1991	50	53
18° ♂ #11	1.0897	44	50	18° ♀ #11	0.9674	51	48
18° ♂ #12	1.1589	45	49	18° ♀ #12	1.1661	53	52
18° ♂ #13	1.1449	45	51	18° ♀ #13	1.3188	53	52
18° ♂ #14	1.0989	46	49	18° ♀ #14	1.1231	56	52
18° ♂ #15	1.0322	47	48	18° ♀ #15	1.2497	59	55
18° ♂ #16	0.9906	48	48				
18° ♂ #17	1.0776	49	51				
18° ♂ #18	1.1129	49	49				
18° ♂ #19	1.1571	50	51				
18° ♂ #20	1.0002	51	49				

Vermont Late Flight 2004 Emergence Data

Pupae placed in emergence chambers 4/8/04

ID	Pupal Wt.	Days to Emerge	Fore Wing Length	ID	Pupal Wt.	Days to Emerge	Fore Wing Length
22° ♂ #1	1.0099	24	48	22° ♀ #1	1.0206	28	51
22° ♂ #2	0.9229	25	47	22° ♀ #2	1.1161	29	53
22° ♂ #3	0.8256	26	47	22° ♀ #3	1.1909	30	51
22° ♂ #4	0.9927	27	49	22° ♀ #4	1.00323	31	52
22° ♂ #5	0.9874	28		22° ♀ #5	0.9683	31	48
22° ♂ #6	1.1853	28	53	22° ♀ #6	1.0187	32	51
22° ♂ #7	0.8833	29	48	22° ♀ #7	1.0828	33	51
22° ♂ #8	1.0132	29	50	22° ♀ #8	1.3412	33	53
22° ♂ #9	1.1293	29	50	22° ♀ #9	1.0228	33	51
22° ♂ #10	0.974	29	48	22° ♀ #10	1.0678	33	51
22° ♂ #11	1.0869	30	50	22° ♀ #11	1.3561	34	53
22° ♂ #12	0.992	32	50	22° ♀ #12	0.9329	34	48
22° ♂ #13	1.0794	33	51	22° ♀ #13	1.0048	36	
22° ♂ #14	0.9945	34	51	22° ♀ #14	1.1676	38	46
22° ♂ #15	1.0172	35	51	22° ♀ #15	1.1612	39	47
22° ♂ #16	0.9105	37		22° ♀ #16	0.9678	39	52
22° ♂ #17	1.0926	38	51	22° ♀ #17	1.2123	40	53
				22° ♀ #18	1.2428	41	52
				22° ♀ #19	1.0902	41	48
				22° ♀ #20	1.1223	45	49

Vermont Late Flight 2004 Emergence Data

Pupae placed in emergence chambers 4/8/04

ID	Pupal Wt.	Days to Emerge	Fore Wing Length	ID	Pupal Wt.	Days to Emerge	Fore Wing Length
26° ♂ #1	0.6829	12	43	26° ♀ #1	1.0896	24	50
26° ♂ #2	1.1102	20	49	26° ♀ #2	1.1348	24	50
26° ♂ #3	1.0456	21	50	26° ♀ #3	0.9876	24	48
26° ♂ #4	0.9519	21	50	26° ♀ #4	1.208	25	52
26° ♂ #5	0.8109	21	48	26° ♀ #5	1.0086	26	50
26° ♂ #6	1.0692	22	50	26° ♀ #6	1.2064	26	52
26° ♂ #7	0.9964	22	51	26° ♀ #7	1.1013	27	53
26° ♂ #8	0.8775	22	48	26° ♀ #8	1.1969	29	54
26° ♂ #9	0.9538	22	50	26° ♀ #9	1.0888	30	49
26° ♂ #10	1.0049	23	50	26° ♀ #10	1.1769	30	54
26° ♂ #11	0.9492	23	49	26° ♀ #11	1.1694	30	51

26° ♂ #12	0.9458	23	49	26° ♀ #12	1.1673	30	50
26° ♂ #13	1.0314	24	50	26° ♀ #13	1.1883	30	54
26° ♂ #14	0.8649	25		26° ♀ #14	0.9874	30	52
26° ♂ #15	1.0587	25	50	26° ♀ #15	1.1146	33	52
26° ♂ #16	0.9894	25	51				
26° ♂ #17	1.1722	25	54				
26° ♂ #18	1.0106	25	45				
26° ♂ #19	1.1116	25	52				
26° ♂ #20	1.0163	26	50				
26° ♂ #21	1.1347	27	50				
26° ♂ #22	1.0072	29	48				
26° ♂ #23	0.883	29	45				
26° ♂ #24	1.1198	31	52				

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